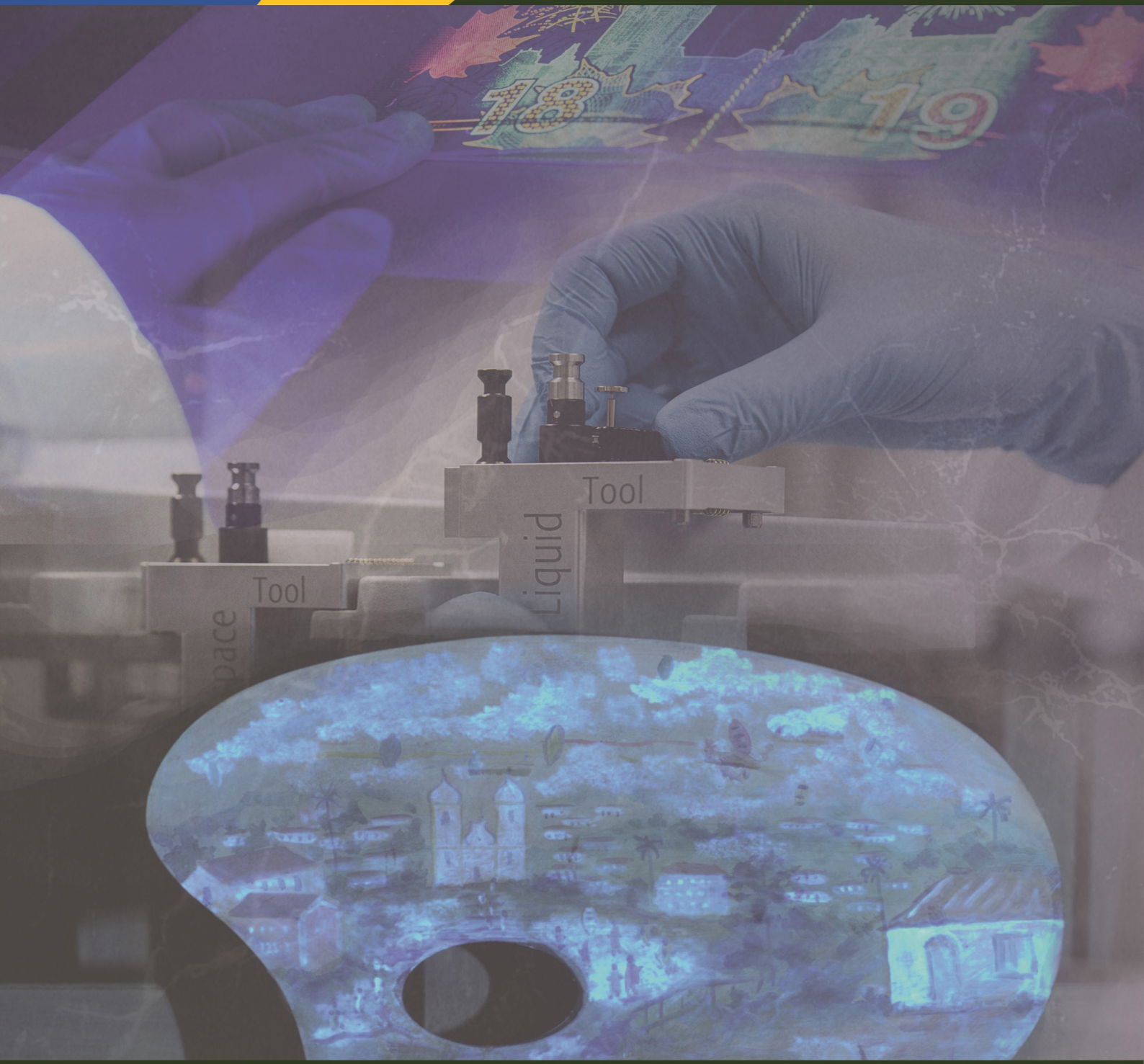
BrJAC

Brazilian Journal of Analytical Chemistry

an International Scientific Journal



New Trends in Analytical Chemistry for the Examination and Interpretation of Traces of Crimes

Jesus Antonio Velho

January – March 2022 Volume 9 Number 34

III IBEROAMERICAN CONFERENCE ON MASS SPECTROMETRY

Rio 2022

www.ibero2022.com

DECEMBER
10-15, 2022

RIO DE JANEIRO - BRAZIL

Sheraton Grand Rio Hotel & Resort

Organization

Sociedade Brasileira de Espectrometria de Massas

Br MASS

Venue

SHERATON
EST. 1937

Brazilian Journal of Analytical Chemistry

VISÃO FOKKA - COMUNICATION AGENCY

The *Braz. J. Anal. Chem.* (BrJAC) is a peer-reviewed, open access, scientific journal intended for professionals and institutions acting mainly in all branches of analytical chemistry.

ISSN 2179-3425 printed ISSN 2179-3433 eletronic

Scope

BrJAC is dedicated to the diffusion of significant and original knowledge in all branches of Analytical Chemistry and Bioanalytics. BrJAC is addressed to professionals involved in science, technology and innovation projects in Analytical Chemistry at universities, research centers and in industry.

BrJAC is a quarterly journal that publishes original, unpublished scientific articles, reviews and technical notes that are peer reviewed in the double-blind way. In addition, it publishes interviews, points of view, letters, sponsor reports, and features related to analytical chemistry. Once published online, a DOI number is assigned to the paper.

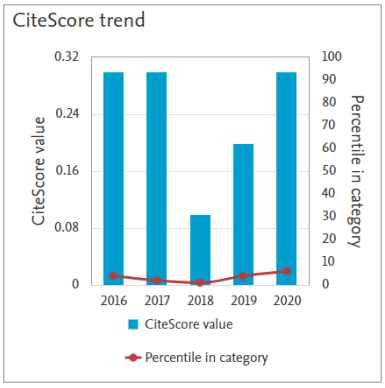
Manuscripts submitted for publication in BrJAC cannot have been previously published or be currently submitted for publication in another journal.

For complete information on ethics and policies on conflicts of interest, copyright, reproduction of already published material and preprints, in addition to the manuscript submission and peer review system, please visit “About us” and Guidelines for Authors at www.brjac.com.br

Indexing Sources

Scopus

CiteScore 2020: 0.3; SJR: 0.131; SNIP: 0.146. CiteScore Tracker 2021: 0.6 Access source details

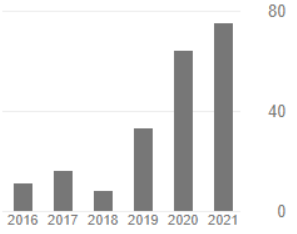


CLARIVATE ANALYTICS
EMERGING SOURCES CITATION INDEX
INDEXED

CAPES QUALIS

Google scholar

	All	Since 2016
Citations	272	210
h-index	6	5
i10-index	3	1



Production Editor
Silvana Odete Pisani

Publisher
Lilian Freitas
MTB: 0076693/ SP
lilian.freitas@visaofokka.com.br

Advertisement
Luciene Campos
luciene.campos@visaofokka.com.br

Art Director: Adriana Garcia

WebMaster: Daniel Letieri

BrJAC’s website: www.brjac.com.br / Contact: brjac@brjac.com.br
Like BrJAC on Facebook: https://www.facebook.com/brjachem

ABEC BRASIL BrJAC is associated to the Brazilian Association of Scientific Editors

VISÃO FOKKA BrJAC is published quarterly by:
Visão Fokka Communication Agency
Av. Washington Luiz, 4300 - Bloco G - 43
13042-105 – Campinas, SP, Brazil
contato@visaofokka.com.br
www.visaofokka.com.br

EDITORIAL BOARD

EDITORIAL ADVISORY BOARD

EDITORIAL ADVISORY BOARD (Continuation)

CONTENTS

CONTENTS

CONTENTS

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 1-2
doi: 10.30744/brjac.2179-3425.editorial.mamsveiga.N34

BrJAC

EDITORIAL

Forensic Analytical Chemistry: Connecting Science and Justice

Márcia Andreia Mesquita Silva da Veiga

Guest Editor of this special edition on Forensic Analytical Chemistry

Associate Professor in the Department of Chemistry at the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo (FFCLRP-USP), Ribeirão Preto, SP, Brazil

Currently, analytical chemistry is more than simply its division into classical and instrumental. It is an interdisciplinary area that involves notions of biology, toxicology, statistics, computer science, and physics, among others. There are several areas of knowledge applied in the development of a chemical analysis, which is configured as all the processes necessary for the identification and quantification of the different components of a sample. When this sample is a trace material from a crime scene, analytical chemistry assumes a central role in the conversion of this sample into material evidence with legal value through consolidated and validated procedures, obtained by exhaustive investigative and methodological studies. The responsibility assumed is in the confidence of the result obtained, which will only be possible with the validation of the method. Although not all methods are perfect, a quantitative determination requires a precise and accurate methodology. Therefore, analytical chemistry is very important to forensic chemistry. Material evidence has a great influence on a trial because it is clothed in technical characteristics, and the expectation is that it will help to unequivocally clarify the truth of the facts. It is this expectation that makes the work of the analytical chemist so important in conducting an analytical procedure for forensic purposes. The result obtained may or may not incriminate someone.

Another analytical challenge in forensic analysis is the collection and preparation of a sample that has a criminal trace profile. Such procedures should preserve as much of the criminal evidence as possible. At a crime scene, several samples can be considered evidence: soils, fibers, glass, gunshot residues, explosives, among others. Locard's principle of exchange states that whenever two objects come into contact, an exchange of materials occurs between them and, thus, a connection is established between the suspect and the crime scene or between the suspect and the victim based on the transfer of fragments of the materials. Once again, analytical rigor will play a relevant role in the preservation and experimental conduct of the traces. A failure in the analytical procedure may make it impossible to use a trace as material evidence in a court of law, jeopardizing its use in the conviction of the judge or jury. It is up to the forensic analysts to provide a result with credibility and legal security, i.e., to rigorously follow the analytical protocols.

Forensic research is dynamic. One example is the demand for analytical methods that encompass the wide variety of newly emerging psychoactive substances (NPS), formerly known as "designer drugs", which must continually be detected and catalogued. In the Interview in this volume, Dr. Barry Logan tells us about this challenge in his career. I want to register my special thanks to Prof. Dr. Bruno Martinis from the Department of Chemistry at the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo (DQ-FFCLRP-USP), who mediated and made possible the interview for BrJAC of one of the most prominent scientists in the area.

Cite: da Veiga, M. A. M. S. Forensic Analytical Chemistry: Connecting Science and Justice. *Braz. J. Anal. Chem.,* 2022, *9* (34), pp 1-2. doi: http://dx.doi.org/10.30744/brjac.2179-3425.editorial.mamsveiga.N34

In his Point of View, Federal Criminal Expert Marcus Andrade tells us about the complexity of forensic chemical analysis of works of art. The analysis of the surface of a painting requires, besides the historical study of the piece, the physical-chemical characterization of its components through an interesting combination of non-destructive analytical techniques such as microscopy, X-ray fluorescence, infrared spectroscopy, among others, which are powerful tools in the process of authenticating a work of art. Nevertheless, the art market, due to the high added value, has attracted organized crime in money laundering in cases of active and passive corruption.

Prof. Dr. Jesus Antônio Velho, Federal Criminal Expert and lecturer at the DQ-FFCLRP-USP, addresses the new trends in analytical chemistry for the examination and interpretation of traces of crimes: the determination of the origin of seized drugs (chemical profile), the investigation of document fraud, and the analysis of the evaluation of works of art. Professor Jesus' letter is the result of his long experience in the field and his exceptional insight into the forensic sciences. The author of several books in the field, his text is a gift to us all.

We hope readers will enjoy this special edition of BrJAC. Forensic professionals, students, professors, and researchers seek to contribute to criminal justice more broadly through tireless dedication. When we apply forensic analytical chemistry to a crime scene, more than an expert report, we give voice to the victims, who, through their traces, reveal to us the truth of the facts.

Márcia Andreia Mesquita Silva da Veiga is currently an Associate Professor in the Department of Chemistry at the Faculty of Philosophy, Sciences, and Letters of Ribeirão Preto, University of São Paulo, Brazil. She has a degree in Chemistry (Federal University of Amazonas, 1991), a master's in physical chemistry, and a doctoral in Analytical Chemistry (Federal University of Santa Catarina, 1996 and 2000), and post-Doc in Analytical Chemistry at the Institute of Chemistry, University of São Paulo (2005). Leads the research group L.Q.A.I.A. (Laboratory of Applied Instrumentation and Analytical Chemistry), and nowadays, is coordinator of the professional master's degree in Chemistry (PROFQUI) at the University of São Paulo. She works mainly with optical techniques for trace and isotopic analysis. Her current research focus is on sample preparation procedures, detection and quantification of nanomaterials and their application, bioaccessibility assays in foods and soils, the potential of high-resolution graphite furnace molecular absorption spectrometry for elemental and isotopic analysis, detection and quantification of gunshot residues, and new technological approaches to chemistry teaching. CV

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 3-9
doi: 10.30744/brjac.2179-3425.interview.blogan

BrJAC

INTERVIEW

Barry Logan, a prominent toxicologist and forensic analytical chemist, kindly spoke with BrJAC about his research into drugs of abuse, the legacy and inspiration for future generations

Barry Logan PhD, F-ABFT, Chief Scientist NMS Labs, and Executive Director at the Center for Forensic Science Research and Education (CFSRE)
Adjunct Professor at Thomas Jefferson University, Forensic Toxicology Program

Dr. Barry Logan is a world leading forensic toxicologist currently serving as Chief Scientist at NMS Labs, and Executive Director at the Center for Forensic Science Research and Education (CSFRE) in Willow Grove, Pennsylvania. He was born and completed his undergraduate and graduate education in Glasgow, Scotland, completed a postdoctoral fellowship at the University of Tennessee in Memphis TN, then served for eighteen years as State Toxicologist for the State of Washington, with an appointment at the University of Washington in Seattle. For nine of those years he also served as Director of the Washington State Crime Laboratory System, which provided services in forensic biology, toxicology, chemistry, document examination, serology, DNA analysis, firearms and crime scene support. In 2008, Logan joined the United States leading forensic toxicology and chemistry reference laboratory – NMS labs – in Pennsylvania to direct their toxicology services. In 2010 he founded the CFSRE and in 2017, established www.NPSDiscovery.org the leading clearing-house for the dissemination of newly emergent drugs in the United States.

He has over 150 publications and 600 presentations in forensic toxicology and analytical chemistry, including work on the effects of methamphetamine, cocaine and marijuana on drivers, and drug caused and related death. His recent work has focused on the analytical and interpretive toxicology of novel psychoactive substances (NPS). Dr Logan's other appointments include Executive Director of the Robert F. Borkenstein course at Indiana University, and academic appointments at Arcadia University, and Thomas Jefferson University in Philadelphia. In recognition of his work and contributions, Dr. Logan has received numerous national and international awards, and in 2013-14 served as President of the American Academy of Forensic Sciences (AAFS). A recent bibliometric analysis of the impact of the world's forensic scientists, positioned him as the leading contributor to research in the field of forensic toxicology in the United States, and sixth in the world.

In the last ten years Dr. Logan has had extensive involvement with forensic scientists in Brazil, hosting graduate students from the Federal University of Rio Grande do Sul, University of São Paulo, and University of Campinas at his laboratory in the United States, and visiting scientists from

Cite: Logan, B. Barry Logan, a prominent toxicologist and forensic analytical chemist, kindly spoke with BrJAC about his research into drugs of abuse, the legacy and inspiration for future generations. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 3-9. doi: https://dx.doi.org/10.30744/brjac.2179-3425.interview.blogan

the Federal Police and State Crime Laboratories. He has presented multiple times at Interforensics, ENQFor, and Brazilian Academy of Forensic Sciences meetings. The CSFRE supports participation of young scientists from Brazil in the AAFS meeting and a reciprocal opportunities for young US scientists to attend Interforensics.

Which factors influenced your education? When did you decide to study chemistry? What motivated you? How was the beginning of your career?

I grew up in western Scotland outside Glasgow in the 1960's and 70's and remember being very excited about my first chemistry classes in secondary school, and the lab aspects of it. The idea of learning by doing experiments, discovering things, putting individual results together to answer bigger questions was very appealing to me. That's eventually what led me to an interest in forensic science. I enjoyed chemistry much more than my other science classes, physics was too mathematical, and biology was lacked the finality of a conclusion. I especially liked the idea of using chemistry to solve problems, and figure things out and remember reading an article in a school science magazine, called "Detectives in White Coats" about forensic science. I cut it out and took it to my career teacher who told me I'd have to go to medical school to get into that field. Undeterred, I read whatever I could about forensic science, not so easy before the internet, and eventually discovered a neighbor, Keith Eynon, who worked for the Strathclyde Police Forensic Laboratory. He told me chemistry was the way to go, so I applied to chemistry degree programs and attended Glasgow University, earning a bachelor's degree in chemistry. I really enjoyed my chemistry courses, both the analytical aspects and the theoretical. I enjoyed quantum chemistry, group theory, and chemical physics too, but the math was too off-putting for me. My interest in analytical chemistry was reaffirmed by the organic chemistry labs where we had to characterize the products we synthesized based on color reaction, UV and NMR data. One of my professors, Charles Brooks, established the first Gas Chromatography-Mass Spectrometry (GCMS) Unit in a UK University with a grant from the Science Research Council, and one of his graduate students, Bob Anderson, was hired to implement the technique at the Department of Forensic Medicine and Science at Glasgow University.

The idea of learning by doing experiments, discovering things, putting individual results together to answer bigger questions was very appealing to me.

After graduating with my chemistry degree, I interviewed in the forensic medicine department for a PhD scholarship. It was a terrible interview. I was so very nervous that I sweated profusely through the whole interview. At one point the interviewer handed me a box of tissues to dry my face. In spite of that performance I was offered the position and spent the next four years working on my PhD. My first forensic toxicology assignment was to collect urine from the greyhounds racing at Shawfield Stadium and test it for doping drugs. I followed the dogs around with a stainless-steel bowl until they were ready to provide a sample. We used thin layer chromatography for alkaloids, UV-visible spectrophotometry for barbiturates, and gas chromatography with nitrogen phosphorus detection for other drugs. GCMS was not used for routine casework at the time it was too cumbersome, subject to breaking down, too expensive, and computers were only just being interfaced with analytical instruments. My PhD program introduced me to postmortem toxicology also for the Procurator Fiscals office, and I went to court to see my boss John Oliver testify in a drunk driving case. That experience confirmed for me that I wanted to be a forensic toxicologist. I headed off to the United States for a postdoctoral position at the University of Tennessee in Memphis, under David Stafford, a former graduate student of Harold McNair an early pioneer of GC in forensic toxicology in the United States.

What has changed in the student profile, ambitions, and performance since the beginning of your career?

Academia has changed markedly since I went to University, so I see many differences. Universities are a business now, due to less support from governments. It seems to me that being a student today is less fun than when I was at school. Getting an education is a rite of passage, and a requirement for getting a good job, as opposed to learning for the love of learning. After my first year, almost every class I took was a chemistry class and they were illuminating – even areas unrelated to where I made my career, like quantum chemistry and group theory. I enjoyed every minute of my undergraduate degree, and the friendships and connections I made; I hope today's students still take the time to enjoy their time. As students, it was much more up to us to keep up and meet our commitments. I think we respected (and feared) our teachers a lot more than students do today. All our professors were researchers first, and teachers second, and their academic success was based more on their accomplishments as a researcher than as a teacher. Today, there is much more emphasis on student success, and academicians are recognized and rewarded for their teaching activities which is much better for the students. Universities are run a lot more like businesses today which is both good and bad. Students often feel that they are customers of the institution, and that the institution owes them an education. I always talk about earning my degree from Glasgow University, not just receiving my degree. There is more accountability today for how both teachers and students spend their time, but less time for pursuing more esoteric, risky and exploratory avenues.

What are your lines of research? What work are you currently developing?

My career took me from my PhD program to a postdoctoral program which was a great track. A postdoc gives you the opportunity to take the lessons you learned from your doctoral program from how to be a scientist, how to perform someone else's research, find what topics interest and fascinate you, explore your own ideas, make your own mistakes, critically evaluate your own theories and perspectives, to forming your own philosophy of your science. My experience as a postdoc gave me a much greater love for research than I got from my PhD program, and a lot more confidence in myself. After my postdoc, I took a path within forensic toxicology that was more service oriented and included the application of science to answering questions in court and with medical professionals about cause and manner of death, addiction, poisoning, overdose, homicide, and impairment. I spent ten years working as the State Toxicologist for the State of Washington in Seattle in the US pacific northwest. The focus at this time was documenting the effects of various drugs on the appearance and behavior of suspected drug impaired drivers, examining, the behavioral observations, driving effects and toxicological findings. I produced over 30 papers on drug driving related findings during this time. Even in that service role, which was extremely rewarding intellectually, and from a community service point of view, I recognized the value of more applied research in learning from real world cases and applying that learning to the resolution of future cases. I started publishing case series and looking for patterns and trends in drug related deaths and impairment, especially in drug-impaired driving. Generating reference data for the interpretation of drug related death is an incredibly important resource for practicing forensic toxicologists and is easily achievable by young scientists in the course of their routine casework. The application of analytical tools and strategies to forensic casework is a unique opportunity for scientists working in this more applied field to contribute and grow. I continue my work on the role of drugs and alcohol in impaired driving, and have recently focused on the creation of guidelines for more consistent best practices by forensic toxicology laboratories, to ensure better data on this significant societal problem, leading to better policies and strategies in reducing alcohol and drug impaired driving.

My experience as a postdoc gave me a much greater love for research than I got from my PhD program, and a lot more confidence in myself.

My current focus on emerging novel psychoactive substances (NPS) started in 2010 with the advent of the research chemical or designer drug movement. I remember attending a conference in Glasgow in 2009 and sitting next to someone at the conference dinner who I hadn't met before who told me about "Spice"- a

new drug showing up in drug seizures. This was very interesting as besides the traditional drugs of abuse, and a slow but steady pipeline of new therapeutics, there was relatively little turnover in new drugs in forensic casework. “Spice” turned out to be the first of the synthetic cannabinoids, JHW-108 and HU-210, drugs that had been pirated from the drug development research literature and synthesized for distribution on the street as “Legal Highs”, novel substances for which there were at the time no controls, allowing them to be widely sold and distributed openly at gas stations, and convenience stores as novelty products. By this time, I was working at NMS Labs, a commercial independent reference laboratory in Philadelphia PA. In the private sector innovation is especially important in order to provide a service that adds to the resources available to the country’s public sector laboratories. We began developing and offering tests for these new “designer drugs”, which later were renamed as novel psychoactive substances (NPS). This included the “Bath Salts” novel stimulants and hallucinogens in the amphetamine, methylenedioxyamphetamine, and pyrovalerone classes, many more synthetic cannabinoids, novel opioids, including many deadly fentanyl analogs, and novel illicit benzodiazepines.

In addition to my work as Chief Scientist at NMS Labs, I was also invited by the company’s owners, Eric and Michael Rieders, to lead their non-profit Foundation, the Fredric Riders Family Foundation and its Center for Forensic Science Research and Education (CFSRE), after their father Fredric Rieders Sr. At the Foundation, I created the program NPS Discovery (www.npsdiscovery.org), an initiative designed to conduct focused research on the identification, surveillance, and monitoring of new substances, and provide early identification and notification of their penetration in the United States Drug Supply. Once identified, the program characterizes and studies to toxicology, chemistry and epidemiology of the drugs, including the preparation of public health alerts, trend reports, new drug monographs, and publications on the analysis, quantitation, metabolomics, and receptor binding and functional activity of these new drugs. This enables the program to provide an informed early warning system to public health and safety agencies in the United States to promote data collection, analysis, harm reduction, interdiction, supply reduction, and scheduling to better monitor and control NPS. NPS Discovery has identified over 75 new substances in the United States in the last three years. This work continues apace, and it has been a great opportunity for our energetic young scientists, including several from Brazil, to contribute to this knowledge.

For you, what have been the most important achievements in the analytical research field recently? Could you briefly comment on recent advances and challenges in analytical chemistry considering your contributions?

In the 1990’s and 2000’s forensic toxicology became very routine. The focus turned appropriately to improving quality, validation, and reliability of the results produced in forensic laboratories. Instrumental platforms had the levels of sensitivity required for detection of most drugs at toxic and therapeutic concentrations, a few new substances were launched each year from the pharmaceutical pipelines, but at a rate where it was easy to keep up. Attention turned to method improvement, to accreditation, better documentation of methods and procedures, and more validation of methods. More laboratories also began determining measurement uncertainty for assays that had critical cut-off points, such as drug impaired driving cases in states where there were legally adopted concentration limits.

Towards the end of the 2000’s more laboratories began to have access to LCMSMS, whereas before, GCMS was the most common technique. LCMSMS allowed for more rapid sample preparation and eliminated the need for chemical derivatization of many polar compounds and metabolites which had been required for GCMS. LCMSMS is best suited to confirmatory quantitative procedures, and GCMS remained the choice for screening, until the increase in availability of economical high-resolution mass spectrometry (HRMS) platforms in the 2010’s.

HRMS has been a revolutionary technique for drug screening and confirmation, as it allows the identification of unknowns based on measurement of the accurate mass of the parent compounds and their fragments, allowing both chemical formula determination and structural determination – especially important for the identification of novel emerging drugs and NPS. In addition, HRMS allows for the prospect

of retrospective data mining of non-targeted acquisition data sets to be able to look back in time for the presence of novel compounds discovered today, in archived data without having to retest those samples. This has greatly enhanced NPS Discovery's ability to do trend analysis of the life cycles of new drugs from the first case through their proliferation, plateau and decline, and the appearance of successor compounds.

There are several meetings of chemistry experts that take place around the world. What is the importance of these meetings to the development of the area?

The first of the five pillars of NPS Discovery is Intelligence gathering. This phase of an effective new drug early warning system is understanding the universe of compounds that may be at large in the world, and rapidly identifying those that are rising to the level of a public health threat. Key to this Intelligence phase is our communication with our colleagues around the world. The scientific staff of the CFSRE attend scientific meetings in the United States and internationally to network with colleagues, create new collaborators, identify funding resources and breaking down international barriers. During 2020 and the impacts of COVID-19, we learned the hard way how much opportunity we lose to make these new connections when we can't travel to meetings or meet face to face. We have found ways to make online meetings and virtual meetings work for keeping up, for checking status, for sharing information, but they are not well suited to getting to know new people, to build the personal relationships and trust relationships that come from talking at a break, going to dinner, or sitting in a bar after a long day of presentations.

Dr. Logan at the 57th Annual Meeting of TIAFT 2019 in Birmingham, UK.

Conferences in the field of forensic toxicology that I have found to be the most dynamic and fruitful are the multidisciplinary meetings like the American Academy of Forensic Sciences, and the American Society for Mass Spectrometry, these are great for the cross-pollination of ideas between fields or areas of analytical science. Subject matter specific meetings are also critical, because of their focus and the strong relevance of the content. My favorites are the international meetings, since its great to be able to travel to a new location, to broaden your horizons, but also to hear from people you wouldn't typically hear from in your own backyard. I enjoy attending The International Association of Forensic Toxicologists (TIAFT) meetings because of the large number of academic programs represented there, more so than in the United States. I have attended several conferences in China and Brazil, and although I speak no Chinese or Portuguese, have been amazed at the capabilities of Google translate to read posters and communicate with many more people than I would ever do if not there in person.

No scientific discipline flourishes when people work in isolation, and collaborations are key. Scientific professional groups and their conferences help facilitate that.

No scientific discipline flourishes when people work in isolation, and collaborations are key. Scientific professional groups and their conferences help facilitate that. I'd encourage all young scientists to find a sponsor, join an organization, volunteer, listen, participate. Never be afraid of asking a senior professor or department chair to support your application, without exception, its an easy thing to do, and may open more channels for communication with your sponsor.

Do you believe that the current graduate programs produce quality researchers in the field of analytical chemistry? Is there need for further integration?

Research is a challenging assignment and not every scientist is cut out for it. As part of an undergraduate or Master's degree program if you are lucky enough to have research exposure, enjoy it for the lessons learned about how difficult it is, and to develop an appreciation for those who choose it as a career. From my perspective, research teaches you about the scientific method, hypothesis testing, critical thinking, time and resource management, and appreciation for bias and uncertainty. All are valuable lessons for every scientist. The research experience may also benefit the academic institution, the student's ultimate employer and the profession, so should be viewed as a means to an end in higher education as much as it is an end in itself.

To be properly prepared for research, I believe that you need either the discipline of a doctoral program, with a number of years dedicated to your research project, or a strong mentor and peers who teach you essentials of the scientific method, the patience to get things right, the skills in critical analysis of your work, and the writing skills to communicate it to your peers. I think the latter path to research is actually more important than the former. I also highly respect scientists for whom research is a subsidiary endeavor to their more routine work. It speaks to their commitment to discovery, and their passion for their science, and always adds value to the field.

Research is a calling, and especially so in forensic toxicology. It is important that researchers have a deep appreciation for the field. In addition to the applied research that falls out of learnings from more routine experience in service work or casework, there is always a need for people who are inspired to follow big ideas, which sometimes succeed and sometimes fail, but that drive forward knowledge and discovery. To dedicate your professional life to that is a calling.

Dr. Logan and his long-time collaborator Fran Diamond.

For you what is the importance of the support of funding agencies for the scientific development of the country?

Research and exploration cost money. There is no way around that. Not every research project results in a discovery, or a product, or an application, but perhaps it presents an opportunity to avoid dead ends or fruitless approaches to solving forensic problems. Research funding is a luxury when countries or governments have other human rights and basic human needs to meet, at least in degree. However, nurturing your country's academies, seeding today's and tomorrow's scientists and researchers, keeping your scientific community up-to-date with the world's technologies and new discoveries is an investment in the future of humankind, and in the advancement of knowledge. Research funding should be consistently

a percentage of a country's gross domestic product, proportionate to what its economy can support, and viewed as an investment its future. Of course, countries need to prioritize where that money should be spent, but investment in scientific support for criminal justice, social justice, and civil society all depend on the resources and objective information that forensic sciences provide to the prosecutors, the defense and the courts, and must not be overlooked. At the CFSRE we seek out like-minded collaborators and invest our own resources in developing proof-of-concept studies to collect pilot data that can support robust and compelling grant applications. These give the funders confidence in our ability to execute and follow through on our proposed objectives.

What sort of a career could someone expect in the field of analytical chemistry could pursue? What advice would you give to a newcomer to this area?

I love the fact that the forensic sciences are both a career in their own right, but also an opportunity to attract young scientists into careers in science in general, and a gateway to other careers in pharmaceutical sciences, environmental sciences, law and medicine. Becoming a scientist is hard! It involves learning the language, concepts, principles, scientific method, technology, and the history of science. Analytical chemistry introduces young scientists to the fundamentals of chemistry, the nature of matter and materials, measurement science, identification. Analytical chemistry services so many other aspects of synthetic, industrial, and forensic disciplines, and should be a part of all undergraduate chemistry curricula.

How would you like to be remembered?

Very few scientists are lucky enough to discover or invent something that has a multi-generational impact, so I hope I have a pragmatic expectation about my legacy. I think that as scientists the opportunity we have to influence beyond what we accomplish in our own work, is to inspire the people closest to us in our agencies, institutions and professional organizations – our students, our young scientists, and our peers. I hope a few of my presentations or publications, or projects I started have inspired some reflection and inspiration to the current and upcoming generations of people working in my field, but mostly I would like to think that there is an echo of my perspective that shows up in the work of my graduate students, or mentees, and that they in turn can accomplish more than I could in the time allotted to me.

Dr. Logan with a group of high school students and mentors in the summer science program at the Center for Forensic Science Research and Education – CFSRE.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 10-12
doi: 10.30744/brjac.2179-3425.point-of-view-mvoandrade

BrJAC

POINT OF VIEW

Forensic Analysis of Artworks
More than a (Complex) Analytical Issue

Marcus Vinicius de Oliveira Andrade

Forensic Expert at Brazilian Federal Police, Belo Horizonte, MG, Brazil

In 2008, Nicolas Eastaugh, founder and chief researcher at Art Discovery, a renowned London company for the analysis and research of artwork, discovered the presence of the white titanium (titanium dioxide) pigment in a painting attributed to the Dutch naturalized expressionist artist Heinrich Capendonk. The work had reached a record value of EUR 2.4 million at an auction in 2006. However, in 1915, the year in which the work was supposed to have been created, white titanium was not even available for use as a pigment, which would happen about 20 to 30 years later. The analytical result achieved by Eastaught revealed one of the biggest schemes of artwork forgeries ever discovered. The forger, Wolfgang Beltracchi, made a fortune, (under)estimated at EUR 30 million, built over 25 years acting in the art market. There are several cases of counterfeiting schemes involving artwork, large fortunes, renowned galleries, museums, collectors, specialists, and masterpieces. Cases like the one revealed by Eastaugh's analyses or the millionaire counterfeit scheme involving the century-old North American Knoedler Gallery [1] are illustrative examples of how the art market is vulnerable to this kind of crime.

The International Monetary Fund (IMF) and the United Nations Office on Drugs and Crime (UNODC) estimated the total annual trade in art and antiques in 2018 at around USD 70 billion, of which about USD 6 billion may have been due to illegal transactions related to theft, counterfeiting, smuggling, and organized crime. Still according to those institutions, half of that amount involved financial crimes and money laundering [2]. In Brazil, within the scope of the *Lava Jato* Operation, the Federal Police seized 842 pieces of art and historical and cultural heritage, including paintings from different historical periods, sculptures, and other pieces, which add up to an estimated value of over BRL 33 million [3]. All the pieces were related to investigations involving money laundering in cases of active and passive corruption.

As other forms of money laundering resulting from various crimes have been curtailed by world authorities through specific legislation, the art market world has become increasingly attractive to crime. This scenario, combined with the great financial relevance of the legitimate art market, caused a very considerable increase in the demand for works by renowned authors and, as a direct consequence, a proportional increase in the number of forgeries and adulterations. As a result, the quality of counterfeits has also experienced a great improvement, requiring a proportional gain in technology and expertise in forensic analysis and authentication fields [4]. Similarly, the high speculation in prices of artworks also increased the interest in new and advanced analytical techniques for determining authenticity, authorship, origin, and materials used by the authors [5].

The refinement of counterfeiting and adulteration techniques has demanded a multidisciplinary and technological approach to the authentication process, and, at this point, we are faced with a considerable degree of complexity in the already difficult process of authenticating works of art. The authorship or authenticity determination of a painting is unavoidably based on a triangle formed by three disciplines: art history, preservation sciences, and materials sciences [1,6]. The voices of our benches and equipment are unlikely to be able, on their own, to unequivocally conclude the authenticity of a work of art. Likewise, the

Cite: Andrade, M. V. O. Forensic Analysis of Artworks: More than a (Complex) Analytical Issue. *Braz. J. Anal. Chem.,* 2022, *9* (34), pp 10-12. doi: http://dx.doi.org/10.30744/brjac.2179-3425.point-of-view-mvoandrade

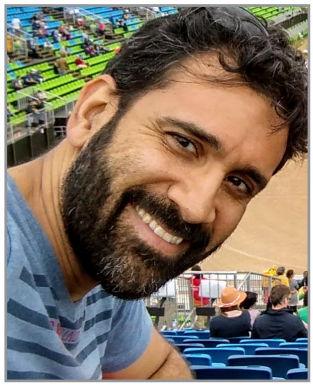
most trained eyes of a professional connoisseur are no longer able to face the most astute counterfeiters. The best results of authenticity studies will always be achieved when these three distinct disciplines come together and complement each other in the search for comprehensive, technical, and artistic knowledge about the work. In addition to the historical study of the piece, the physical–chemical characterization of materials and components or elementary and multispectral imaging become powerful tools for fraud detection and even characterization and individualization of the authentic piece [6].

The simplest techniques, generally used for initial documentation, to the more complex, analytical resources are used to extract the greatest amount of information from the different parts that make up a painting. In its diverse and complex layers, from the support to the final coating, paintings are composed of multilayers of heterogeneous mixtures of varied organic and inorganic compounds. A thorough investigation of this scenario always requires the use of advanced and combined techniques to better understand each case, depending on their nature [7]. The analysis of the painting surface by a stereo-microscope will reveal genuine – or artificially produced – craquelure or brushwork patterns compatible with the artistic style proposed by the author of the work. Likewise, the UV fluorescence properties of the painting may differentiate between old and new additions of paint to the piece. However, it is in the deepest layers of an artwork that the most sophisticated analytical techniques contribute most incisively. X-ray radiography and infrared reflectography, coupled with ultra-sensitive charge-coupled devices (CCD), began to detect underdrawings that were invisible to the previously available methods. Several works have demonstrated the usefulness of tools such as synchrotron radiation, microimaging by X-ray fluorescence (XRF), and Raman or Fourier-transform infrared (FTIR) spectroscopies, combined with the versatility of portable spectrometric identification techniques, where pigment particles less than 1 micrometer in diameter can be analyzed. Even the power of pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) to identify novel synthetic organic polymers, which greatly assists in the analyses of pigments, coatings, binders, and other painting components, can, in many cases, reveal anachronisms present in the counterfeits. Other techniques such as laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), isotope-ratio mass spectrometry (IRMS), and accelerator mass spectrometry (AMS) allow for the determination of isotopic ratios of heavy and light elements, which are decisive to determining the geographical origin of some pigments and the age of a painting canvas or its wooden frame. Such techniques used in association with multivariate analysis tools, artificial intelligence, and machine learning help achieve increasingly conclusive results, requiring less time and effort from the entire team of researchers [1]. Each of the analytical techniques, evaluated for their versatility, resolution power, type of information generated, portability, and employment, have the potential for greater use in combined and multimodal work. This approach makes the description of the artwork much more precise and richer in details since it individualizes not only its components, support, and coating materials, but also the context in which they were used. The wisdom in better bringing together the analytical resources available and performing the analyses required by each case determines the success of a forensic examination of a forgery or the authenticity of a work of art [8].

It is not hard to see that it is unlikely that a single company or laboratory, whether public, private, academic, or not, will own all the technological resources to exhaust such an analysis. Furthermore, it is not uncommon for crimes involving works of art to be transnational. All this leads us to the last step of our range of complexity: laboratories, museums, forensic, and research institutes involved in examining the authenticity of artworks should operate on secure and integrated networks, generating data that is widely shared between partner institutions. Several institutes in the world already work in this way, such as INTERPOL, the INTERNATIONAL COUNCIL OF MUSEUMS (ICOM) [9], the Federal Bureau of Investigation (FBI), UNODC, and the Integrated Platform for the European Research Infrastructure on Cultural Heritage (Iperion CH). This has proven to be one of the most effective ways to curb this type of crime, which, sneakily, erodes our history and cultural heritage [10].

REFERENCES

1. Ragai, J. *The Scientist and The Forger – Probing a turbulent art world*, 2nd Ed., Vol. 1. World Scientific Publishing Europe Ltd, New Jersey, **2018**.
2. Mashberg, T. "The Art of Money Laundering: The loosely regulated art market is rife with opportunities for washing illicit cash" *International Monetary Fund e-Library*, **2019**. Available at: https://www.elibrary.imf.org/view/journals/022/0056/003/article-A009-en.xml [Accessed April 12, 2021].
3. Polícia Federal, Ministério da Justiça e Segurança Pública do Brasil. "Operação Lava Jato." Available at: www.pf.gov.br/imprensa/ [Accessed April 12, 2021].
4. Hwang, S.; Song, H.; Cho, S. W.; Kim, C. E.; Kim, C. S.; Kim, K. *PLoS ONE*, **2017**, *12* (2) (http://dx.doi.org/10.1371/journal.pone.0171354).
5. López-Ramírez, M. R.; Navas, N.; Rodríguez-Simón, L. R.; Otero, J. C.; Manzano, E. *Anal. Methods*, **2015**, *7* (4), pp 1499–1508 (http://dx.doi.org/10.1039/c4ay02365j).
6. Schossler, P.; de Figueiredo Júnior, J. C. D. A.; Fortes, I.; Cruz Souza, L. A. *Sci. Justice*, **2014**, *54* (6), pp 465–469 (http://dx.doi.org/10.1016/j.scijus.2014.06.013).
7. Rodriguéz-Simón, L. R.; Navas, N.; Manzano, E. *Is the painting authentic? A multi-method approach to investigate the provenance and the authenticity of two 20th century canvas paintings with the signature of 'Picasso'*. In: International Conference of Education, Research and Innovation Proceedings (ICERI), Nov. **2009**, pp 3782–3793.
8. Radvan, R.; Ratoiu, L.; Cortea, I. M.; Chelmus, A.; Angheluta, L.; Marinescu, D. *Multi-step Approach for Characterization of Artworks Based on Hyperspectral Imaging and Complementary Techniques*. 11th International Conference on Developments in eSystems Engineering (DeSE), **2018**, pp 117-122 (http://dx.doi.org/10.1109/DeSE.2018.00025).
9. International Council of Museums, "Object ID - ICOM." Available at: https://icom.museum/en/resources/standards-guidelines/objectid/ [Accessed Feb. 10, 2021].
10. United Nations Office on Drugs and Crime. *Acting together against destruction and trafficking of cultural property by terrorist and organized crime groups protecting cultural heritage an imperative for humanity*, **2016**. Available at: https://www.unodc.org/documents/publications/SRIUN_Protecting_Cultural_Heritage_2016.09.12_LR.pdf [Accessed April 18, 2021].



Marcus V. O. Andrade is a senior forensic expert and holds his M.Sc. (2003) in pharmaceutical sciences from the Federal University of Minas Gerais. Since 2003, he has worked for the Brazilian Federal Police, where he has performed in several areas, such as forensic chemistry, bombs and explosives, crime scene investigations, counterfeiting artworks, and environmental crimes. In the last few years, he coordinated forensic responses to complex crimes, such as the dam collapse in Mariana (2015) and Brumadinho (2019). His work in forensic sciences includes contributing to the development of important partnerships between the Federal Police and Universities, such as the Network of Forensic Sciences of Minas Gerais, the National Network of Forensic Isotopes, and the Integrated Network of Forensic Laboratories in Works of Art and Historical and Cultural Heritage. He is also a member of the board of directors of the Brazilian Academy of Forensic Sciences.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 13-14
doi: 10.30744/brjac.2179-3425.letter-javelho

BrJAC

LETTER

New Trends in Analytical Chemistry for the Examination and Interpretation of Traces of Crimes

Jesus Antonio Velho[1,2]

[1]*Departamento de Química da Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo. Av. Bandeirantes, 3900, Ribeirão Preto, SP, 14040-900, Brazil*
[2]*Instituto Nacional de Criminalística, Polícia Federal, SAIS Quadra 07 Lote 23, 70610-200, Brasília, DF, Brazil*

Forensic sciences are generally described as the application of the scientific method to the analysis of traces in order to identify the authorship and materiality of a crime (Figure 1). Forensic scientists evaluate different types of materials, and the type of scientific method and techniques employed depend on the questions to be answered within a given context [1,2].

Forensic chemistry is one of the most far-reaching areas within the forensic science field. With the increase in technology and the development of analytical techniques, chemistry has been used more and more to elucidate legal controversies. Therefore, knowledge in chemistry is indispensable to solve crimes [3]. In this letter, the applications of analytical chemistry will be discussed within emerging forensic themes: the determination of the origin of seized drugs (chemical profiling), the investigation of document fraud, and the valuation analysis of pieces of art.

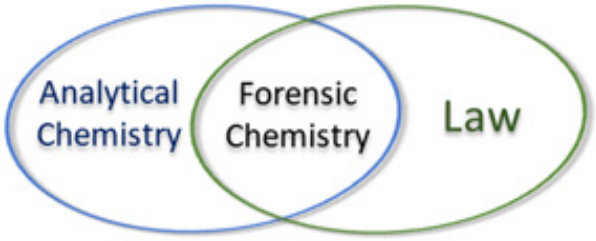


Figure 1. Techniques in analytical chemistry are used for the resolution of legal matters through the forensic sciences.

Chemical profiling consists of a series of chemical analyses that provide the concentration of the components present in the seized drugs, present as major or minor components, or event those present only in trace levels [4]. Using different analytical methods, complex chemical profiles are obtained for each drug sample analyzed, giving these samples a chemical “signature” based on the presence of impurities of natural origin and added diluents/adulterants. Therefore, such studies generate relevant data that make it possible to establish connections between samples and materials of different seizures, classifying them into chemically correlated groups. Through these connections, it is possible to establish specific links among suppliers, drug dealers, and users, designing distribution network patterns and providing subsidies for the identification of the origin of the drug, including its geographical origin [5].

Another striking forensic application of analytical chemistry is age determination and the authenticity of papers and inks. Once an ink is deposited on a support (paper), it is exposed to air, light, and moisture, and the following physical–chemical processes occur: coloration degradation, solvent evaporation,

Cite: Velho, J. A. New trends in analytical chemistry for the examination and interpretation of traces of crimes. *Braz. J. Anal. Chem.,* 2022, *9* (34), pp 13-14. doi: http://dx.doi.org/10.30744/brjac.2179-3425.letter-javelho

and hardening (polymerization) of the resins. These processes have been used in the complex task of determining the absolute or relative (comparative) age of manuscripts on paper. The largest number of publications refer to ballpoint pen inks. Ezcurra and collaborators [6] published a comprehensive review on the dating of paints by modern instrument writers. Analytical paint dating exams essentially consist of quantifying how paint components vary over time [7].

Last but not least, the authentication of pieces of art used as a tool to fight crime is a recent area of activity of analytical chemistry in Brazil. Operation “Lava Jato” shed light on the possibility that criminal use of the art market is a widespread method among agents of corruption and that it is much more complex and structured than previously thought. It is up to criminal experts to determine the authenticity of a piece of art. In general, the analytical investigations are guided by the identification and quantification of substances used in the production of the art piece, using non-destructive methodologies, such as Raman spectroscopy [8].

Acknowledgments

I dedicate this work to the victims of crimes in Brazil. I would like to thank Dr. Eduardo Geraldo Campos for reviewing this letter.

REFERENCES

1. Cockbain, E.; Laycock G. Crime Science. In: *Oxford Research Encyclopedia of Criminology and Criminal Justice*. Oxford University Press, **2017** (https://doi.org/10.1093/acrefore/9780190264079.013.4).
2. Velho, J. A.; Costa, K. A; Damasceno, C. T. M. *Locais de Crime*. Editora Millennium, Campinas, **2019**.
3. Bruni, A. T.; Velho, J. A.; Oliveira, M. F. *Fundamentos de Química Forense*. Editora Millennium, Campinas, **2019**.
4. United Nations Office on Drugs and Crime. Drug Characterization / Impurity Profiling, Background and Concepts, United Nations, New York, **2001**.
5. United Nations Office on Drugs and Crime. Methods for Impurity Profiling of Heroin and Cocaine, Laboratory and Scientific Section, United Nations, New York, **2005**.
6. Ezcurra, M; Gongora, J. M. G.; Maguregui, I.; Alonso, R. *Forensic Sci. Int.*, **2010**, *197* (1-3) pp 1-20 (https://doi.org/10.1016/j.forsciint.2009.11.013).
7. Jones, R. W.; Cody, R. B.; McClelland, J. F. *J. Forensic. Sci.*, **2006**, *51* (4), pp 915-918 (https://doi.org/10.1111/j.1556-4029.2006.00162.x).
8. de Faria, D. L. A.; Massabni, A. C. *A espectroscopia Raman revelando a química das obras de arte*. Conselho Regional de Química da 4ª Região (CRQ IV), Seção Especial da Química Viva, *2019*, pp 1-5, São Paulo. Available at: https://www.crq4.org.br/default.php?p=texto.php&c=quimicaviva_quimica_e_arte_espec_raman

Jesus Antonio Velho has a PhD in pathophysiology from the Faculty of Medicine of the State University of Campinas (UNICAMP) and studied Pharmacy-biochemistry at the State University of Londrina (UEL). He is a Federal Criminal Expert, working in the areas of Crime Scene Analysis, Forensic Chemistry, and Forensic Ballistics. In relation to teaching activities, he is a doctoral professor in the area of criminalistics at the University of São Paulo (USP Ribeirão Preto) and a guest professor at the Federal Police Academy (ANP). He is Director of the Board of the Brazilian Society of Forensic Sciences, 2019/2021. He is a member of the Editorial Board and organizing author of the “Criminalística Premium” series of books by Editora Millennium, in addition to acting as editor of RBC (Revista Brasileira de Criminalística) and RBCP (Revista Brasileira de Ciências Policais).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 15-34
doi: 10.30744/brjac.2179-3425.RV-19-2021

BrJAC

REVIEW

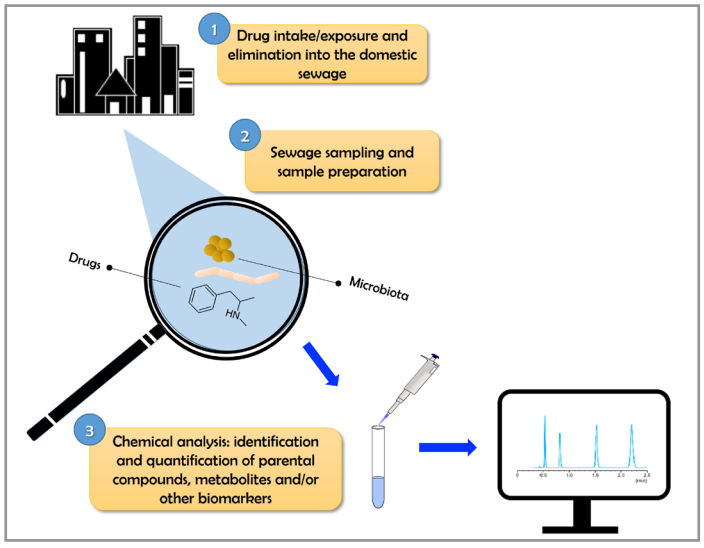
Forensic Analysis of Illicit Drugs and Novel Psychoactive Substances in Wastewater

A review of toxicological, chemical and microbiological aspects

Eduardo Geraldo de Campos[1], Elaine Cristina Pereira De Martinis[1], Bruno Spinosa De Martinis[2]*

[1]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, Ribeirão Preto, 14040-903, São Paulo, Brazil

[2]Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, Ribeirão Preto, 14040-901, São Paulo, Brazil



Wastewater-based epidemiology has emerged as a new analytical strategy for monitoring licit and illicit drug use in a population by measuring the levels of biomarkers in wastewater. The main concept of this approach is that chemical substances ingested by the population will be excreted in urine and feces, which will be discarded into the sewage network and may accumulate at the wastewater treatment plant. Several licit and illicit substances such as ethanol, nicotine, cocaine, amphetamine, methamphetamine and morphine have been investigated and reported in wastewater in worldwide. In recent years, this approach has also been explored for environmental monitoring of novel psychoactive substances (NPS) as well, since analyses of wastewater represent a fast and cost-effective way to evaluate collectively drug intake in a given population served by a sewage network. In this paper, a comprehensive and interdisciplinary review of the forensic, toxicological, chemical and microbiological aspects of the analysis of "traditional" drugs of abuse and NPS in wastewater and examples of applications reported in recently published papers is provided. Wastewater analysis is a very promising strategy in monitoring drug use in the context of Forensic Chemistry and Toxicology, and has been implemented by many researchers in the analysis of drugs of abuse, as supported by many recent literature reports.

Keywords: wastewater-based epidemiology, illicit drugs, novel psychoactive substances, forensic toxicology, forensic chemistry

Cite: Campos, E. G.; De Martinis, E. C. P.; De Martinis, B. S. Forensic Analysis of Illicit Drugs and Novel Psychoactive Substances in Wastewater: A review of toxicological, chemical and microbiological aspects. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 15-34. doi: http://dx.doi.org/10.30744/brjac.2179-3425.RV-19-2021

Submitted 04 February 2021, Resubmitted 12 June 2021, Accepted 15 June 2021, Available online 09 August 2021.

INTRODUCTION

According to the United Nations Office on Drugs and Crime (UNODC) (2020), drug use has increased, from estimated 210 million users in 2009 to 269 million users in 2018 [1]. The illicit drug market is also becoming more complex [1], with the emergence of novel psychoactive substances (NPS). UNODC defines NPS as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat" [2]. NPS are new drugs of abuse that are usually produced to avoid legislation and scheduling controls [3], which can lead to the emergence of new, "legal" synthetic drugs, if they are not prohibited or scheduled yet. Many classes of NPS have been reported, such as novel stimulants, designer opioids, designer benzodiazepines, synthetic cannabinoids and novel hallucinogens [3]. Until December 2019, more than 950 NPS were reported to UNODC [2]. Since many of these drugs are unknown, information regarding their mechanisms of action, effects, metabolism and poorer. This is a significant challenge for forensic chemists and toxicologists, physicians, coroners, law enforcement agents and several other professionals involved in this field, as these new synthetic drugs represent a great threat in public health and safety. Therefore, several strategies are required to endure the NPS problem.

The analysis of drug residues in wastewater is an innovative analytical and epidemiological approach used in the estimation of drug use proposed in 2001 [4], explored for the first time in 2005 [5], which has been utilized in several other studies since then [6]. This approach called wastewater-based epidemiology (WBE) can provide useful public health information by measuring human biomarkers of drug use excreted in urine [7]. Any chemical substance consumed by humans may be excreted into urine and feces in the unmodified form and/or as metabolite(s) and eliminated into a particular sewage network or directly into surface waters [4,8]. Drugs and/or metabolites accumulated at the wastewater treatment plant (WWTP) during certain period should represent the compounds excreted by a given population into the sewage network that reached the WWTP in the same period, if these substances are stable in wastewater and efficiently transported through sewage networks [9,10]. Considering drug's pharmacokinetics and environmental fate, these amounts of drugs and/or their major metabolites can be used for estimating drug intake by a given population [5]. Therefore, the analysis of drugs in wastewater is a valuable analytical strategy that can aid in the assessment of drugs consumed by a community covered by a particular sewage network [8–10], providing anonymous population-normalized data [4,11], in a non-invasive [4,5] and timely approach [12].

DRUG TESTING IN WASTEWATER AND FORENSIC APPLICATIONS

The analysis of illicit drugs in wastewater can be an alternative/complementary approach to monitoring drug use in a given population in Forensic Chemistry and Toxicology [13]. For example, combining the analysis of wastewater with chemical profiling of seized materials can be a valuable strategy to expand the knowledge regarding the illicit drug use and market [14]. These studies can be used for the direct analysis of wastewater to monitor variations of drug use due to special events [8], which has been evidenced by some studies during music festivals [15], holidays [16], sports competitions [14] and, more recently, the pandemic of COVID-19 [17]. For example, WBE has been explored in the study of spatial and temporal trends of alcohol (ethanol), tobacco and illicit drugs use [7]. The investigation of clandestine laboratories may also be supported by the analysis of chemical waste in the sewage, such as the specific chemical profile of wastewater due to the disposal of waste from illicit production of stimulants [18]. Analysis of chemical markers in wastewater provide association to specific synthetic routes of amphetamine [18]. The advantages of wastewater analysis over other epidemiological approaches include more objective estimations, reduced costs [8], guarantee of the anonymity and privacy of people [4], and almost real-time assessment of drug use [14], with no need to collect biological specimens from individuals. However, wastewater analysis is associated to some uncertainties that should always be considered. The analysis in wastewater itself cannot inform data on drug use pattern and prevalence and purity of drugs [19]. Especially in Forensic Chemistry and Toxicology applications, it is noteworthy that drugs present in wastewater may

reach the sewer and WWTP from different sources besides human excretion, including direct disposal of the drugs and other synthesis products [4]. For example, drugs in the form of powders, tablets or vegetable materials, usually forms they are available for illicit use, can be discarded through the sewer, dissolve in wastewater and reach the WWTP. In this context, it is important to consider this possibility and a recommended approach is to include products of the human metabolism of these drugs in the scope of the method, to avoid biases [9]. On the other hand, metabolism studies may be required for some drugs, especially new synthetic drugs. In addition, enantiomeric profiling needs to be used in the analysis of chiral compounds, to obtain information related to the source of a particular drug (e.g., illicit use, metabolism or direct disposal), such as in the analysis of amphetamine-like drugs [9,20]. Several countries and agencies have been already implementing monitoring tools through wastewater analyses, for example, the National Wastewater Drug Monitoring Program (NWDMP) [21], the Sewage Analysis CORe group Europe [22] and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [19].

WBE has potential as a promising to support forensic intelligence strategies, including drug enforcement and control [23]. In general, studies on drug testing in wastewater have shown agreement and correlation with other epidemiological data, being able to complement data from reports on drug seizures, population surveys and more [8]. In the field of drugs of abuse, epidemiological analysis in wastewater can be applied in different aspects, such as monitoring and rapidly assessing drug trends, efficacy of drug abuse control programs and comparison with population surveys [9]. Data provided by wastewater analysis can lead to a comprehension of the size and the changes of drug market, making possible an indirect assessment of the impact of specific criminal groups after dismantled by law enforcement [23]. From a forensic intelligence perspective, the combination of all these data, including results from wastewater analysis, can support a better understanding of the drug abuse problem, supporting strategic control and treatment initiatives [13,23]. Data obtained by law enforcement operations and investigations can strengthen the results obtained by wastewater analysis, which are subjected to uncertainties [23]. Considering the emergence of NPS, the approach of combining data provided by wastewater analysis, law enforcement investigations/operation, drug seizures and toxicological analysis of intoxication cases could be a very useful and interesting strategy [23]. The analysis of wastewater can be an additional early warning system, to communicate potential new abuse substances available in the drug market. Nonetheless, for the analysis of NPS in wastewater, the toxicokinetics and the drug fate in wastewater need to be known and reference materials are required, in particular for targeted methods; alternatively, high resolution mass spectrometry (HRMS) can be used for NPS identification in non-targeted methods, including retrospective analysis [12]. These aspects will be discussed in details in the following sections.

GENERAL CONSIDERATIONS

Quantification of parent drugs or metabolites in wastewater can provide data related to the amount of compounds reaching the WWTP and to the mass load, which can provide useful information on the estimated amount of drugs being consumed by a population, considering toxicokinetics and fate of each drug and characteristics of the sewage network [9]. Combining target analytes concentration in influent wastewater and toxicokinetics, environmental and WWTP data, back-calculation can be done to estimate drug consumption, in mass/day or doses/day, and further normalized to 1,000 inhabitants, based on the population served by the WWTP [4,9,19], enabling the comparison of data from different locations [9].

The back-calculation of drug intake based on wastewater levels is based on the concentration, which is defined as the amount of the parent compound or one of its metabolites found in wastewater (in ng/L), the flow rate corresponding to that of the sewage network (in L/day), the correction factor related to the metabolism/excretion of each analyte and the population is the total population served by the WWTP [6,12]. The correction factor is based on drug's toxicokinetics/pharmacokinetics, accounting for the drug's excretion rate and the molecular mass ratio between parent drug and its metabolite [12], in the case of using a metabolite as biomarker.

The toxicokinetics of each target drug (absorption, distribution, metabolism and excretion) is one of the key factors determining the amount of each drug/metabolite that will be eliminated into the sewage network [4]. Toxicokinetics, in turn, is influenced by some factors such as the type of drug, dose, route of administration and individual characteristics (e.g., age and health conditions). Moreover, the gut microbiota may play a role in the biotransformation of xenobiotics in the body, influencing the formation of metabolites that might be excreted into urine/feces and eventually reach the sewage [4]. It is important to consider that potential biases in the estimation of drug intake by a population may influence interpretation of results and there is a need for minimizing the uncertainty of this estimate [24]. Information on chemical identity of relevant metabolites, excretion rates in parental or metabolite forms and proportion parent/metabolites especially in urine need to be considered [25], for the selection of the target analytes in the sample and for further calculations. In back-calculation, specific correction factors account for the metabolism and excretion (mainly urinary) of a drug [12]. Some authors recommend ~~is~~ to refine the correction factors by extensive review and study of pharmacokinetics data available, in order to select the proper correction factor data for estimations [12]. The selection of metabolites (instead of the parent drug) as biomarkers in wastewater analysis is also very important since it may distinguish human active consumption of a drug from direct disposal or synthesis [4], such as in case of cocaine (parent)/benzoylecgonine (BE) (major metabolite). The estimation of drug intake based on doses may also have some associated uncertainties since the “standard dose” is highly variable according to the drug, administration route and use patterns (chronic, occasional and heavy users) [9]. Therefore, pharmacokinetics data are required. A challenge in the analysis of drugs of abuse in wastewater is that human toxicokinetics/pharmacokinetics data on traditional drugs of abuse are limited and for NPS, data are even scarcer [25]. Pharmacokinetics studies involving drugs of abuse are very complex due to safety and ethical constraints and are conducted only in authorized research centers [12].

Processes that may cause structural modifications of the drug/metabolite from the point of disposal of excreta to the point of sampling is another aspect to consider [26]. Processes of mass transfer (including sorption, partitioning and transportation), besides chemical and biological reactions, can occur in wastewater and define the fate of each target drug, affecting their final concentrations in wastewater [24]. The adsorption of drugs into suspended particulate material may also affect the overall concentration of these drugs in wastewater [24]. Furthermore, it should be taken into account that wastewater samples are usually adjusted to acidic pH at the time of collection and that acidification potentially modifies the partitioning of drugs between liquid phase and particulate matter [27]. Therefore, when the particulate fraction of wastewater is not analyzed, the intake can be underestimated for some drugs, such as reported for methadone and cannabis [28].

Abiotic and biotic processes occurring in the environment can be responsible for the conversion of emerging pollutants into transformation products (TP) [29], which may also apply to illicit drugs and metabolites present in the aquatic environment, particularly in the sewage. Known processes primarily inducing the formation of TP are reactions of oxidation, hydroxylation, hydrolysis, conjugation, cleavage, dealkylation, methylation and demethylation [29]. Biotransformation occurring within human, animal and microbial metabolisms, in natural or engineered systems, are considered biotic processes whereas abiotic processes include hydrolysis and photolysis occurring in the natural environments and WWTP [29]. The microbiome of wastewater can induce the biotransformation of drug metabolites excreted by humans, directly affecting the interpretation of analytical findings [30]. For example, in-sewage biotransformation has a role in the final concentration of cocaine and its biomarkers and thus in the back-calculation of cocaine use [26]. In influent wastewater, there is a high diversity of bacteria [31] and the high diversity of wastewater microbiome leads to many potential microorganisms being responsible for the transformation of drugs and their metabolites, and having a role in the overall microbial metabolome [30]. Microorganisms can also affect pharmacological active substances exhibiting chiral properties [32]. Illicit drugs can undergo microbial biotransformations in wastewater under aerobic or anaerobic conditions, which may be mediated by bacterial enzymes [24]. Enzymatic reactions occurring in microbial metabolism include hydroxylations,

N-oxidations, *S*-oxidations, dealkylations, dehalogenations, nitro reductions and hydrolysis of amides and carboxylesters, among others [29]. For example, a recent study studied showed that the biotransformation of pyrrolidinophenone-type psychoactive substances in incubation with a *Pseudomonas putida* strain isolated from wastewater, reporting that a similar TP was formed also when the drug was incubated with wastewater as inoculum [30]. It is noteworthy though that human metabolites and microbial TP may present common metabolic pathways, converging into the formation of similar compounds, which makes challenging to discern the origin of metabolites [29]. Another consideration is that drugs and metabolites may be subjected to wildlife biotransformation as well [29].

There are other factors that are considered in wastewater analysis. The features and conditions of the sewage network/WWTP need to be assessed and considered in WBE studies as well [9]. The daily flow rate, dissolved oxygen concentration, pH, presence of sediments and temperature can model the conditions and composition of wastewater [24], which ultimately may affect the stability of drugs. For calculations, the total population covered by the WWTP and the flow rate are required [9]. A limitation is assessing the total population served by a given WWTP [8], which can be challenging since the population may exhibit fluctuations in specific seasons [9,19]. Two combined approaches, the estimation based on chemical markers in wastewater or census and sewage capacity data, can be used for determining the population served by a sewage network and WTTP [12]. Census and sewage capacity data may not account for seasonal fluctuations in the total population served by a given WWTP [33]. The parameters of water quality (e.g., chemical oxygen demand, biological oxygen demand, total nitrogen and total phosphorus) may be used but non-anthropogenic sources can affect the estimations of population based on these markers [33]. Another marker recently proposed is ammonium ion, which could be less sensitive to non-human sources [33]. However, more research is required to establish biomarkers to calculate the population served by the WWTP and to monitor eventual fluctuations [12].

Another factor is that processes occurring within the WWTP might play a role in the fate of drugs and metabolites in wastewater, particularly when the analysis is performed in effluent (treated) wastewater. A conventional WWTP is designed to provide the removal of any pathogens and coliforms present in wastewater and to reduce loads of carbon, nitrogen and phosphorus [34]. Chemical treatment in WWTP and hydrolysis/photolysis naturally occurring in the environment thus can also lead to TP [29]. For example, a study explored the effects of photolysis by simulated sunlight or UV irradiation to cocaine and metabolites, unveiling the formation of several TP [35]. Usually, wastewater is not exposed to sunlight or UV radiation but two products from cocaine and benzoylecgonine identified in photolysis experiments were also detected in influent and effluent wastewater samples in that study [35]. The authors concluded some of these products might be result from *in vivo* elimination, but other products might be derived of other processes occurring in sewage, such as bacterial biotransformation [35]. In another study, the effects of hydrolysis, chlorination and photolysis to 11-Nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) were investigated [36]. The identified products of transformation of THC-COOH were not found in influent wastewater samples but hydrolysis and photolysis products were detected in effluent wastewater and surface water samples [36]. Some of the WWTP use processes of UV irradiation, chlorination and ozonation [36], which might lead to physicochemical transformations of drugs and metabolites.

ANALYTICAL CONSIDERATIONS

Chemical analysis of illicit drugs and metabolites in wastewater is paramount for WBE studies, since the quantification of these compounds in the wastewater is needed for back-calculations and drug intake estimations [37]. Thus, Analytical Chemistry is one of the bases of WBE [12]. Wastewater is a high-complexity matrix, which contains solids, dissolved and particulate matter, microorganisms, nutrients, metals and micro pollutants [24,38]. Drugs and metabolites are usually present at very low concentrations in wastewater, in the range of ng L^{-1}, much lower than in human biological fluids, which adds another level of complexity to the chemical analyses of this matrix [9,37]. Therefore, the analysis of chemical substances in this type of specimen may be challenging and requires analytical techniques

of high sensitivity combined with sample preparation prior the analysis. Another important consideration is the quality control in wastewater analysis. Method validation is of greater importance, using reference materials and quality controls, assessing figures of merit (e.g., limit of detection and limit of quantification) and confirming positive findings, to assure the quality of the results [12]. In addition, it is recommended to include chemical markers of wastewater in the scope of the method, as a quality control and normalization factor [4]. These markers can be either indicators of either human use of substances (as caffeine or nicotine) or human activity (as coprostanol present in feces or creatinine present in urine) [4]. The use of high sensitivity, accuracy and precision, validated analytical methods is needed. Inter-laboratory exercises are also recommended in order to standardize analytical procedures and calculations [12,24].

Wastewater sampling is a critical step in WBE studies and the selection of a proper type and frequency of collection can avoid misinterpretation of the findings [12]. The active collection of wastewater samples can be performed mainly by composite or grab sampling [9,39]. Composite wastewater samples consist in a pool of influent, raw wastewater collected during 24 h, to be representative of an entire day of elimination into the sewage [9,39]. Composite samples are representative of the average daily conditions of the wastewater during the period when sampling was occurring [40]. Grab samples are collected as a single sample or a set of samples collected over a period no longer than 15 minutes, and it should reflect the conditions of wastewater at the moment of sampling [40]. The major limitation of using grab samples is that these samples may be biased by fluctuations in concentrations, especially due to special events or environmental conditions [4].

Another possibility consists in a passive sampling, in which a polymeric-based sorbent material is deployed at the WWTP for longer periods (days or weeks) and provides a long-term accumulation of chemical substances present in sewage [11]. An example of this approach is the use of polar organic integrative samplers (POCIS) [9]. This approach is particularly interesting for monitoring NPS considering some of these drugs might be used in a low rate by a population [11] or the prevalence fluctuations related to cycles of emergence-disappearance common to some NPS. However, passive sampling methods required calibration and quantification, for a better understanding on the mechanism by which compounds are collected and potential variability [11].

Sample preparation is a very important step in wastewater analysis. It is used to concentrate the target analytes and to reach low limits of detection (LOD) and of quantification (LOQ), besides acceptable recoveries [9], since the levels in wastewater are usually in reduced magnitude in comparison to the levels at the moment of excretion (caused by dilution, microbial degradation/biotransformation and sorption to particulate material) [4]. Preparation of wastewater samples is also required to remove matrix interferences that can affect the analysis, especially considering the ionization in liquid chromatography coupled to mass spectrometry (LC-MS) based methods [9].

After the collection, wastewater samples are kept and stored at low temperatures (4 ºC or -20 ºC, according to the estimated time) [9]. Usually, the pH of wastewater samples is adjusted by acidification, right after sampling [9] or prior to sample preparation. This procedure is recommended to improve sample stability, by decreasing bacterial activity [27]. For example, it has been reported the acidification of filtered or non-filtered wastewater samples increases the stability of many classes of NPS [11]. In addition, acidification of wastewater samples is also required if a solid phase extraction (SPE)-based method using mixed-mode cation exchange phase is performed to extract basic drugs [27]. However, acidification of wastewater samples can promote the biotransformation of THC-COOH [37]. The addition of sodium metabisulfite has also been explored for preserving wastewater specimens [37], such as to improve the stability of cocaine [24] and synthetic cannabinoids [11]. Therefore, stability studies are required to assess the optimal conditions for storing wastewater samples, to avoid degradation of target compounds and misinterpretation of results.

For sample preparation, several studies described in the recent literature have combined filtration, centrifugation and solid phase extraction (SPE). Filtration with membrane of glass fiber filters or centrifugation is required in order to remove all solid components present in wastewater samples [9,37,39]. SPE is well

known as a high selectivity extraction technique, which provides both good clean-up and pre-concentration of target compounds. In addition, SPE is a traditional technique adopted by many forensic laboratories. Offline and online SPE have been used in many studies for extracting target drugs and metabolites from wastewater samples, but offline SPE is the most commonly used approach in wastewater sample preparation reported in the literature [12,37]. Other solid phase-based sample preparation techniques have also been used, such as solid phase microextraction (SPME) and molecular imprinted polymers (MIPs)-SPE [41]. MIPs-based SPE resulted in high selectivity, accuracy and precision for the analysis of amphetamines and methylenedioxy derivatives in wastewater [41]. In another study, SPME was used for extracting Δ9-tetrahydrocannabinol (THC) and THC-COOH from wastewater samples, obtaining satisfactory precision and accuracy [42]. Liquid-liquid extraction (LLE) has also been used in some studies, and, for example, in comparison with SPE, minimal differences in the recovery of cannabinoids from wastewater have been reported for both methods [43]. Wastewater samples collected using POCIS are usually processed using a different approach. POCIS present sorbents similar to those of SPE cartridges in the membrane, enabling the collection of hydrophilic drugs [9]. In a recent study published in the literature, the extraction was performed from POCIS using methanol, two times, and combined both extracts, which were further analyzed by LC-MS/MS [44]. According to the authors, findings obtained from samples collected with POCIS showed an underestimation in comparison to 24-h composite collected samples, which could be explained by the potential blockage of the POCIS surface with solid materials during filtration and consequent reduced trapping of drugs [44].

Drug testing in wastewater has become plausible due to great advancements in analytical technologies, making it possible to detect trace levels of drugs and metabolites in this type of sample [8]. Coupling chromatography and mass spectrometry is the best analytical strategy for chemical analysis of wastewater, in order to reach the needed sensitivity and selectivity [37]. Several studies have used LC-MS techniques for detection and quantification of drugs in wastewater samples [12], with recent studies reporting both high-performance and ultra-performance liquid chromatography (HPLC and UPLC, respectively). Ionization in LC-MS methods are usually performed by electrospray (ESI) in both positive and negative modes, depending on the type of analyzed compounds. For example, in recent studies, ESI in a negative mode was used in the detection of ethyl sulfate (EtS), metabolite of ethanol, whereas other illicit drugs were detected using ESI in positive mode [17,45]. Several detection systems in mass spectrometry (MS) have been used, including hybrid and high-resolution systems. Low-resolution MS including ion trap and triple quadruple analyzers are the most used techniques in quantification of illicit drugs and metabolites in wastewater [37]. However, the use of HRMS has been increasingly explored in recent years [37]. Some examples include triple quadrupole mass spectrometry (e.g. [45–47]), quadrupole-ion trap mass spectrometry (QTrap) (e.g. [16,17,48]), quadrupole-orbitrap mass spectrometry (e.g. [49,50]) and quadrupole-time-of-flight mass spectrometry (QTOF) (e.g. [49]). These techniques can provide high sensitivity and selectivity, reaching good results, especially considering the complexity of wastewater samples. Particularly LC-HRMS-based methods can provide a comprehensive screening of illicit drugs and NPS, their metabolites and TP [37]. Direct analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) systems (without extraction) has also been reported. In a study published in the literature, wastewater samples were directly injected into LC-MS/MS after filtration without any extraction, reaching limits of detection (LOD) between 0.05 and 30 ng L^{-1} and median limit of quantification (LOQ) of 31 ng L^{-1} [51]. The authors found that using SPE for clean-up did not increase the sensitivity of the method in comparison to direct injection and exhibited decreased sample throughput, adding more time to the process [51].

Gas chromatography-mass spectrometry (GC-MS) is another technique of high selectivity and sensitivity but it requires a derivatization step for many compounds of forensic interest, which can extend the analytical workflow [37]. In the literature, GC-MS has been used by some studies. For example, GC-MS with an ion trap detector was recently used in enantiomeric profiling of several compounds including amphetamine, methamphetamine, 3,4-methylenedioxy-methamphetamine (MDMA) and norketamine [52]. Wastewater samples were filtered, acidified and further extracted using SPE followed by chiral derivatization

with (R)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride ((R)-MTPA-Cl) [52]. Authors obtained a good separation for several diastereomers of the target analytes, without using a chiral column [52]. In another study, GC-Ion trap-MS/MS was used in combination with SPE and MSTFA derivatization for the determination of illicit drugs in grab samples collected from 5 WWTPs [53]. Cocaine and its metabolite, benzoylecgonine, THC and its metabolite THC-COOH, codeine and morphine were unequivocally detected in wastewater by GC-MS/MS [53]. However, the authors stated that the method used was not able to detect the methadone metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and requires more time for analysis than LC-MS-based methods, considering the derivatization step of 90 min [53]. In addition to chromatographic-spectrometric techniques, other techniques have been explored in wastewater analysis. A recent example is in which the application of surface-enhanced Raman spectroscopy (SERS) sensor was used in the detection of methamphetamine in wastewater [54]. According to the authors, the SERS-based method exhibited results comparable to those obtained by a LC-MS-based method, high sensitivity, good selectivity to methamphetamine and good reproducibility [54].

The use of HRMS has introduced many possibilities in forensic analyzes. The many features of HRMS instrumentations including mass accuracy and sensitivity allows the investigation of a high number of analytes, including additional compounds present in the sample that were not previously targeted [37]. Time-of-flight (TOF) and Orbitrap are the most frequently used analyzers for HRMS [37]. Based on HRMS techniques, another approach in the analysis of wastewater is the suspect screening/analysis, in which any compound that is in the instrument's library is qualitatively analyzed in a sample [11,55]. In this approach, there is no need for selecting analytes and using reference materials for method development [11]. However, each entry in the instrument's library requires data of each compound (e.g., exact mass, retention time and fragments), which depends on the availability of reference materials; if there is no reference material available, the data for including the compound in the library is more limited but the tentative identification might still be possible by inspecting the high resolution mass spectra [37]. In addition, *in silico* models can be used for predicting chemical structure and properties of unknown compounds, which can include or exclude potential chemical structures and increase the level of confidence in the identification, in case no reference materials or data are available [11].

The application of the techniques mentioned above can be used for targeted and non-targeted analysis. Targeted analyses are performed for a limited number of compounds present in the scope of the method [55], which leads to high sensitivity and selectivity but do not detect any other compounds present in the sample that were not included in the scope of the method [37]. In general, targeted analyzes are performed using GC-MS or LC-MS techniques [37]. In wastewater samples, LC-MS/MS, with triple quadrupole or ion trap analyzers (low-resolution mass spectrometry), have been successfully used for analysis of drugs and NPS in wastewater samples [11,37]. On the other hand, non-targeted analyses consist in the investigation of any compound present in the sample detectable by the analytical technique in use [47], without selecting any analytes [11] and providing any information of the analyte of interest prior to the analysis [37]. In this approach, both chromatographic profile and accurate mass spectrum are thoroughly investigated for tentative identification [11]. This approach is difficult when applied to wastewater due to the complex chemical composition of the samples, the potentially high number of compounds present, and at low concentration [37].

In addition to parent drugs, human metabolites and TP may also be investigated in wastewater as biomarkers of drug use, in the absence of the parent or as a complement to the parent compound. Combining toxicokinetics and stability in wastewater data on drugs of abuse is a critical step to perform WBE studies since these data will be used for calculation and estimations [25]. The selection of the appropriate biomarker for a specific drug is paramount for chemical analysis of wastewater as well as for further back-calculations and estimations. In general, parent drugs or their metabolites present in urine are selected as biomarkers for illicit drugs use in the analysis of wastewater [37]. Data on human metabolism and identity of major metabolites is available for many known drugs of abuse whereas for NPS such information is much more limited [37]. In this context, studies on biotransformation occurring in-sewage are needed [12].

If the identity of a metabolite or a TP is known then it can be added to the scope of the method and in the library of an analytical instrument, to be considered for analysis in wastewater, routinely or retrospectively [29,37]. However, if the identities of those compounds are not described, alternative approaches need to be adopted. The structural elucidation of metabolites or TP might be done by the study of high-resolution mass spectra, based on fragmentation patterns of the parent drug and unknown compounds, but also on the chromatographic profile, especially if there are additional peaks at retention times corresponding to the fragments under investigation [37]. This can also be performed retrospectively, reanalyzing other compounds that were not initially targeted in previous data acquisition [37].

In vitro studies may be explored for characterization of potential human metabolites, which can further be used as biomarkers of drug intake in wastewater. *In vitro* models can be used to assess toxicokinetics data for classic and novel drugs of abuse, predicting the human metabolism of drugs [25]. There are several *in vitro* models available including cells, cell fractions and organs, such as human hepatocytes and human liver microsomes. In these models, the target parent drug is incubated within systems containing liver enzymes and samples are further analyzed to characterize potential metabolites [25]. Using *in vitro* models, there are no concerns regarding ethical and safety issues, in contrast to *in vivo* studies, and the costs are usually lower. On the other hand, for *in vitro* assays, reference materials of the drug of interest and high resolution analytical techniques are required (e.g. HRMS). In regards to NPS, especially recently emerged ones, reference materials may not be available yet [25]. *In silico* studies may also be performed to predict human metabolites or TP [37]. For example, the software SMARTCyp of the University of Copenhagen is an *in silico* platform that predicts the molecular sites where a potential Cytochrom P450 metabolic reaction can occur [25,56]. Another example is the EAWAG-BBD Pathway Prediction System, which predicts the microbial biotransformation of chemical substances based on its database and it has been successfully used for predicting the fate of environmental contaminants [25,57]. Once potential metabolites and TP are predicted, these compounds can be investigated in data obtained by HRMS, extracting the exact mass from chromatographic data based on several positive identification criteria and tentatively characterizing the structure based on fragmentation data [37]. Although both models (*in vitro* and *in silico*) are very useful to predict potential human metabolites, it is still possible they are not formed *in vivo* or excreted in urine, in a real user scenario. Nonetheless, these models are considered powerful tools to predict a list of potential metabolites that can be eventually be tested as target compounds using high-resolution mass spectrometric methods [25].

Research on drug stability in wastewater has been conducted over the recent years and there is information available in the literature for some drugs and their metabolites. In-sample stability has been assessed for some drugs and metabolites but in-sewage stability subjected to different conditions is not well-understood [24]. Therefore, studies assessing biotransformation/biodegradation kinetics, microbial kinetics and stability can be very powerful tools to characterize the fate of drugs, metabolites and TP in wastewater. In addition, characterization of the microbiome present in wastewater through metagenomics can support the investigation of the functional potential of the microbiome for the biotransformation/ biodegradation of drugs. The combination of *in vitro* or *in vivo* metabolic profiling and biotransformation/ degradation studies of drugs is a suitable analytical strategy to obtain data on known and unknown metabolites and TP, which might be further investigated in wastewater samples and assessed as potential biomarkers for WBE studies [37].

The analysis of drugs, metabolites and TP in real sewage would be ideal for understanding the biological, physical and chemical behavior of these substances, under sewage networks in normal operation conditions; however, this is a complex and limited approach that would require several studies to obtain accurate data [24]. In-laboratory studies on drugs' stability in wastewater should consider: (1) inclusion of biofilms present in sewage during stability assessments; (2) physical-chemical characterization of wastewater to assure the reproducibility; (3) effective spiking concentrations considering the purpose of the study; (4) quality controls (positive control, negative control and abiotic controls); (5) suitable experimental design and sampling [14]. A detailed discussion on each of these recommendations can be found elsewhere [24].

DRUGS REPORTED IN WASTEWATER

The applications of WBE in forensic research has been performed since the middle-2000s, with one the first studies on the analysis of cocaine and BE in wastewater published in 2005 [5]. Since then, many researchers have evaluated licit and illicit drugs in wastewater around the globe. Among licit substances, nicotine is a substance highly available in the world, which can be measured itself in wastewater. Additionally, nicotine can be used as a marker of human consumption, for quality control and data normalization purposes [4]. In wastewater samples, there are several studies available in the recent literature detecting nicotine metabolites cotinine or trans-3′-hydroxycotinine to assess nicotine use (e.g. [17,45,46,50,59–65]). Ethanol is one of the most commonly consumed substances in the world [59] and ethanol intake can also be measured in wastewater. In the body, ethanol is mainly metabolized to acetaldehyde and acetic acid, with minor fractions metabolized to ethyl sulfate (EtS) and ethyl glucoronide (EtG) [66]. EtS and EtG are common metabolites of ethanol found in urine after alcohol intake [59,66]. It has been suggested that EtS is a more recommended metabolite for ethanol consumption estimation based on wastewater levels, since EtG instability in effluent wastewater has been previously reported [67]. Additionally, it is important to consider that the source of ethanol present in wastewater can be the direct disposal of alcoholic beverages and other products (e.g. hand sanitizers) [67]. This might be a factor for wastewater research conducted during the pandemic of COVID-19, since there are many ethanol-based hand sanitizers being used. However, in the presence of ethanol in sewage, the probability of formation of EtS in wastewater is minimal, thus not affecting the selection of EtS as a biomarker [67]. In wastewater samples, EtS has been proposed for estimating ethanol consumption (e.g. [17,45,61,64]).

Cannabis is the most used drug reported by UNODC, with 192 million users around the world in 2018 [1]. Trends in cannabis use have been influenced by its legalization in some countries, and according to the UNODC, it will take time to assess the impacts of non-medical use legalization measures and the cannabis market should be under close monitoring [1]. In regards to cannabinoids, THC-COOH is the THC metabolite commonly used as biomarker of cannabis use in wastewater [43], usually at greater concentrations in influent wastewater in comparison to effluent wastewater [36]. THC-COOH has been detected in wastewater, in several studies (e.g. [15,17,43–45,47,49,50,61,64,65,68–73]). In the literature, the detection of THC itself was also reported (e.g. [63,71]). Another metabolite of THC, THC-OH has also been reported in wastewater (e.g. [68,71]). It is important to consider that due to their lipophilicity, metabolites of THC may be eliminated through the feces, adsorb and deposit to particulate content present in wastewater [43]. THC-COOH may interact with particulate material present in wastewater and failure in measuring its content in this fraction of wastewater may lead to underestimations [28]. CBD is another cannabinoid present in cannabis, with therapeutic but not psychoactive properties. CBD is excreted in urine mostly in the parent form [74]. In a recent study, CBD and the metabolites CBD-7-OH and CBD-7-COOH were searched in wastewater but only CBD was detected [43]. Considering the current landscape of legalization of cannabis for medical and/or recreational purposes, the concentrations of THC-COOH could increase in environmental waters [74], and this could eventually be observed with other cannabinoids present in cannabis.

In the literature, stimulants are a class of drugs frequently detected and reported in wastewater. In recent days, cocaine is still one of the most largely produced drugs, with estimated 19 million users in 2018 [1]. In wastewater samples, cocaine and/or benzoylecgonine have been detected (e.g. [13,17,44–46,49,51,60–65,68–71,73,75–78]. Other cocaine metabolites have been reported in some studies: a few examples include ecgonine methyl ester (EME) [63], norcocaine [68,71], anhydroecgonine methyl ester (AME) [60] and cocaethylene [60,68,69,71]. Another group of stimulants, amphetamines seized between 2009 and 2018 has significantly increased [1]. The estimated number of amphetamines and prescription stimulants users in 2018 was 27 million [1]. In wastewater samples, amphetamine and/or methamphetamine have been detected (e.g. [13,15–17,44–52,54,60–65,68,70,71,73,75–81]. However, these drugs are metabolites of others such as fenproporex, selegeline and famprofazone, and these findings in wastewater can overestimate the use of amphetamine or methamphetamine [9,82]. Therefore,

it is noteworthy to highlight that by enantiomeric analysis it is possible to differentiate between legal and illegal sources of amphetamine-like compounds [82]. The amphetamine-like drugs MDMA and MDA have also been determined in wastewater samples (e.g. [13,15–17,44–47,50–52,60–65,68–71,75–78,81]. According to the UNODC, in 2018 21 million people have used ecstasy [1]. MDMA metabolites, HMMA and HMA, have been reported in wastewater samples in some studies as well (e.g. [7,60,83]). Some authors even recommend including HMMA [83,84] and HMA [83] in the scope of analytical methods to estimate MDMA use. 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA), another illicit stimulant similar to MDMA, has been also reported in some studies (e.g. [7,68,71]). An important consideration regards to the chirality of methylenedioxy derivatives, such as MDMA and MDA. For example, chiral analytical methods can help to understand the source in wastewater of MDMA (MDMA use vs. direct disposal) and MDA (MDA use vs. MDMA metabolism) [20].

Opioids are a class of substances largely used in the treatment of moderate to severe pain [85]. This class includes pain reliever prescribed drugs such as oxycodone, hydrocodone, codeine, morphine and others, as well as heroin (an illegal drug) and other synthetic opioids such as fentanyl [86]. In 2018, it was estimated that 57.8 million people used opioids, including opiates and pharmaceutical opioids [1]. Morphine (e.g. [17,44,45,48,60–64,68,71,73,75,76]), codeine (e.g. [44,48,60–63,65,71,75,81]), methadone and its metabolite EDDP (e.g. [13,17,44,50,60,62,63,65,68,69,71,75,76,81]) are some of the most commonly detected opioids in wastewater, which in turn is one of the main sources of opioids present in superficial waters [39]. In two recent studies, normorphine [60,63] was also targeted and reported in wastewater analysis. Heroin was also measured in wastewater in some studies (e.g. [15,71]) and, although 6-MAM is a unique heroin metabolite, this compound may not be commonly reported in wastewater samples due to its low levels in these samples [9]. For example, concentrations of 6-MAM in wastewater were recently as low as 15.4 ng/L [50]. 6-MAM was also reported in wastewater in other studies (e.g. [60,64,70,71,76]). In wastewater analysis, it is challenging to estimate heroin use based on morphine, considering that morphine can be present in wastewater resulting from therapeutic use of morphine and codeine [9] or illicit use of morphine. When estimating heroin use based on morphine levels, the amount of morphine used therapeutically and the amount of morphine from codeine metabolism need to be taken into account [9,60]. It is also important to consider that the ingestion of poppy seeds can result in the formation and excretion of morphine in urine [87]. Norcodeine, a codeine metabolite, has not been usually targeted in the wastewater codeine testing, which can be explained due to its low levels in wastewater specimens, requiring high sensitive analytical techniques [88]. However, examples of studies detecting norcodeine in wastewater are available (e.g. [60,63]). Another metabolite of methadone, 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP), was also targeted [6].

In addition to morphine, codeine and methadone, other opioids have been reported in wastewater studies. Hydromorphone is available in the pharmaceutical market, which poses an analytical challenge to assess whether the source of hydromorphone present in urine is hydromorphone intake or hydrocodone metabolism [89]. The detection of hydromorphone in urine though is not necessarily an indicator of hydromorphone use [90]. Therefore, this should be considered in the analysis and interpretation of hydrocodone and hydromorphone levels in wastewater and consumption estimates. Hydromorphone has been detected and reported in several recent studies in the literature (e.g. [44,60,68,71]). Dihydrocodeine, a metabolite of hydrocodone, and dihydromorphine, a hydromorphone metabolite, were also detected in wastewater samples [60]. Similarly, hydrocodone and norhydrocodone were detected in wastewater samples, according to some studies available in the literature (e.g. [44,63,68,71,75]). Oxycodone is another semisynthetic opioid, derived from codeine [91]. In wastewater specimens, oxycodone, noroxycodone (major metabolite of oxycodone) and oxymorphone (a minor metabolite) [92], have been detected in some studies (e.g. [44,50,51,60–62,65,68,71,75]). Tramadol is an orally active, synthetic opioid [93], analog of codeine [94], with pharmaceutical use. The illicit (non-medical) use of tramadol has also been reported, such as in some countries in West, Central and North Africa [1]. Tramadol, in its parent form, has also been found in wastewater samples (e.g. [17,44,50,60,62,65,76,79]). The metabolites *N*-desmethyltramadol and

O-desmethyltramadol have been detected in wastewater samples as well (e.g. [63,75]). A compound named *O*-*N*-bisdesmethyltramadol was also reported in wastewater [63] but no additional information on this compound was found, and it may be the metabolite *O*-*N*-didesmethyltramadol of tramadol reported elsewhere [95]. An analog of tramadol, tramadol-*N*-oxide was also reported [63]. Buprenorphine is a semisynthetic opioid, derived from thebaine, medically used in pain management and in the treatment of opioid dependence [96]. Buprenorphine and/or its metabolite norbuprenorphine have been determined in wastewater (e.g. [44,46,61,68,71,75]). Additionally, the conjugated metabolite norbuprenorphine-glucoronide was detected in wastewater samples [50,65]. Finally, another opioid highly relevant in Forensic Toxicology and Chemistry is fentanyl, characterized by its high potency (80 times higher than morphine) and reduced duration of action [97]. However, the illicit use of fentanyl and the emergence of illicit analogs have been causing public health problems in many regions, including the US and Europe [98]. In 2018, fentanyl was associated with two thirds of 67,367 deaths by overdose in the USA [1]. In recent studies available in the literature, fentanyl and/or its metabolite norfentanyl have been detected in wastewater (e.g. [61,63,68,71]). Fentanyl detection in wastewater can be analytically challenging. Its elimination occurs in urine and feces, mainly in the form of the inactive metabolites (primarily norfentanyl) [99,100], with small fractions corresponding to the unchanged and free forms of fentanyl [99–101]; thus this drug might not be detected in wastewater samples due to its low levels. However, in case fentanyl is detected in wastewater, it might be present as a result of direct disposal, similarly to other drugs. In addition, fentanyl can also be present in other drugs (e.g., heroin, cocaine, methamphetamine and MDMA) as adulterant [86], which is particularly important when estimating drug intake and comparing it with other epidemiological or seized data.

Benzodiazepines comprise a number of drugs, which includes diazepam, oxazepam, temazepam, alprazolam and more [102]. The frequent prescription of benzodiazepines is made due to their pharmacological properties, useful in the treatment of anxiety, insomnia, convulsions, as sedative, amnesic and relaxant agent [102,103]. However, misuse of benzodiazepines has also been reported [104]. One of these drugs is diazepam, which exhibits a complex metabolism, with other active metabolites, including nordiazepam and temazepam (minor), which can be both further metabolized to oxazepam, another active compound that is conjugated in Phase II metabolism (oxazepam glucuronide) [102,103]. The metabolism of oxazepam occurs mainly by glucuronidation [105]. Oxazepam and temazepam are also pharmaceutical drugs. In recent literature, the detection of diazepam and nordiazepam have been reported in wastewater (e.g. [51,63,68,71]). Oxazepam and/or temazepam have been detected in wastewater as well in some studies (e.g. [17,44,46,50,51,60,62,63,65,68,71,75]). Alprazolam is another benzodiazepine drug, with short-duration action [106], used mainly in the treatment of anxiety and panic disorders [102]. Alprazolam has been detected in wastewater samples in several studies (e.g. [63,68,71]). The metabolite α-OH-alprazolam was also quantified in wastewater samples [107]. Clonazepam is a benzodiazepine prescribed in the treatment of anxiety and seizures [108]. A few studies have reported the detection of clonazepam in wastewater, [34,65]. Clonazepam's metabolite, 7-aminoclonazepam, was also detected in WBE studies (e.g. [75]). In blood, clonazepam and other nitrobenzodiazepines exhibit instability, which is especially remarkable in postmortem blood contaminated with bacteria [108]. In a similar context, the microbiome of sewage might play a role in the stability of clonazepam in wastewater, similarly to biological fluids such as blood. Although it is not a benzodiazepine, zolpidem exhibits a mechanism of action similar to benzodiazepines [105] and it is used therapeutically as hypnotic [109], being part of the group called "*Z*-drugs" [110]. These drugs, including zolpidem, have been associated with several cases of misuse, dependence and even fatal intoxications [110]. In the literature, zolpidem and its metabolite zolpidem 4-phenyl carboxylic acid were detected in wastewater in some studies [79,111].

Other substances eventually involved in forensic casework have also been reported in wastewater. Lysergic acid diethylamide (LSD) is a semi-synthetic hallucinogen, derived from lysergic acid present in fungus ergot *Claviceps purpurea* [112]. In wastewater, both substances, the parent and its metabolite 2-oxo-3-OH-LSD, have been detected in studies available in the recent literature (e.g. [50,65]). Ketamine

is a derivative of phencyclidine (PCP) and shows anesthetic, analgesic, hypnotic and amnesic properties [113]. It is well described that ketamine is responsible for inducing dissociative anesthesia [114]. However, similarly to other drugs, the illicit use of ketamine for recreational purposes is well known. Ketamine and norketamine have been determined in studies recently published on wastewater (e.g. [44,48–51,60,65,70,75,76,81]). It is important to highlight that clinical and veterinary prescriptions, as well as illicit use of ketamine can all contribute for its release into the environment [115], which includes into the sewage. gamma-hydroxybutyric acid (GHB) is a chemical substance endogenously produced, resulting from the gamma-aminobutyric acid (GABA) metabolism [116]. However, GHB has been used illicitly, as a drug of abuse, since the 1990s [117] and as a dietary supplement and sleep inducer [118]. GHB has also been used as a chemical agent in drug-facilitated crimes (DFC) [119]. In wastewater, as expected, GHB can be excreted into sewage as a product of endogenous metabolism, as a component of dietary supplements or as an illicit drug [120]. In a recent study, GHB has been detected in wastewater and authors concluded that the GHB present is probably from endogenous metabolism, based on its levels [120].

In the context of Forensic Chemistry and Toxicology, NPS represent a challenge in clinical, toxicological, public health and public safety aspects. As a result of its emergence in the drugs of abuse market, this group of “new” drugs have been frequently reported in biological samples collected from intoxication cases and in seized materials. Therefore, it is not surprising that some of these substances also started to be reported in wastewater. Examples of recent studies reporting NPS are summarized in Table I. However, the detection of NPS in wastewater can be especially challenging for many reasons. Some NPS may be present in wastewater either after being directly disposed through the sewage network or also as a contaminant within “traditional” drugs. For example, fentanyl analogs can be used as adulterants of heroin, cocaine and other drugs and also as fake pharmaceutical opioids [1]. Another example is 4-ANPP, which can be either a metabolite or a precursor of fentanyl analogs in synthetic processes [121]. Since the metabolism of some new drugs is still unknown, it is difficult to target potential human metabolites in wastewater, as it has been done with other classical drugs. Some NPS or their metabolites may not have been reported until now in wastewater samples due to their unknown identity. Thus they are not known and not currently being targeted, or because of unavailability of reference materials for identification and method development purposes.

Table I. Examples of studies in the recent literature covering the detection of NPS in wastewater

List of NPS reported in wastewater	Reference
Ethylone, mephedrone and N-ethyl-pentylone	[16]
Methcathinone, 4-methyl-pentedrone, 1-(3-chlorophenyl) piperazine (mCPP), 4-methyl-amphetamine and 4-ANPP	[68]
4-methylethcathinone (4-MEC), methedrone and mephedrone	[51]
Methylone	[61]
Mephedrone	[60]
Carfentanil, methoxyacetylfentanyl, furanylfentanyl, MAB-CHMINACA, methcathinone, 4-methylpentedrone, 2-methyl-4′-(methylthio)-2-morpholinopropiophenone (MMMP), mCPP and 5-(2-Aminopropyl) Indole (5IT)	[122]
25-iP-NBoMe, 3,4-dimethylmethcathinone (3,4-DMMC), 4,4’-Dimethylaminorex (4,4-DMAR), α-methyltryptamine, buphedrone, methcathinone, mephedrone and ephenidine (NEDPA) *Detected only:* 2-phenethylamine, 25E-NBOMe, 4-chloro-α-PPP and 2,5-dimethoxy-4-isopropylamphetamine (DOiP)	[49]

Table I. Examples of studies in the recent literature covering the detection of NPS in wastewater (Continuation)

List of NPS reported in wastewater	Reference
Mephedrone	[70]
Cathinone, mephedrone and 1,3-benzodioxolyl-N-methylbutanamine (MBDB)	[50]
2C-D, dimethoxyamphetamine (3,4-DMA), 4-methyl-pyrrolidino-propiophenone (MPPP), cathine/ norpseudoephedrine and para-fluorofentanyl	[63]
Methylone, ethylone, butylone and mephedrone	[64]
Cathinone, mephedrone and MBDB	[65]
5.6-methylenedioxy-2-aminoindane (MDAI), AB-CHMINACA, methoxetamine, 4′-Methyl-α-pyrrolidinopropiophenone (MePPP), methedrone, 5-OH-DMT, dimethyltryptamine (DMT), 2-phenetylamine, *N*-ethylamphetamine, methoxyphenamine, methylbenzylpiperazine (MBZP) and ethylphenidate	[120]
5-fluoro-APINACA, JWH-073 (4-hydroxypentyl) and MDMB-CHMICA	[43]
Dipentylone	[78]
3-MMC, 4-FA, 4-MEC, Alpha-PVP, butylone, ethylone, mephedrone, methiopropamine, methoxetamine, methylone, *N*-ethylpentylone, pentedrone, pentylone, PMA and eutylone	[123]
3-MMC, 4-FA, 4-MEC, ethylone, methylenedioxypyrovalerone (MDPV), mephedrone, methcathinone, methylone, N-ethylpentylone and pentylone. 4-chloromethcathinone, 4-fluoromethamphetamine, acetyl fentanyl, mitragynine and eutylone	[124]

CONCLUDING REMARKS

Wastewater analysis is very promising in Forensic Chemistry and Toxicology. Much information can be extracted from the analysis of these specimens and data obtained from WBE studies can support multiple strategies in public health and security. Although the goal of this study is not a systematical and exhaustive review of the literature, rather it is a comprehensive review on this topic, many reports in the literature support that classic and novel drugs of abuse could be monitored in wastewater. However, studies involving the analysis of wastewater need to address many considerations and there are current limitations and uncertainties, which require further research. It is important to consider the stability and fate of drugs in wastewater, features of the sewage network and WWTP and environmental conditions. Although data required for calculations and estimations is available for some drugs such as excretion rates and parent/metabolite ratios, data availability is still very limited for new synthetic drugs, indicating that gathering more data will aid in estimations based on wastewater levels.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors are grateful to the Ph.D. candidate Otávio Guilherme Gonçalves de Almeida, B.Sc. (FCFRP-USP) for his helpful discussions on possible microbiome-related degradation of drugs.

REFERENCES

1. United Nations Office on Drugs and Crime (UNODC). *World Drug Report 2020 - Booklet 1*. Vienna, Austria: UNODC, **2020**.
2. https://www.unodc.org/LSS/Page/NPS [Accessed on 13 January 2021].
3. Krotulski, A. J.; Varnum, S. J.; Logan, B. K. *J. Forensic Sci.*, **2020,** *65* (2), pp 550–562 (https://doi.org/10.1111/1556-4029.14184).
4. Daughton, C. G. Illicit Drugs in Municipal Sewage. In: Daughton, C. G., Jones-Lepp, T. L. (Ed.). *Pharmaceuticals and Care Products in the Environment*. American Chemical Society, **2001**, pp 348–364.
5. Zuccato, E.; Chiabrando, C.; Castiglioni, S.; Calamari, D.; Bagnati, R.; Schiarea, S.; Fanelli, R. *Environ. Heal.*, **2005,** *4* (1), pp 14 (https://doi.org/10.1186/1476-069X-4-14).
6. Baker, D. R.; Barron, L.; Kasprzyk-Hordern, B. *Sci. Total Environ.*, **2014,** *487* (1), pp 629–641 (https://doi.org/10.1016/j.scitotenv.2013.11.107).
7. Castrignanò, E.; Lubben, A.; Kasprzyk-Hordern, B. *J. Chromatogr. A*, **2016,** *1438*, pp 84–99 (https://doi.org/10.1016/j.chroma.2016.02.015).
8. Castiglioni, S.; Bijlsma, L.; Covaci, A.; Emke, E.; Hernández, F.; Reid, M.; Ort, C.; Thomas, K. V.; van Nuijs, A. L. N.; de Voogt, P.; et al. *Environ. Sci. Technol.*, **2013,** *47* (3), pp 1452–1460 (https://doi.org/10.1021/es302722f).
9. van Nuijs, A. L. N.; Castiglioni, S.; Tarcomnicu, I.; Postigo, C.; de Alda, M. L.; Neels, H.; Zuccato, E.; Barcelo, D.; Covaci, A. *Sci. Total Environ.*, **2011,** *409* (19), pp 3564–3577 (https://doi.org/10.1016/j.scitotenv.2010.05.030).
10. Zuccato, E.; Chiabrando, C.; Castiglioni, S.; Bagnati, R.; Fanelli, R. *Environ. Health Perspect.*, **2008,** *116* (8), pp 1027–1032 (https://doi.org/10.1289/ehp.11022).
11. Bijlsma, L.; Bade, R.; Been, F.; Celma, A.; Castiglioni, S. *Anal. Chim. Acta*, **2021,** *1145*, pp 132–147 (https://doi.org/10.1016/j.aca.2020.08.058).
12. Castiglioni, S.; Thomas, K. V.; Kasprzyk-Hordern, B.; Vandam, L.; Griffiths, P. *Sci. Total Environ.*, **2014,** *487* (1), pp 613–620 (https://doi.org/10.1016/j.scitotenv.2013.10.034).
13. Bannwarth, A.; Morelato, M.; Benaglia, L.; Been, F.; Esseiva, P.; Delemont, O.; Roux, C. *Forensic Sci. Res.*, **2019,** *4* (2), pp 141–151 (https://doi.org/10.1080/20961790.2018.1500082).
14. Sodré, F. F.; Souza, G. B.; Feitosa, R. S.; Pereira, C. E. B.; Maldaner, A. O. *J. Braz. Chem. Soc.*, **2017,** *28* (11), pp 2146–2154 (https://doi.org/10.21577/0103-5053.20170063).
15. Benaglia, L.; Udrisard, R.; Bannwarth, A.; Gibson, A.; Béen, F.; Lai, F. Y.; Esseiva, P.; Delémont, O. *Forensic Sci. Int.*, **2020,** *309*, pp 1–8 (https://doi.org/10.1016/j.forsciint.2020.110148).
16. Bade, R.; White, J. M.; Gerber, C. *Sci. Total Environ.*, **2021,** *757*, 143728 (https://doi.org/10.1016/j.scitotenv.2020.143728).
17. Reinstadler, V.; Ausweger, V.; Grabher, A. L.; Kreidl, M.; Huber, S.; Grander, J.; Haslacher, S.; Singer, K.; Schlapp-Hackl, M.; Sorg, M.; et al. *Sci. Total Environ.*, **2021,** *757*, 144006 (https://doi.org/10.1016/j.scitotenv.2020.144006).
18. Emke, E.; Vughs, D.; Kolkman, A.; de Voogt, P. *Forensic Sci. Int.*, **2018,** *286*, pp e1–e7 (https://doi.org/10.1016/j.forsciint.2018.03.019).
19. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). *Wastewater Analysis and Drugs: A European Multi-City Study*. EMCDDA, Lisbon, Portugal, **2020**.
20. Kasprzyk-Hordern, B.; Baker, D. R. *Environ. Sci. Technol.*, **2012,** *46* (3), pp 1681–1691 (https://doi.org/10.1021/es203113y).
21. https://www.acic.gov.au/publications/national-wastewater-drug-monitoring-program-reports [Accessed on 27 May 2021].
22. https://score-cost.eu/ [Accessed on 27 May 2021].
23. Been, F.; Esseiva, P.; Delémont, O. *Forensic Sci. Int.*, **2016,** *266*, pp 215–221 (https://doi.org/10.1016/j.forsciint.2016.05.032).

24. McCall, A.-K.; Bade, R.; Kinyua, J.; Lai, F. Y.; Thai, P. K.; Covaci, A.; Bijlsma, L.; van Nuijs, A. L. N.; Ort, C. *Water Res.*, **2016,** *88*, pp 933–947 (https://doi.org/10.1016/j.watres.2015.10.040).
25. Reid, M. J.; Baz-Lomba, J. A. A.; Ryu, Y.; Thomas, K. V. *Sci. Total Environ.*, **2014,** *487* (1), pp 651–658 (https://doi.org/10.1016/j.scitotenv.2013.12.057).
26. Plósz, B. G.; Reid, M. J.; Borup, M.; Langford, K. H.; Thomas, K. V. *Water Res.*, **2013,** *47* (7), pp 2129–2140 (https://doi.org/10.1016/j.watres.2012.12.034).
27. Baker, D. R.; Kasprzyk-Hordern, B. *J. Chromatogr. A*, **2011,** *1218* (44), pp 8036–8059 (https://doi.org/10.1016/j.chroma.2011.09.012).
28. Senta, I.; Krizman, I.; Ahel, M.; Terzic, S. *Anal. Bioanal. Chem.*, **2013,** *405* (10), pp 3255–3268 (https://doi.org/10.1007/s00216-013-6720-9).
29. Bletsou, A. A.; Jeon, J.; Hollender, J.; Archontaki, E.; Thomaidis, N. S. *TrAC Trends Anal. Chem.*, **2015,** *66*, pp 32–44 (https://doi.org/10.1016/j.trac.2014.11.009).
30. Mardal, M.; Bischoff, M.; Ibáñez, M.; Ruffing, U.; Hernández, F.; Meyer, M. R. *Drug Test. Anal.*, **2017,** *9* (10), pp 1522–1536 (https://doi.org/10.1002/dta.2165).
31. Lee, S.-H.; Kang, H.-J. ; Park, H.-D. *Water Res.*, **2015,** *73*, pp 132–144 (https://doi.org/10.1016/j.watres.2015.01.014).
32. Evans, S. E.; Davies, P.; Lubben, A.; Kasprzyk-Hordern, B. *Anal. Chim. Acta*, **2015,** *882*, pp 112–126 (https://doi.org/10.1016/j.aca.2015.03.039).
33. Been, F.; Rossi, L.; Ort, C.; Rudaz, S.; Delémont, O.; Esseiva, P. *Environ. Sci. Technol.*, **2014,** *48* (14), pp 8162–8169 (https://doi.org/10.1021/es5008388).
34. Loos, R.; Carvalho, R.; António, D. C.; Comero, S.; Locoro, G.; Tavazzi, S.; Paracchini, B.; Ghiani, M.; Lettieri, T.; Blaha, L.; et al. *Water Res.*, **2013,** *47* (17), pp 6475–6487 (https://doi.org/10.1016/j.watres.2013.08.024).
35. Bijlsma, L.; Boix, C.; Niessen, W. M. A.; Ibáñez, M.; Sancho, J. V.; Hernández, F. *Sci. Total Environ.*, **2013,** *443*, pp 200–208 (https://doi.org/10.1016/j.scitotenv.2012.11.006).
36. Boix, C.; Ibáñez, M.; Bijlsma, L.; Sancho, J. V.; Hernández, F. *Chemosphere*, **2014,** *99*, pp 64–71 (https://doi.org/10.1016/j.chemosphere.2013.10.007).
37. Hernández, F.; Castiglioni, S.; Covaci, A.; de Voogt, P.; Emke, E.; Kasprzyk-Hordern, B.; Ort, C.; Reid, M.; Sancho, J. V.; Thomas, K. V.; et al. *Mass Spectrom. Rev.*, **2018,** *37* (3), pp 258–280 (https://doi.org/10.1002/mas.21525).
38. Warwick, C.; Guerreiro, A.; Soares, A. *Biosens. Bioelectron.*, **2013,** *41* (1), pp 1–11 (https://doi.org/10.1016/j.bios.2012.07.012).
39. Campos-Mañas, M. C.; Ferrer, I.; Thurman, E. M.; Agüera, A. *Trends Environ. Anal. Chem.*, **2018,** *20* (https://doi.org/10.1016/j.teac.2018.e00059).
40. U.S. Environmental Protection Agency (EPA). *Wastewater Sampling*. Athens, United States: EPA, **2017**.
41. González-Mariño, I.; Quintana, J. B.; Rodríguez, I.; Rodil, R.; González-Peñas, J.; Cela, R. *J. Chromatogr. A*, **2009,** *1216* (48), pp 8435–8441 (https://doi.org/10.1016/j.chroma.2009.09.069).
42. Racamonde, I.; Villaverde-de-Sáa, E.; Rodil, R.; Quintana, J. B.; Cela, R. *J. Chromatogr. A*, **2012,** *1245*, pp 167–174 (https://doi.org/10.1016/j.chroma.2012.05.017).
43. Pandopulos, A. J.; Bade, R.; O'Brien, J. W.; Tscharke, B. J.; Mueller, J. F.; Thomas, K.; White, J. M.; Gerber, C. *Talanta*, **2020,** *217*, pp 121034 (https://doi.org/10.1016/j.talanta.2020.121034).
44. Bishop, N.; Jones-Lepp, T.; Margetts, M.; Sykes, J.; Alvarez, D.; Keil, D. E. *Sci. Total Environ.*, **2020,** *745*, pp 140697 (https://doi.org/10.1016/j.scitotenv.2020.140697).
45. Asicioglu, F.; Genc, M. K.; Bulbul, T. T.; Yayla, M.; Simsek, S. Z.; Adioren, C.; Mercan, S. *Water Res.*, **2021,** *190*, pp 116729 (https://doi.org/10.1016/j.watres.2020.116729).
46. Kasprzyk-Hordern, B.; Proctor, K.; Jagadeesan, K.; Lopardo, L.; O'Daly, K. J.; Standerwick, R.; Barden, R. *Environ. Int.*, **2021,** *147*, pp 106331 (https://doi.org/10.1016/j.envint.2020.106331).

47. Daglioglu, N.; Guzel, E. Y.; Atasoy, A.; Gören, I. E. *Environ. Sci. Pollut. Res.*, **2021,** *28* (12), pp 15076–15089 (https://doi.org/10.1007/s11356-020-11404-9).
48. Yuan, S.; Wang, X.; Wang, R.; Luo, R.; Shi, Y.; Shen, B.; Liu, W.; Yu, Z.; Xiang, P. *Water Sci. Technol.*, **2020,** *82* (9), pp 1771–1780 (https://doi.org/10.2166/wst.2020.445).
49. Bijlsma, L.; Celma, A.; Castiglioni, S.; Salgueiro-González, N.; Bou-Iserte, L.; Baz-Lomba, J. A.; Reid, M. J.; Dias, M. J.; Lopes, A.; Matias, J.; et al. *Sci. Total Environ.*, **2020,** *725* (https://doi.org/10.1016/j.scitotenv.2020.138376).
50. Bírošová, L.; Lépesová, K.; Grabic, R.; Mackuľak, T. *Environ. Sci. Pollut. Res.*, **2020,** *27* (12), pp 13501–13511 (https://doi.org/10.1007/s11356-020-07950-x).
51. Ng, K. T.; Rapp-Wright, H.; Egli, M.; Hartmann, A.; Steele, J. C.; Sosa-Hernández, J. E.; Melchor-Martínez, E. M.; Jacobs, M.; White, B.; Regan, F.; et al. *J. Hazard. Mater.*, **2020,** *398*, 122933 (https://doi.org/10.1016/j.jhazmat.2020.122933).
52. Gonçalves, R.; Ribeiro, C.; Cravo, S.; Cunha, S. C.; Pereira, J. A.; Fernandes, J. O.; Afonso, C.; Tiritan, M. E. *J. Chromatogr. B*, **2019,** *1125*, 121731 (https://doi.org/10.1016/j.jchromb.2019.121731).
53. González-Mariño, I.; Quintana, J. B.; Rodríguez, I.; Cela, R. *J. Chromatogr. A*, **2010,** *1217* (11), pp 1748–1760 (https://doi.org/10.1016/j.chroma.2010.01.046).
54. Mao, K.; Yang, Z.; Zhang, H.; Li, X.; Cooper, J. M. *Water Res.*, **2021,** *189*, 116559 (https://doi.org/10.1016/j.watres.2020.116559).
55. Rentsch, K. M. *TrAC - Trends Anal. Chem.*, **2016,** *84*, pp 88–93 (https://doi.org/10.1016/j.trac.2016.01.028).
56. Olsen, L.; Montefiori, M.; Tran, K. P.; Jørgensen, F. S. *Bioinformatics*, **2019,** *35* (17), pp 3174–3175 (https://doi.org/10.1093/bioinformatics/btz037).
57. Gao, J.; Ellis, L. B. M. M.; Wackett, L. P. *Nucleic Acids Res.*, **2010,** *38* (1), pp D488–D491 (https://doi.org/10.1093/nar/gkp771).
58. Moyer, T. P.; Charlson, J. R.; Enger, R. J.; Dale, L. C.; Ebbert, J. O.; Schroeder, D. R.; Hurt, R. D. *Clin. Chem.*, **2002,** *48* (9), pp 1460–1471 (https://doi.org/10.1093/clinchem/48.9.1460).
59. van Wel, J. H. P.; Gracia-Lor, E.; van Nuijs, A. L. N.; Kinyua, J.; Salvatore, S.; Castiglioni, S.; Bramness, J. G.; Covaci, A.; Van Hal, G. *Drug Alcohol Depend.*, **2016,** *162*, pp 170–175 (https://doi.org/10.1016/j.drugalcdep.2016.03.002).
60. Rice, J.; Kannan, A. M.; Castrignanò, E.; Jagadeesan, K.; Kasprzyk-Hordern, B. *Sci. Total Environ.*, **2020,** *735*, 139433 (https://doi.org/10.1016/j.scitotenv.2020.139433).
61. Bade, R.; White, J. M.; Nguyen, L.; Pandopulos, A. J.; Gerber, C. *Drug Alcohol Depend.*, **2020,** *216*, 108315 (https://doi.org/10.1016/j.drugalcdep.2020.108315).
62. McKay, S.; Tscharke, B.; Hawker, D.; Thompson, K.; O'Brien, J.; Mueller, J. F.; Kaserzon, S. *Sci. Total Environ.*, **2020,** *704* , 135891 (https://doi.org/10.1016/j.scitotenv.2019.135891).
63. Gago-Ferrero, P.; Bletsou, A. A.; Damalas, D. E.; Aalizadeh, R.; Alygizakis, N. A.; Singer, H. P.; Hollender, J.; Thomaidis, N. S. *J. Hazard. Mater.*, **2020,** *387*, 121712 (https://doi.org/10.1016/j.jhazmat.2019.121712).
64. Fallati, L.; Castiglioni, S.; Galli, P.; Riva, F.; Gracia-Lor, E.; González-Mariño, I.; Rousis, N. I.; Shifah, M.; Messa, M. C.; Strepparava, M. G.; et al. *Sci. Total Environ.*, **2020,** *698*, 134207 (https://doi.org/10.1016/j.scitotenv.2019.134207).
65. Mackuľak, T.; Grabic, R.; Špalková, V.; Belišová, N.; Škulcová, A.; Slavík, O.; Horký, P.; Gál, M.; Filip, J.; Híveš, J.; et al. *Environ. Sci. Pollut. Res.*, **2019,** *26* (31), pp 31812–31821 (https://doi.org/10.1007/s11356-019-06290-9).
66. Helander, A.; Beck, O. *J. Anal. Toxicol.*, **2005,** *29* (5), pp 270–274 (https://doi.org/10.1093/jat/29.5.270).
67. Reid, M. J.; Langford, K. H.; Mørland, J.; Thomas, K. V. *Alcohol. Clin. Exp. Res.*, **2011,** *35* (9), pp 1593–1599 (https://doi.org/10.1111/j.1530-0277.2011.01505.x).

68. Montgomery, A. B.; O'Rourke, C. E.; Subedi, B. *Sci. Total Environ.*, **2021,** *752* , 141712 (https://doi.org/10.1016/j.scitotenv.2020.141712).
69. Devault, D. A.; Peyré, A.; Jaupitre, O.; Daveluy, A.; Karolak, S. *Forensic Sci. Int.*, **2020,** *314* (https://doi.org/10.1016/j.forsciint.2020.110355).
70. Sulej-Suchomska, A. M.; Klupczynska, A.; Dereziński, P.; Matysiak, J.; Przybyłowski, P.; Kokot, Z. J. *Sci. Rep.*, **2020,** *10* (1), pp 81–87 (https://doi.org/10.1038/s41598-020-61628-5).
71. Croft, T. L.; Huffines, R. A.; Pathak, M.; Subedi, B. *J. Hazard. Mater.*, **2020,** *384*, 121306 (https://doi.org/10.1016/j.jhazmat.2019.121306).
72. Devault, D. A.; Amalric, L.; Bristeau, S.; Cruz, J.; Tapie, N.; Karolak, S.; Budzinski, H.; Lévi, Y. *Environ. Sci. Pollut. Res.*, **2021,** *28* (9), pp 10940–10966 (https://doi.org/10.1007/s11356-020-10868-z).
73. Cruz-Cruz, C.; Vidaña-Pérez, D.; Kalb, M. M.; Martínez-Ruiz, M. J.; Olaiz-Fernández, G.; Hernández-Lezama, L. F.; Hernández-Ávila, M.; Barrientos-Gutiérrez, T. *Salud Publica Mex.*, **2019,** *61* (4), pp 461–469 (https://doi.org/10.21149/9819).
74. Apul, O. G.; Rowles, L. S.; Khalid, A.; Karanfil, T.; Richardson, S. D.; Saleh, N. B. *Environ. Int.*, **2020,** *137*, 105586 (https://doi.org/10.1016/j.envint.2020.105586).
75. Lemas, D. J.; Loop, M. S.; Duong, M.; Schleffer, A.; Collins, C.; Bowden, J. A.; Du, X.; Patel, K.; Ciesielski, A. L.; Ridge, Z.; et al. *Sci. Total Environ.*, **2021,** *764* , 143963 (https://doi.org/10.1016/j.scitotenv.2020.143963).
76. Du, P.; Liu, X.; Zhong, G.; Zhou, Z.; Thomes, M. W.; Lee, C. W.; Bong, C. W.; Zhang, X.; Hao, F.; Li, X.; et al. *Int. J. Environ. Res. Public Health*, **2020,** *17* (3), pp 1–11 (https://doi.org/10.3390/ijerph17030889).
77. González-Mariño, I.; Baz-Lomba, J. A.; Alygizakis, N. A.; Andrés-Costa, M. J.; Bade, R.; Bannwarth, A.; Barron, L. P.; Been, F.; Benaglia, L.; Berset, J. D.; et al. *Addiction*, **2020,** *115* (1), pp 109–120 (https://doi.org/10.1111/add.14767).
78. Celma, A.; Sancho, J. V.; Salgueiro-González, N.; Castiglioni, S.; Zuccato, E.; Hernández, F.; Bijlsma, L. *J. Chromatogr. A*, **2019,** *1602*, pp 300–309 (https://doi.org/10.1016/j.chroma.2019.05.051).
79. Kim, K. Y.; Oh, J. E. *J. Hazard. Mater.*, **2020,** *396*, 122622 (https://doi.org/10.1016/j.jhazmat.2020.122622).
80. Shao, X. T.; Liu, Y. S.; Tan, D. Q.; Wang, Z.; Zheng, X. Y.; Wang, D. G. *Environ. Sci. Pollut. Res.*, **2020,** *27* (8), pp 8157–8165 (https://doi.org/10.1007/s11356-019-07504-w).
81. Zhang, X.; Huang, R.; Li, P.; Ren, Y.; Gao, J.; Mueller, J. F.; Thai, P. K. *Environ. Sci. Pollut. Res.*, **2019,** *26* (23), pp 23593–23602 (https://doi.org/10.1007/s11356-019-05575-3).
82. Kasprzyk-Hordern, B.; Baker, D. R. *Sci. Total Environ.*, **2012,** *423*, pp 142–150 (https://doi.org/10.1016/j.scitotenv.2012.02.019).
83. González-Mariño, I.; Zuccato, E.; Santos, M. M.; Castiglioni, S. *Water Res.*, **2017,** *115*, pp 1–8 (https://doi.org/10.1016/j.watres.2017.01.063).
84. Mardal, M.; Kinyua, J.; Ramin, P.; Miserez, B.; Van Nuijs, A. L. N.; Covaci, A.; Meyer, M. R. *Drug Test. Anal.*, **2017,** *9* (1), pp 106–114 (https://doi.org/10.1002/dta.1957).
85. Sarhill, N.; Walsh, D.; Nelson, K. A. *Support. Care Cancer*, **2001,** *9* (2), pp 84–96 (https://doi.org/10.1007/s005200000183).
86. https://www.drugabuse.gov/drug-topics/opioids [Accessed on 15 January 2021].
87. Smith, M. L.; Nichols, D. C.; Underwood, P.; Fuller, Z.; Moser, M. A.; LoDico, C.; Gorelick, D. A.; Newmeyer, M. N.; Concheiro, M.; Huestis, M. A. *Forensic Sci. Int.*, **2014,** *241*, pp 87–90 (https://doi.org/10.1016/j.forsciint.2014.04.042).
88. Thai, P. K.; Lai, F. Y.; Bruno, R.; van Dyken, E.; Hall, W.; O'Brien, J.; Prichard, J.; Mueller, J. F. *Environ. Int.*, **2016,** *94*, pp 307–314 (https://doi.org/10.1016/j.envint.2016.05.033).
89. Valtier, S.; Bebarta, V. S. *J. Anal. Toxicol.*, **2012,** *36* (7), pp 507–514 (https://doi.org/10.1093/jat/bks058).

90. McDonough, P. C.; Levine, B.; Vorce, S.; Jufer, R. A.; Fowler, D. *J. Forensic Sci.*, **2008,** *53* (3), pp 752–754 (https://doi.org/10.1111/j.1556-4029.2008.00730.x).
91. Moore, K. A.; Ramcharitar, V.; Levine, B.; Fowler, D. *J. Anal. Toxicol.*, **2003,** *27* (6), pp 346–352 (https://doi.org/10.1093/jat/27.6.346).
92. Lugo, R. A.; Kern, S. E. *J. Pain Palliat. Care Pharmacother.*, **2004,** *18* (4), pp 17–30 (https://doi.org/10.1300/J354v18n04_03).
93. Wu, W. N.; McKown, L. A.; Codd, E. E.; Raffa, R. B. *Eur. J. Drug Metab. Pharmacokinet.*, **2002,** *27* (3), pp 193–197 (https://doi.org/10.1007/BF03190457).
94. Ardakani, Y. H.; Rouini, M. R. *J. Pharm. Biomed. Anal.*, **2007,** *44* (5), pp 1168–1173 (https://doi.org/10.1016/j.jpba.2007.04.012).
95. Barbosa, J.; Faria, J.; Queirós, O.; Moreira, R.; Carvalho, F.; Dinis-Oliveira, R. J. *Drug Metab. Rev.*, **2016,** *48* (4), pp 577–592 (https://doi.org/10.1080/03602532.2016.1229788).
96. Rouguieg, K.; Picard, N.; Sauvage, F.-L. L.; Gaulier, J.-M. M.; Marquet, P. *Drug Metab. Dispos.*, **2010,** *38* (1), pp 40–45 (https://doi.org/10.1124/dmd.109.029546).
97. Poklis, A.; Backer, R. *J. Anal. Toxicol.*, **2004,** *28* (6), pp 422–425 (https://doi.org/10.1093/jat/28.6.422).
98. Logan, B. K.; Mohr, A. L. A.; Friscia, M.; Krotulski, A. J.; Papsun, D. M.; Kacinko, S. L.; Ropero-Miller, J. D.; Huestis, M. A. *J. Anal. Toxicol.*, **2017,** *41* (7), pp 573–610 (https://doi.org/10.1093/jat/bkx031).
99. Wilde, M.; Pichini, S.; Pacifici, R.; Tagliabracci, A.; Busardò, F. P.; Auwärter, V.; Solimini, R. *Front. Pharmacol.*, **2019,** *10*, pp 1–16 (https://doi.org/10.3389/fphar.2019.00238).
100. Watanabe, S.; Vikingsson, S.; Roman, M.; Green, H.; Kronstrand, R.; Wohlfarth, A. *AAPS J.*, **2017,** *19* (4), pp 1102–1122 (https://doi.org/10.1208/s12248-017-0070-z).
101. Armenian, P.; Vo, K. T.; Barr-Walker, J.; Lynch, K. L. *Neuropharmacology*, **2018,** *134*, pp 121–132 (https://doi.org/10.1016/j.neuropharm.2017.10.016).
102. Mandrioli, R.; Mercolini, L.; Raggi, M. *Curr. Drug Metab.*, **2008,** *9* (8), pp 827–844 (https://doi.org/10.2174/138920008786049258).
103. Temte, V.; Kjeldstadli, K.; Bruun, L. D.; Birdal, M.; Bachs, L.; Karinen, R.; Middelkoop, G.; Øiestad, E.; Høiseth, G. *J. Anal. Toxicol.*, **2019,** *43* (2), pp 104–111 (https://doi.org/10.1093/jat/bky062).
104. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). *The misuse of benzodiazepines among high-risk opioid users in Europe*. EMCDDA, Lisbon, Portugal, **2018**.
105. Mandrioli, R.; Mercolini, L.; Raggi, M. A. *Curr. Drug Metab.*, **2011,** *11* (9), pp 815–829 (https://doi.org/10.2174/138920010794328887).
106. United Nations Office on Drugs and Crime (UNODC). *Recommended Methods for the Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens*. UNODC, New York, United States, **1997**.
107. Gushgari, A. J.; Driver, E. M.; Steele, J. C.; Halden, R. U. *J. Hazard. Mater.*, **2018,** *359*, pp 437–444 (https://doi.org/10.1016/j.jhazmat.2018.07.073).
108. Steentoft, A.; Linnet, K. *Forensic Sci. Int.*, **2009,** *184* (1–3), pp 74–79 (https://doi.org/10.1016/j.forsciint.2008.12.004).
109. Chouinard, G.; Lefko-Singh, K.; Teboul, E. *Cell. Mol. Neurobiol.*, **1999,** *19* (4), pp 533–552 (https://doi.org/10.1023/A:1006943009192).
110. Schifano, F.; Chiappini, S.; Corkery, J. M.; Guirguis, A. *Int. J. Neuropsychopharmacol.*, **2019,** *22* (4), pp 270–277 (https://doi.org/10.1093/ijnp/pyz007).
111. Oliveira, T. S.; Murphy, M.; Mendola, N.; Wong, V.; Carlson, D.; Waring, L. *Sci. Total Environ.*, **2015,** *518–519* , pp 459–478 (https://doi.org/10.1016/j.scitotenv.2015.02.104).
112. Marta, R. F. L. O. *Drug Metab. Rev.*, **2019,** *51* (3), pp 378–387 (https://doi.org/10.1080/03602532.2019.1638931).
113. Craven, R. *Anaesthesia*, **2007,** *62* (s1), pp 48–53 (https://doi.org/10.1111/j.1365-2044.2007.05298.x).
114. Dinis-Oliveira, R. J. *Forensic Sci. Res.*, **2017,** *2* (1), pp 2–10 (https://doi.org/10.1080/20961790.2017.1285219).

115. Lin, A. Y. C.; Lee, W. N.; Wang, X. H. *Water Res.*, **2014,** *53*, pp 351–360 (https://doi.org/10.1016/j.watres.2014.01.022).
116. Bosman, I. J.; Lusthof, K. J. *Forensic Sci. Int.*, **2003,** *133* (1–2), pp 17–21 (https://doi.org/10.1016/S0379-0738(03)00044-6).
117. Busardo, F. P.; Kyriakou, C. *Recent Pat. Biotechnol.*, **2015,** *8* (3), pp 206–214 (https://doi.org/10.2174/1872208309666150504143155).
118. Mason, P. E.; Kerns, W. P. *Acad. Emerg. Med.*, **2002,** *9* (7), pp 730–739 (https://doi.org/10.1111/j.1553-2712.2002.tb02154.x).
119. Haller, C.; Thai, D.; Jacob, P.; Dyer, J. E. *J. Anal. Toxicol.*, **2006,** *30* (6), pp 360–364 (https://doi.org/10.1093/jat/30.6.360).
120. Diamanti, K.; Aalizadeh, R.; Alygizakis, N.; Galani, A.; Mardal, M.; Thomaidis, N. S. *Sci. Total Environ.*, **2019,** *685*, pp 1058–1065 (https://doi.org/10.1016/j.scitotenv.2019.06.173).
121. Freni, F.; Pezzella, S.; Vignali, C.; Moretti, M.; Cisini, S.; Rossetti, C.; Ravizza, R.; Motta, M.; Groppi, A.; Morini, L. *Forensic Sci. Int.*, **2019,** *304*, pp 109915 (https://doi.org/10.1016/j.forsciint.2019.109915).
122. O'Rourke, C. E.; Subedi, B. *Environ. Sci. Technol.*, **2020,** *54* (11), pp 6661–6670 (https://doi.org/10.1021/acs.est.0c00250).
123. Bade, R.; White, J. M.; Nguyen, L.; Tscharke, B. J.; Mueller, J. F.; O'Brien, J. W.; Thomas, K. V.; Gerber, C. *Sci. Total Environ.*, **2020,** *731*, 139209 (https://doi.org/10.1016/j.scitotenv.2020.139209).
124. Bade, R.; White, J. M.; Chen, J.; Baz-Lomba, J. A.; Been, F.; Bijlsma, L.; Burgard, D. A.; Castiglioni, S.; Salgueiro-Gonzalez, N.; Celma, A.; et al. *Water Res.*, **2021,** *193*, 116891 (https://doi.org/10.1016/j.watres.2021.116891).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 35-51
doi: 10.30744/brjac.2179-3425.RV-32-2021

BrJAC

REVIEW

Chemometric Approaches in Questioned Documents

Roberta Petry Gorziza[1*], Marina González[1*], Carina Maria Bello de Carvalho[2], Rafael Scorsatto Ortiz[2,3], Marco Flores Ferrão[4,5], Renata Pereira Limberger[1,3]

[1]*Departamento de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, Azenha, 90610-000, Porto Alegre, RS, Brazil*

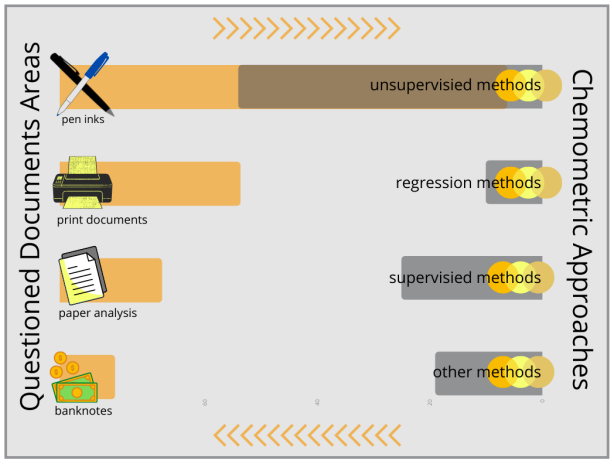
[2] *Polícia Federal, Divisão Técnico-Científica do Rio Grande do Sul, Av. Ipiranga, 1365, Azenha, 90610-093, Porto Alegre, RS, Brazil*

[3]*Instituto Nacional de Ciência e Tecnologia Forense (INCT Forense), Porto Alegre, RS, Brazil*

[4]*Departamento de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS Brazil*

[5]*Instituto Nacional de Ciência e Tecnologia em Bioanalítica (INCT Bioanalítica), Campinas, SP, Brazil*

*These authors contributed equally.



Questioned documents comprehend analysis of identity theft, forged signatures or texts, documents alterations and falsification of security documents or banknotes. Questions involving inks or paper require chemical analysis, and multivariate analysis or chemometrics has been an emerging tool for data evaluation and interpretation after instrumental data collection in this area. The goal of this study is to identify previous articles that applied multivariate analysis within questioned documents for forensic purposes. The search for articles was performed in four databases (Google Scholar, Science Direct, Pubmed and Scopus). Sixty studies, published in the last ten years, were selected. Thirty-four articles described pen inks analysis; fourteen articles comprehended printed documents studies; eight articles evaluated paper analysis, and four articles included banknotes analysis. Spectroscopy, mass spectrometry, chromatography, thermo gravimetric analysis and multivariate image analysis were the analytical methods applied to collect chemical data. Chemometrics methods included mainly unsupervised pattern recognition techniques, regression methods, and supervised pattern recognition techniques, amongst other methods. This review summarized and discussed multivariate analysis techniques applied in different questioned documents sub-areas, highlighting the importance of this knowledge for forensic analysts. In addition, it shows new research topics such as different printing and pen inks, papers and security documents analysis herein not included.

Keywords: questioned documents, inks, paper, chemometrics, multivariate analysis.

Cite: Gorziza, R. P.; González, M.; de Carvalho, C. M. B.; Ortiz, R. S.; Ferrão, M. F.; Limberger, R. P. Chemometric Approaches in Questioned Documents: A Review. *Braz. J. Anal. Chem.,* 2022, *9* (34), pp 35-51. doi: http://dx.doi.org/10.30744/brjac.2179-3425.RV-32-2021

Submitted 04 March 2021, Resubmitted 27 April 2021, Accepted 24 May 2021, Available online 11 June 2021.

INTRODUCTION

Questioned documents is an important area of Forensic Sciences, comprehending document fraud analysis such as identity theft, forged signatures or texts, documents alterations and falsification of security documents or banknotes. While physical analysis is widely applied in questioned documents, many situations demand the chemical analysis of inks and support paper [1–3].

Writing and printing inks are formulas composed by pigments or dyes, resins, solvents, driers and drying oils, extenders and additives, such as surfactants, conductive salts, biocides, composite carriers, or adhesion promoters [4]. Writing inks comprises different types of pens, such as ballpoint pens (oil-based inks composed of dyes or pigments and organic solvents), gel pens (water-based inks composed mainly of pigments), and rollerball, fountain and felt-tip pens (water-based inks composed of dyes and ethylene glycol) [4]. The most prevalent printing inks are inkjets and toners. Inkjet inks can be solvent-based, water-based, UV curable, and phase-change, depending on the printer instrument; toner inks are dry powders or liquid-dispersed powders, mainly constituted of pigments and resins [4].

The paper production from wood demands pulping and bleaching process, using chemical agents such as sodium sulfide (Na_2S), sodium hydroxide (NaOH), chlorine monoxide (Cl_2O), calcium carbonate ($CaCO_3$), ozone or oxygen, with metallic oxides. Thus, the final paper products contain cellulose and particular compounds, specific of each production set [1].

In questioned documents forensic examination, inks and papers characterization and transformation are often required for differentiation and age estimation, respectively [2]. In addition, counterfeit security documents and banknotes must be differentiated from the authentic ones. In this regard, numerous different analytical methods have been studied [1,3,5–8]. Many analytical methods, for the chemical analysis of inks and paper, produce a huge amount of data outputs, demanding an efficient and accurate method for results interpretation. Multivariate analysis in chemistry, or chemometrics, are statistical approaches for chemical data analysis [9,10]. Additionally, it can be used for planning and simulations of chemical experiments [9,10]. Chemometrics offer trustworthy tests for classification, discrimination or models development for different chemical samples datasets [11]. Thus, chemometrics are emerging tools in Forensic Sciences , as mathematical and statistical tools are potential methods to enrich and to correlate forensic analytical data in many areas besides questioned documents (biological, physical and chemical sciences, toxicology, ballistic, anthropology) [11]. While many analytical methods are applied for inks and paper examination, chemometrics can improve results interpretation and data presentation towards a forensic document investigation. Chemometrics increases the data analysis objectivity in a comparison study, and it is a powerful tool to perform databases research. Hence, chemometrics is a growing trend in questioned documents data analysis, and this knowledge is important for forensics analysts [3].

The aim of this work is to review questioned documents topics which were studied by multivariate analysis or chemometrics approaches, highlighting its importance in the field. This review summarizes research studies that applied chemometrics in questioned documents analysis, and if offers a brief explanation of the most applied chemometrics techniques. It also identifies the most covered research topics in questioned documents, and the analytical methodologies performed prior to chemometrics analysis. Therefore, this review detects areas and methodologies that could be explored in further research.

MATERIALS AND METHODS

Figure 1 summarizes the articles search method. The search for articles was performed in November and December 2020, on four different databases, using the descriptor *"multivariate analysis" or "chemometrics" and questioned documents*. The search was performed in the last ten years (2010-2020). The initial search from Google Scholar (1610 results/55 articles selected), Science Direct (190 results/16 articles selected), Pubmed (70 results/10 articles selected) and Scopus (21 results/18 articles selected) retrieved 62 articles, when analyzing titles and abstracts. The inclusion criteria consisted of any study within the questioned documents area, as long as it applied multivariate analysis or chemometric data analysis. Questioned documents articles that did not describe these techniques were excluded of the search. Only original

articles written in English or Portuguese were included. After the exclusion of repeated and review papers, 50 articles were selected and organized by area (pen inks, printed documents, paper and banknotes analysis).

In order to find as many articles as possible, complementary searches were performed on Google Scholar. For these searches, the term “questioned documents” was substituted from the descriptor *“multivariate analysis” or “chemometrics” and questioned documents*, by using specific terms related to each area, one at a time: “pen inks”, “printed”, “prints”, “printing”, “inkjet”, “toners”, “stamps”, “packages”, “packaging”, “banknotes”, and “paper analysis”. Finally, references from questioned documents review papers [1,3] were also analyzed, to retrieve any missing article applying chemometric approaches in the last ten years.

A total of 60 studies applying multivariate analysis/chemometrics in the questioned documents field were selected.

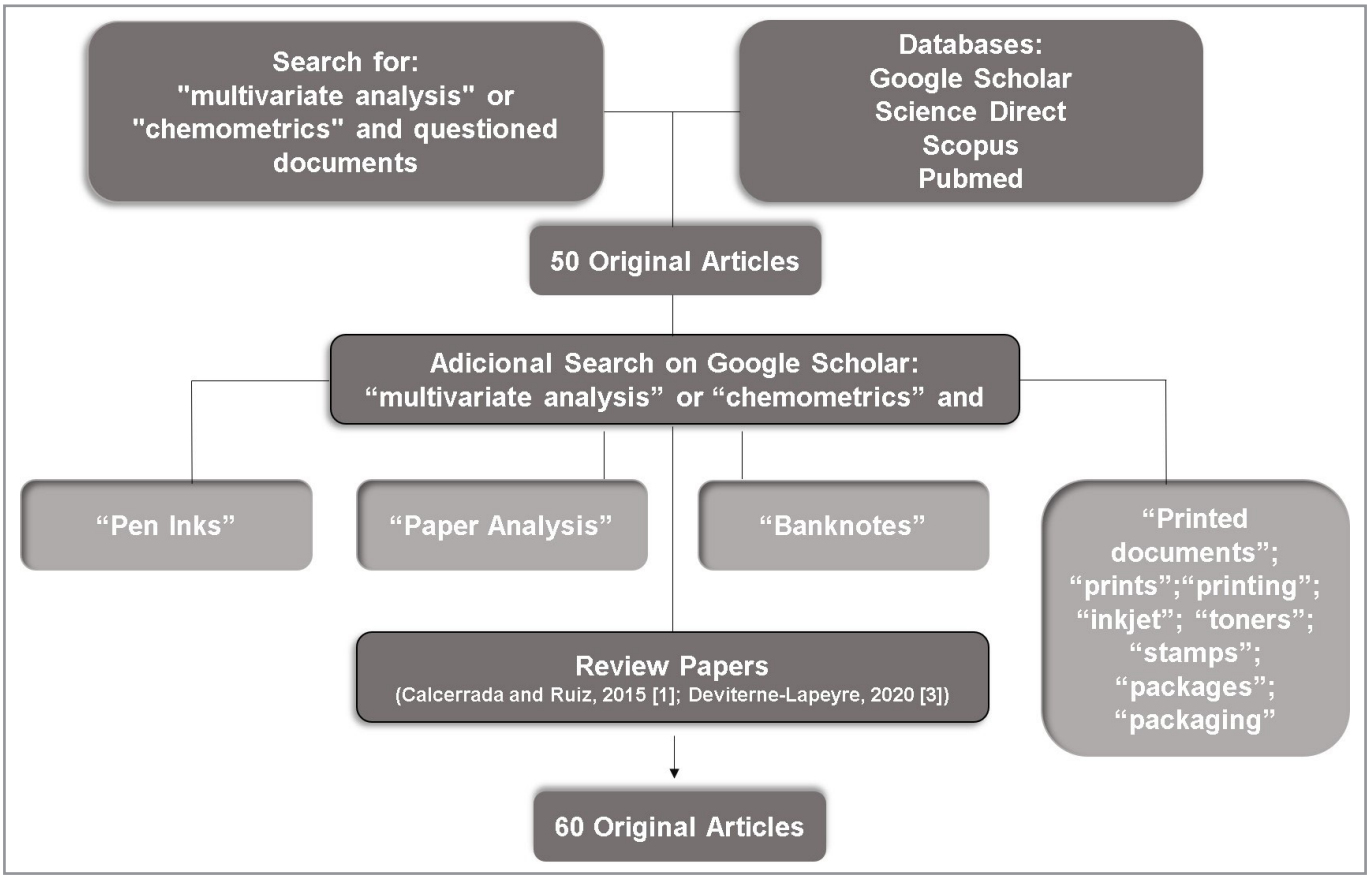


Figure 1. Articles search strategy.

RESULTS AND DISCUSSION

Four major questioned documents areas were contemplated with multivariate analysis/chemometrics studies. Figure 2 shows the articles distribution among the different areas.

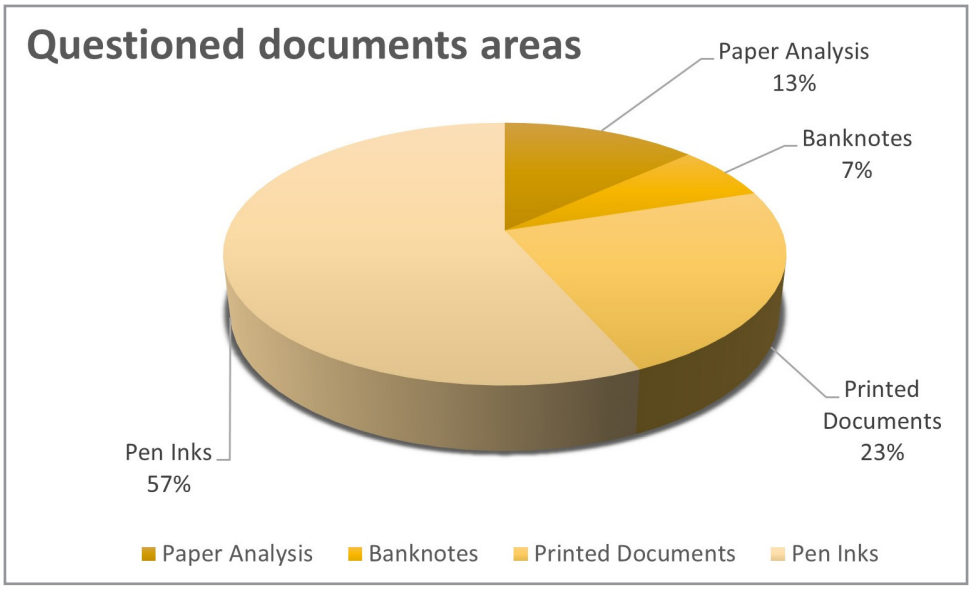


Figure 2. Multivariate analysis/chemometric articles distribution among different questioned documents areas.

Pen inks analysis comprehended 34 articles (57% from total studies). Table I summarizes these studies. Among these, 7 articles utilized mainly spectroscopy techniques to study ballpoint pen ink dating; 16 articles utilized spectroscopy, mass spectrometry and multivariate image analysis to study ballpoint pen inks differentiation; 4 studies applied spectroscopy techniques for marker pen inks, gel pen inks and fiber tip pen inks differentiation, and 4 studies evaluated different types – or classes – of pens, using multivariate image analysis, mass spectrometry and spectroscopy methods. Thus, 4 studies analyzed pen ink crossing lines, between ballpoint pens, between gel pens or between ballpoint and gel pens, using spectroscopy and mass spectrometry techniques.

Table II shows 14 studies applied on printed documents and printer inks, representing 23% from total studies. These documents comprised food packages, pharmaceutical packages, and stamps. Printed inks included toners, inkjets, off set and intaglio inks. Also, crossing lines studies involving printing inks, papers and pen inks were conducted. Although spectroscopic methods were the most applied techniques in this area, mass spectrometry and multivariate image analysis were also studied.

Table III shows 8 studies applied on paper analysis, which represents 13% from total studies. Among these studies, 3 articles aimed to characterize or to discriminate different types of paper, while 5 articles focused on paper age estimation. Spectroscopy methods were the most prevalent techniques applied for paper analysis, along with analytical pyrolysis combined with gas chromatography/mass spectrometry and thermo gravimetric analysis.

The last questioned documents area comprehended banknotes analysis. Table IV shows 4 studies (7% of total studies) of banknotes – mainly Brazilian – classification and counterfeit/authentic bills differentiation, applying spectroscopy techniques, spectrometry, and multivariate image analysis.

Table I. Studies concerning pen inks analysis and multivariate analysis/chemometrics approaches

Main Objectives	Samples	Analytical Method	Multivariate Analysis	Software	Reference
Ballpoint pen ink dating	11 blue ballpoint pens from 6 brands	Vis-MSP[1]	PCA[20], HCA[21], and OPLS[22]	SIMCA 15.0.2	Ortiz-Herrero et al. [12]
Ballpoint pen ink dating	505 ballpoint pens inks (384 blue and 121 black)	DI-MS[2]	ULT[23]	Analyst® TF 1.6	Costa et al. [13]
Ballpoint pen ink dating	37 blue and 27 black ballpoint pens	ATR-FTIR[3]	PCA[20] and HCA[21]	Chemostat®	Bello de Carvalho et al. [14]
Ballpoint pen ink dating	4 black and 1 blue ballpoint pens	UV-Vis-NIR[4]	PLS[24]	SIMCA 13.0	Ortiz-Herrero et al. [15]
Ballpoint pen ink dating	Blue ballpoint pen	UV-Vis[5]	PCA[20] and MLR[25]	SPSS®	Sharma & Kumar. [16]
Ball tip pen inks dating	35 blue ball tips pens	UV-Vis[5]	PCA[20], LDA[26] and PLSR[27]	Unscrambler X 10.4	Sauzier et al. [17]
Ballpoint pen inks characterization and dating	10 blue ballpoint pens	UV-Vis[5], IR[6] and HPTLC[7]	PCA[20]	Microsoft Excel	Senior et al. [18]
To differentiate ballpoint pen inks	12 blue ballpoint pens from 9 brands	MIA[8]/Smartphone	PCA[20], HCA[21], and PLS-DA[28]	PhotometrixPRO®	Gorziza et al. [19]
To differentiate ballpoint pen inks	7 different blue ballpoint pens	Raman Imaging	MF-ICA[29]	MATLAB®	Teixeira et al. [20]
To differentiate ballpoint pen inks	33 blue and 36 black ballpoint pens	Orbitrap MS[9]	PCA[20] and HCA[21]	Chemostat®	Bello de Carvalho et al. [21]
To differentiate ballpoint pen inks	11 unbranded black ballpoint pens	FTIR[10]	PCA[20] and HCA[21]	Minitab®	Kamil et al. [22]
To differentiate ballpoint pen inks	36 black ballpoint pens from 6 brands	PS-MS[11]	PLS[24]	MATLAB®	Amador et al. [23]
To differentiate ballpoint pen inks	57 blue ballpoint pens	ATR-FTIR[3] and HPTLC[7]	PCA[20]	SPSS-20®	Sharma and Kumar. [24]
To differentiate ballpoint pen inks	30 ballpoint pens (blue and red) from 5 brands	Raman Spectroscopy	PCA[20]	Minitab®	Asri et al. [25]
To differentiate ballpoint pen inks	24 black ballpoint pens of 6 different brands	micro-ATR-FTIR[3]	PCA[20]	SPSS-15®	Lee et al. [26]
To differentiate ballpoint pen inks	155 black ballpoint pens of 9 different brands	micro-ATR-FTIR[3]	PCA[20]	SPSS-15®	Lee et al. [27]
To differentiate ballpoint pen inks	57 blue ballpoint pens	UV-Vis-NIR[4]	PCA[20]	SPSS-20®	Kumar and Sharma [28]
To differentiate ballpoint pen inks	24 blue ballpoint pens from 6 brands	FTIR[10]	PCA[20] and HCA[21]	XLSTAT 2011	Halim et al. [29]
To differentiate ballpoint pen inks	14 pen ink classes of blue ballpoint pens	Raman Spectroscopy	PCA[20], HCA[21] and PLS-DA[28]	Not informed	Borba et al. [30]
To differentiate ballpoint pen inks	21 blue ballpoint pens from 10 brands	LA-ICP-MS[12]	MANOVA[30]	SPSS®	Alamilla et al. [31]

Table I. Studies concerning pen inks analysis and multivariate analysis/chemometrics approaches (Continuation)

Main Objectives	Samples	Analytical Method	Multivariate Analysis	Software	Reference
To differentiate model variation of Papermate® pens	37 black ballpoint pens	micro-ATR-FTIR[3]	PCA[20]	SPSS-12®	Lee et al. [32]
To differentiate marker pen inks	24 markers pen inks	UV-Vis[5] and UV-NIR[13]	PCA[20] and DA[31]	SPSS-16®	Sharma et al. [33]
To differentiate gel pens	45 gel pen inks (blue, red and black) from 5 brands	HSI[14]	PCA[20]	Minitab®	Asri et al. [34]
To differentiate gel pens	10 gel pen black inks	LIBS[15]	PCA[20]	N/I	Ballah and Nassef [35]
To differentiate gel pens	45 black gel pen inks	HSI[14]	PCA[20], HCA[21] and SAM[32]	Statistica	Chlebda et al. [36]
To differentiate fiber tip pens	48 fiber-tip pens (black, red, green and blue)	ATR-FTIR[3]	PCA[20] and LDA[26]	Unscrambler X 10.5.1	Yadav et al. [37]
To differentiate black pens	55 different classes of black pens	VSC®6000[16] and LC/MS-TOF[17]	PLS-DA[28]	MATLAB®	Silva et al. [38]
To differentiate blue pens	25 different classes of blue pens	VSC®6000[16]	PLS-DA[28]	MATLAB®	Silva et al. [39]
To differentiate pen inks	42 blue pen inks from different types and brands	MIA[8]/iPhone®	PLS-DA[28]	MATLAB®	Valderrama and Valderrama [40]
To differentiate pen inks	16 black pen inks from different types and brands	HSI-NIR[18]	PCA[20] and PP[33]	MATLAB®	Pereira et al. [41]
To determine ballpoint pens crossing lines order	3 black ballpoint pens	ToF-SIMS[19]	PCA[20] and MCR[34]	MATLAB®	Goacher et al. [42]
To determine ballpoint pens crossing lines order	7 blue ballpoints pen brands	VSC6000®[16]	MCR-ALS[35]	MATLAB®	Martins et al. [43]
To determine gel pens crossing lines order	8 blue and black gel pens from different brands	Raman Spectroscopy	MCR-ALS[35]and PLS-DA[28]	MATLAB®	Brito et al. [44]
To determine ballpoint and gel pens crossing lines order	21 black ballpoint and gel pens from different brands	HSI-NIR[18]	PCA[20], MCR-ALS[35] and PLS-DA[28]	MATLAB®	Brito et al. [45]

N/I: not informed; [1]**Vis-MSP:** Visivel-Microspectrophotometry; [2]**DI-MS:** Direct-Injection Mass Spectrometry; [3]**ATR-FTIR:** Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy; [4]**UV-Vis-NIR:** Ultraviolet-Visible-Near-Infrared Spectroscopy; [5]**UV-Vis:** Ultraviolet-Visible Spectroscopy; [6]**IR:** Infrared; [7]**HPTLC:** High-Performance Thin Layer Chromatography; [8]**MIA:** Multivariate Image Analysis; [9]**Orbitrap MS:** Orbitrap Mass Spectrometry; [10]**FTIR:** Reflectance Fourier-Transform Infrared Spectroscopy; [11]**PS-MS:** Paper Spray Mass Spectrometry; [12]**LA-ICP-MS:** Laser Ablation Inductively Coupled Plasma Mass Spectrometry; [13]**UV-NIR:** Ultraviolet-Near-Infrared Spectroscopy; [14]**HSI:** Hyperspectral imaging; [15]**LIBS:** Laser-Induced Breakdown Spectroscopy; [16]**VSC®6000:** Video Spectral Comparator; [17]**LC/MS-TOF:** Liquid Chromatography Quadrupole Time-of-Flight; [18]**HSI-NIR:** Hyperspectral Imaging Near-Infrared; [19]**ToF-SIMS:** Time-of-Flight Secondary Ion Mass Spectrometry; [20]**PCA:** Principal Component Analysis; [21]**HCA:** Hierarchical Cluster Analysis; [22]**OPLS:** Orthogonal Partial Least Squares; [23]**ULT:** Unsupervised Linkage Threshold; [24]**PLS:** Partial Least Squares; [25]**MLR:** Multiple-linear Regression; [26]**LDA:** linear discriminate analysis; [27]**PLSR:** Partial Least Squares Regression; [28]**PLS-DA:** Partial Least Squares-Discriminant Analysis; [29]**MF-ICA:** Mean-field Approach Independent Component Analysis; [30]**MANOVA:** Multivariate Analysis of Variance; [31]**DA:** Discriminant Analysis; [32]**SAM:** Spectral Angle Mapper; [33]**MCR:** Multivariate Curve Resolution; [33]**PP:** Projection Pursuit; [34]**MCR-ALS:** Multivariate Curve Resolution with Alternating Least Squares.

Table II. Studies concerning printed documents analysis and multivariate analysis/chemometrics approaches

Main Objectives	Samples	Analytical Method	Multivariate Analysis	Software	Reference
To characterize lard on food packages inks	Not specified	FTIR[1]	PCA[15] and SIMCA[16]	Unscrambler X 10.3	Ramli et al. [46]
To identify counterfeit pharmaceutical packages	124 paperboard packages representing the secondary packaging of 6 pharmaceutical products	LIBS[2] and ATR-FTIR[3]	PCA[15], KNN[17], and LDA[18]	JMP Pro 14	Haase et al. [47]
To discriminate authentic and counterfeit stamps	8 counterfeits revenue stamps	XRF[4] Spectroscopy	PCA[15]	Pirouette 3.11	Perez et al. [48]
To classify pigments and inks	10 blue and black inks on paper	Raman Spectroscopy and LIBS[2]	PCA[15], SIMCA[16], PLS-DA[19], and SVM[20]	Unscrambler X 9.8 and 10.1	Hoehse et al. [49]
To create a database of 76 toners, 78 inkjets inks, 79 offset inks, and 86 intaglio inks	319 specimens representing four major types of printing inks	FTIR[1], SEM-EDS[5],LA-ICP-MS[6], DART-MS[7], and Py-GC-MS[8]	PLS-DA[19]	SYSTAT, JMP, Excel 2011, Plot for mac OSX, Mathematica and MATLAB®	Trejos et al. [50]
To discriminate among printing devices from laser, inkjet, and photocopier machines	45 printout samples	ATR-FTIR[3]	PCA[15], HCA[21], and LDA[18]	SPSS-20®	Kumar et al. [51]
Classification of inkjet prints	22 different printers	FT-NIR[9]	DA[22], LDA[18], and QDA[23]	Unscrambler X 10.3	Oravec et al. [52]
To correlate toners of unknown origin	10 black toners	NIR[10]	PCA[15]	V-PARVUS 2009 package	Materazzi et al. [53]
To discriminate and classify toners	40 different black toners sources each for laser printer and photocopier machines	FE-SEM-EDS[11]	PCA[15], HCA[21] and LDA[18]	Microsoft Excel and SPSS-20®	Verma et al. [54]
To discriminate and classify toners	100 samples from printouts taken from laser printers and photocopier machines	UV-Vis[12]	PCA[15], DP[24] and Varimax Rotation	SPSS-20®	Verma et al. [55]
To differentiate black toners	49 types of laser printers of latter brands	FTIR[1]	PCA[15] and MANOVA[25]	Unscrambler X	Gál et al. [56]
To discriminate paper brands and crossing lines order	12 different paper brands,2 toners and 3 blue ballpoint pens	FTIR[1] and AFM[13]	PCA[15]	Unscrambler X 10.3	Farid et al. [57]
To distinguish documents by papers and colorants	8 printer papers, marker pen inks, inkjets and printer toners	NIR[10]	PCA[15]	MATLAB®	Sugawara et al. [58]

Table II. Studies concerning printed documents analysis and multivariate analysis/chemometrics approaches (Cont)

Main Objectives	Samples	Analytical Method	Multivariate Analysis	Software	Reference
To determine the chronological order of crossing lines	1 inkjet, 1 toner, 20 blue ballpoint pens, 16 rollerball pens, 16 felt-tip pens and 8 gel pen inks	MIA[14]/iPhone®	PLS-DA[19]	MATLAB®	Valderrama et al. [59]

[1]**FTIR:** Fourier-Transform Infrared Spectroscopy; [2]**LIBS:** Laser-Induced Breakdown Spectroscopy; [3]**ATR-FTIR:** Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy; [4]**XRF:** X-Ray Fluorescence; [5]**SEM-EDS:** Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy; [6]**LA-ICP-MS:** Laser Ablation Inductively Coupled Plasma Mass Spectrometry; [7]**DART-MS:** Direct Analysis in Real Time Mass Spectrometry; [8]**Py-GC-MS:** Analytical Pyrolysis combined with Gas Chromatography-Mass Spectrometry**;** [9]**FT-NIR:** Fourier Transform Near-Infrared; [10]**NIR:** Near-Infrared; [11]**FE-SEM-EDS:** Field Emission Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy; [12]**UV-Vis:** Ultraviolet-Visible Spectroscopy; [13]**AFM:** Atomic Force Microscopy; [14]**MIA:** Multivariate Image Analysis; [15]**PCA:** Principal Component Analysis; [16]**SIMCA:** Soft Independent Modelling by Class analogy; [17]**KNM**: K-nearest neighbors; [18]**LDA:** Linear Discriminant Analysis; [19]**PLS-DA:** Partial Least Squares Discriminant Analysis; [20]**SVM:** Support Vector Machines; [21]**HCA:** Hierarchical Cluster Analysis; [22]**DA:** Discriminant Analysis; [23]**QDA:** Non-Linear (Quadratic) Classification Analyses; [24]**DP:** Discrimination Power; [25]**MANOVA:** Multivariate Analysis of Variance.

Table III. Studies concerning paper analysis and multivariate analysis/chemometrics approaches

Main Objectives	Samples	Analytical Method	Multivariate Analysis	Software for Analysis	Reference
To characterize and to discriminate paper relics	15 types of paper	ATR-FTIR[1]	PCA[4], SIMCA[5], PLS-DA[6], LS-SVM[7], PCA-LDA[8], and PLS-LDA[9]	MATLAB®	Xia et al. [60]
To discriminate papers	24 different kinds of writing/printing papers	Thermogravimetric Analysis	PCA[4]	N/I	Kumar et al. [61]
To characterize and to discriminate papers.	24 different kinds of writing/printing papers	ATR-FTIR[1]	PCA[4]	SPSS-16®	Kumar et al. [62]
Paper dating	45 books from 1940 to 1980	FTIR[2]	PCA[4], LS-SVM[7],and sPLS[10]	N/I	Xia et al. [63]
Paper dating	3 types of paper (white, recycled and notebook)	Py-GC/MS[3]	PCA[4]	SIMCA 13.0	Ortiz-Herrero et al. [64]
Paper dating	Reports from 15 different years	FTIR[2]	PLS[11] and PCA[4]	MATLAB®	Silva et al. [65]
Paper dating	6 samples of common papers	ATR-FTIR[1]	CE[12], MLR[13] and PLSR[14]	SPSS-20®	Sharma et al. [66]
Paper dating	Several types of historic paper	THz time-domain spectroscopy	PLS[11]	Unscrambler v.9.7	Trafela et al. [67]

N/I: not informed; [1]**ATR-FTIR:** Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy; [2]**FTIR:** Fourier-Transform Infrared Spectroscopy; [3]**Py-GC-MS:** Analytical Pyrolysis combined with Gas Chromatography/Mass Spectrometry; [4]**PCA:** Principal Component Analysis; [5]**SIMCA:** Soft Independent Modelling by Class Analogy; [6]**PLS-DA:** Partial Least Squares with Discriminant Analysis; [7]**LS-SVM:** Least squares support vector machines; [8]**PCA-LDA:** Principal Component Analysis-Linear Discrimination Analysis; [9]**PLS-LDA:** Partial Least Squares-Linear Discrimination Analysis; [10]**sPLS:** Sparse Partial Least Squares; [11]**PLS:** Partial Least Squares; [12]**CE:** Curve Estimation; [13]**MLR:** Multiple Linear Regression; [14]**PLSR:** Partial Least Squares Regression.

Table IV. Studies concerning banknotes analysis and multivariate analysis/chemometrics approaches

Main Objectives	Samples	Analytical Method	Multivariate Analysis	Software	Reference
Classification of banknotes	4 authentic and 12 falsified Brazilian banknotes	MIA[1]/ Smartphone	PCA[2]	Photometrix PRO®	Vittorazzi et al. [68]
Characterization of banknotes	42 counterfeit Brazilian banknotes	Portable X-ray fluorescence and Raman Spectroscopy	PCA[2] and PLS[3]	MATLAB®	Rodrigues et al. [69]
Characterization of banknotes	1 Dollar bill, 1 Euro bill and 6 Real bills	Portable X-ray Fluorescence	PCA[2]	Not informed	Appoloni et al. [70]
To differentiate authentic and counterfeit banknotes	Original and counterfeit Brazilian banknotes	Raman Spectroscopy	PLS-DA[4]	MATLAB®	Almeida et al. [71]

[1]**MIA:** Multivariate Image Analysis; [2]**PCA:** Principal Component Analysis; [3]**PLS:** Partial Least Squares; [4]**PLS-DA:** Partial Least Squares with Discriminant Analysis.

Overall, spectroscopic techniques are the most studies methods to acquire chemical data from questioned documents, prior to multivariate analysis (Figure 3). Mass spectrometry, chromatography, thermo gravimetric and x-ray-based techniques were also studied. Besides a few exceptions, most of these methods are non-destructive techniques, which is an advantage in the questioned documents area, maintaining documents integrity for counterproof. In addition, the method variability allows for many possibilities of documents analysis across different Laboratories. However, it is necessary for Forensic Experts to understand the chemometrics data analysis to conduct appropriate results interpretation and data presentation in reports.

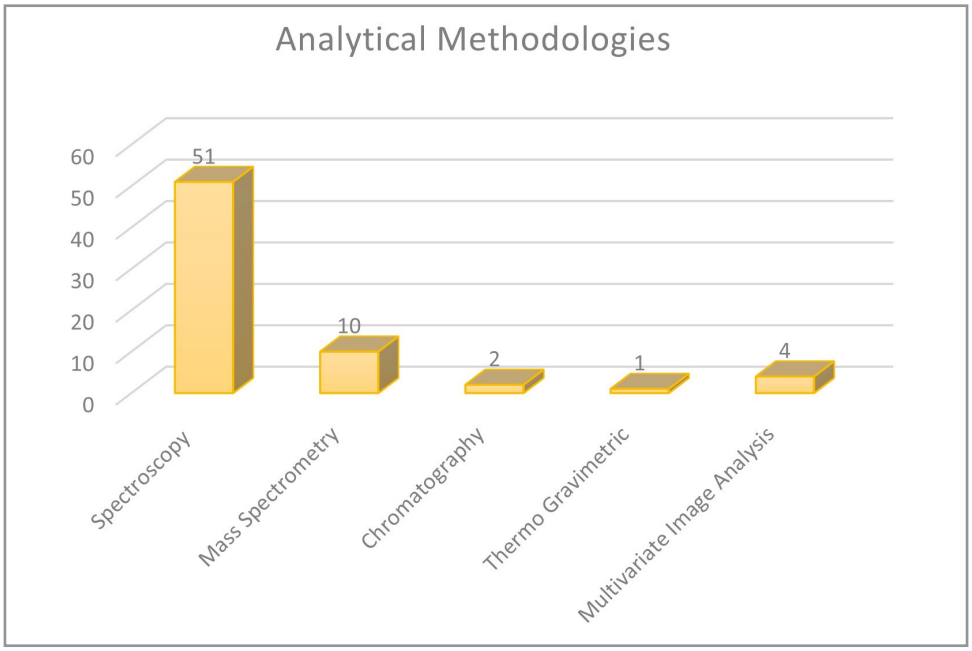


Figure 3. Analytical methodologies applied on questioned documents to capture chemical data.

Prior to multivariate analysis, all the chemical data produced with analytical methodologies (or multivariate image analysis) need to be organized into a matrix. For instance, this matrix associates each sample in lines, while the variables are displayed in columns [72,73]. Thus, sample pre-processing can be performed to minimize undesirable variables, which could rise during data acquisition and interfere with the analysis results [72,73]. Figure 4 shows the most applied multivariate analysis techniques following analytic methodologies.

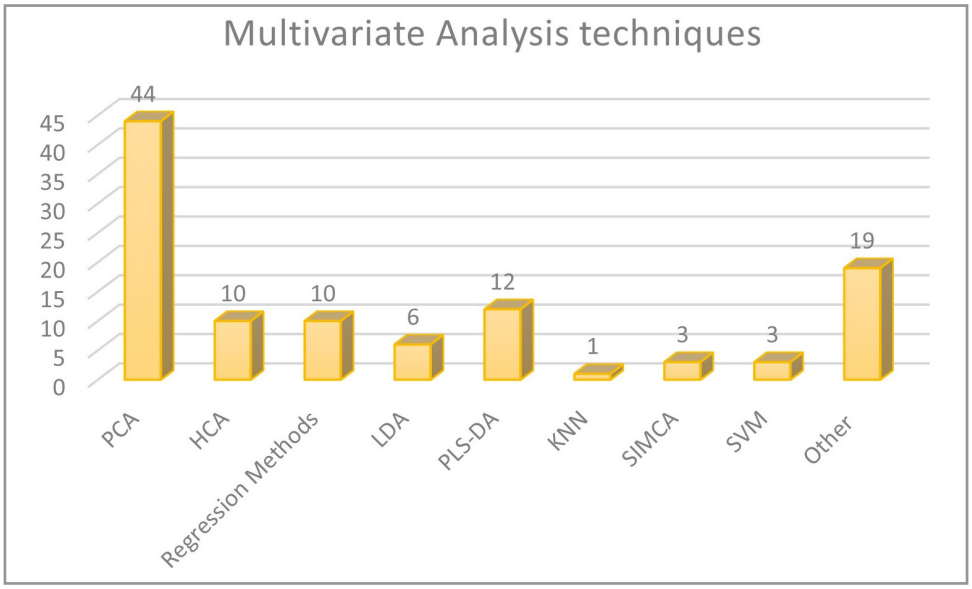


Figure 4. Multivariate analysis techniques involved on data examination from questioned documents.

Multivariate analysis/chemometric techniques can be divided at: a) unsupervised pattern recognition techniques, including principal component analysis (PCA) and hierarchical cluster analysis (HCA); b) regression methods, including partial least squares (PLS) and multiple linear regression (MLR); c) supervised pattern recognition techniques, including linear discriminant analysis (LDA), partial least squares discriminant analysis (PLS-DA), k-nearest neighbor (kNN), soft independent modeling of class analogy (SIMCA) and support vector machine (SVM). Other techniques included mean-field approach independent component analysis (MF-ICA), multivariate analysis of variance (MANOVA), spectral angle mapper (SAM), multivariate curve resolution (MCR) and multivariate curve resolution with alternating least squares (MCR-ALS), among others. A summary of these techniques is shown in Table V.

Table V. Concepts of the main chemometric techniques applied in questioned document analysis

Method Classification	Multivariate Analysis	Concept
UNSUPERVISED PATTERN RECOGNITION TECHNIQUES	PCA	This method projects multivariate data into a smaller space, reducing the spatial dimensionality of the original data set, without affecting the relation between samples. It is a type of controlled loss of information that is compensated by a better understanding of the data inside the data set. This method allows for visualizing and interpreting the differences between the variables and examining the relations that may exist between the samples. The method is also capable to detect samples with an anomalous behavior: the data projection makes the differences evident [72].
	HCA	This method is originated from numerical taxonomy. It is useful for reducing the dimensionality of large data sets by gathering samples into groups of samples that are most similar to each other. Thus, both internal homogeneity within groups and heterogeneity between groups are maximized. The results are presented in a hierarchical tree denominated dendrogram, and the tree branches show a degree of similarity between the samples. This similarity is calculated over a distance: similar samples from one group have a shorter distance between them when compared to samples from other groups [73].
REGRESSION METHODS	PLS	It is a biased method that applies factor analysis, and it is the most popular regression method in chemometrics. The purpose of this technique is to estimate the space of original measures into one of reduced size. The model is built in a single step, in which the information from matrix “X” and values of interest are considered during the data decomposition and compression. Some restriction is imposed on the decomposition of matrix “X” (of samples and variables), directing it to a solution whose target is the property of interest. This is justifiable, if the values of the property of interest are reliable [73].
	MLR	The general purpose of this method is to quantitate the relationship between several independent or predictor variables and a dependent variable. The MLR model is built with descriptive variables using the least squares methods to minimize the residuals [74].
SUPERVISED PATTERN RECOGNITION TECHNIQUES	LDA	it is a linear combination of the sample’s set original attributes, characterized by producing the maximum separation between two populations. The main objectives of this method are a) to confirm whether the groups are correctly discriminated, b) to classify unknown observations, and c) to verify which are the most important variables for the discrimination of these groups. It takes a different approach to consider the existence of classes for the data; it involves projecting the data distribution probability on the graphic’s axes. Therefore, the method not only maintains but it also highlights a linear separation of the data, if it exists [75].
	PLS-DA	It is a method that determines which class an unknown sample belongs, based on the information provided to the system. The method applies multivariate regression by partial least squares (PLS), which has been previously discussed. PLS is an inverse calibration method, in which a direct relationship is sought between the instrumental response (matrix X) and the property of interest (matrix Y or vector y). The classification model is built using the same PLS model. However, in PLS-DA the property of interest is a categorical variable that describes the sample’s class assignment. Generally, a value of 1 is assigned to the class of interest and a value of 0 is assigned to the other class [76].
	kNN	This deterministic model is defined based on a training set, followed by the classification model construction. When building the model, each sample of the training set is deleted and then classified based on the remaining samples. The distances between the excluded sample and the remaining samples of the training set are calculated in the dimensional space. The excluded sample is then classified according to a majority of “votes” from its closest neighbors, and the sample is attributed to the most voted class. This process is applied to all training samples, with a summary of successful analyzes and errors at the end [77].

Table V. Concepts of the main chemometric techniques applied in questioned document analysis (Continuation)

Method Classification	Multivariate Analysis	Concept
SUPERVISED PATTERN RECOGNITION TECHNIQUES	SIMCA	This method assumes that the measured values for a group of similar samples will tend towards a uniform and modellable distribution. By increasing the number of samples, the uniform distribution becomes more visible. The probabilistic distribution allows for estimating the degree of certainty in the classification. This model allocates the main component model to be adjusted to each class in the training set, giving rise to a classifier for each one. The number of factors, suitable for modeling each class, can be determined by doing a cross-validation to maximize the predictive capacity of the individual models in relation to the training set. If it is necessary to include a new class, it is possible to build an independent model for each class by adding it to the existing model, without having to repeat the entire modeling process [77].
	SVM	It is a supervised machine learning algorithm that can be employed for both classification and regression purposes. It is based on the idea of finding a hyperplane that best divides a dataset into two classes. Intuitively, the further from the hyperplane the data points lie, the more confident the results are, which means they are correctly classified. Therefore, the goal is for the data points as far away from the hyperplane as possible, while still stands on the correct side of it. When adding new testing data, the side of the hyperplane in which it lands will define the data class [78].
OTHER METHODS	MF-ICA	Independent component analysis (ICA) is a computational method for separating a multivariate signal into additive subcomponents. This is done by assuming that the subcomponents are non-Gaussian signals and that they are statistically independent from each other. In MF-ICA we derive an expectation-maximization algorithm, in which the expectation step is performed using different medium field (MF) approaches: variational, linear response, and adaptive TAP (Thouless, Anderson and Palmer). The MF theories produce estimates of later source correlations of increasing quality, needed for the maximization step in the estimate for the multivariate signal and the noise [79].
	MANOVA	It is a test to perform the relationship analysis between several response variables and a common set of predictors at the same time. It requires continuous response variables and categorical predictors. MANOVA has several important advantages over performing several ANOVAs, one response variable at a time [80].
	SAM	It is an algorithm that allows for the measurement of spectral similarity between two spectra. This is expressed as a numerical scale (from 0–no similarity to 1–identical spectra). In this approach, selected spectra are treated as vectors in n-dimensional space, in which the number of dimensions is equal to the number of recorded spectral lines. This allows for the calculation of the spectral angle. It is worth mentioning that the SAM method is resistant to illumination variation [36].
	MCR	The MCR method was first created for process analysis purposes, but nowadays it is also applied to non-evolutionary multicomponent systems. Thus, the MCR method was created and classified as a two-way data analysis method, i.e., a valid method to analyze single data matrices. However, the general applications of MCR relates to the possibility to work with multi-way and multiset data structures, i.e., with several data tables simultaneously [81].
	MCR-ALS	It is an algorithm that solves the MCR basic bilinear model, using a constrained Alternating Least Squares algorithm. The constraints improve the profiles interpretability in pure spectra matrices and the related concentration profiles for each of the compounds in the system [81].

As we can observe in Tables I, II, III and IV, different software can applied to perform multivariate analysis or chemometrics, and MATLAB®, SPSS-20®, Unscrambler®, SIMCA®, Minitab®, Microsoft Excel®, Chemostat®, and Photometrix PRO® are the most applied.

In this review, a substantial number of papers applying chemometrics approaches in questioned documents were identified and summarized. These studies show a growing trend for the chemometrics importance to the field, over the past ten years. In this matter, it is crucial for questioned documents experts to understand how to perform data analysis and how to present the results for forensic purposes. For this reason, this review included a brief summary of the most applied chemometric techniques, that should be known by forensic experts. Deviterne-Lapeyre [3] have discussed the challenge of chemical data evaluation for a document examiner scientist. The author emphasized that experts should understand principles and theories about chemometrics, to better explain the results.

Most of the studies herein presented used chemometrics approaches for classification and/or differentiation of samples. Considering the comparison examination, while analytical methods can provide accurate inks and paper chemical data, chemometrics analysis can increase the discriminating power of these techniques [3]. However, research articles should also include intra-variability analysis as a goal, in order to demonstrate the methods limitations as well [3]. Another important role of chemometrics in questioned documents area is the development of databases [3], especially for samples identification, such as pen or paper brands. However, only one of the 60 articles in this review have aimed for a database creation, for printing inks [50]. This is a promising topic for research in questioned documents applying chemometrics. A few articles applied chemometrics for ageing studies of pen inks [12–18] and paper [63–67], but chemical document dating remains as a research topic until a complete standardization is performed [3].

Overall, ballpoint pen inks are the most studied topic of chemometric approaches in questioned documents. Although different types of pen inks, printing inks, paper and banknotes were also studied, these topics are not completely explored and further research using different analytical methodologies and chemometrics data analysis can be performed.

CONCLUSION

This review compiled a significant number of papers that applied chemometrics in the questioned documents area, describing a brief summary of the most applied chemometrics techniques. These studies show different analytical methodologies applied in pen inks, printed documents, paper, and banknotes analysis. Regression methods, unsupervised and supervised pattern recognition techniques were applied for data analysis with different purposes in forensic science, such as discrimination and classification of samples, ageing estimation, determination of crossing lines chronological order and counterfeit banknotes identification. Under light of these studies, the importance of chemometrics in questioned documents is highlighted, and this knowledge should be included in forensic experts training. The chemometric approach for databases development and implementation is a promising research topic for questioned documents, as well as inks and paper ageing studies and new analytical methods for non-ballpoint pen inks, printing inks, paper, banknotes and different security documents.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES) and “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq) Brazilian Institutions. This study is a part of CAPES PRO-FORENSES PROJECT (Processo 23038.006845/2014-91) and part of MCTI/CNPQ/CAPES/FAPS nº 16/2014 – Programa INCT, CNPq 465450/2014-8 Brazilian Public Call Project, entitled “Instituto Nacional de Ciência e Tecnologia Forense”. The authors wish to thank these institutions.

REFERENCES

1. Calcerrada, M.; Garcia-Ruiz, C. *Anal. Chim. Acta*., **2019**, *853*, pp 143–166 (https://doi.org/10.1016/j.aca.2014.10.057).
2. LaPorte, G. M. *Forensic Chem*., **2015**, pp 318–353 (https://doi.org/10.1002/9781118897768.ch8).
3. Deviterne-Lapeyre, C. M. *Forensic Sci. Int. Synerg*., **2020**, *2*, pp 429–441 (https://doi.org/10.1016/j.fsisyn.2020.01.012).
4. Trejos, T.; Almirall, J. R. *Forensic Sci. Handb*., **2020**, pp 425–463 (https://doi.org/10.4324/9781315119939-7).
5. Chen, H.; Meng, H.; Cheng, K. *Forensic Sci. J.*, **2002**, *1* (1), pp 1–14.
6. Ezcurra, M.; Góngora, J. M. G.; Maguregui, I.; Alonso, R. *Forensic Sci. Int.,* **2010**, *197*, pp 1–20 (https://doi.org/10.1016/j.forsciint.2009.11.013).
7. Brito, L. R.; Martins, A. R.; Braz, A.; Chaves, A. B.; Braga, J. W.; Pimentel, M. F. *TrAC - Trends Anal. Chem.,* **2017**, *94*, pp 54–69 (https://doi.org/10.1016/j.trac.2017.07.005).
8. Gorziza, R. P.; Carvalho, C. M. B.; González, M.; Leal, L. B.; Korndörfer, T.; Ortiz, R. S.; Trejos, T.; Limberger, R. P. *Brazilian Journal of Forensic Sciences / Medical Law and Bioethics,* **2019**, *8*, pp 113–138 (https://doi.org/10.17063/bjfs8(3)y2019113).
9. Kowalski, B. R. *J. Chem. Inf. Comput. Sci*., **1975**, *15*, pp 201–203 (https://doi.org/10.1021/ci60004a002).
10. International Union of Pure and Applied Chemistry (IUPAC). *Compendium of Chemical Terminology*, **2009** (https://doi.org/10.1351/goldbook).
11. Kumar, R.; Sharma, V. *TrAC - Trends Anal. Chem.,* **2018**, *105*, pp 191–201 (https://doi.org/10.1016/j.trac.2018.05.010).
12. Ortiz-Herrero, L.; de Almeida, A. C. A.; Bartolomé, L.; Alonso, M. L.; Maguregui, M. I.; Alonso, R. M.; de Melo, J. S. S. *Chemom. Intell. Lab. Syst.,* **2020**, *207*, pp 104187 (https://doi.org/10.1016/j.chemolab.2020.104187).
13. Costa, K. F. F.; Brand, G. D.; Grobério, T. S.; Braga, J. W. B.; Zacca, J. J. *Microchem. J.*, **2019**, *147*, pp 1123–1132 (https://doi.org/10.1016/j.microc.2019.04.034).
14. Carvalho, C. M. B.; Ortiz, R. S.; dos Reis, M.; Ferrão, M. F.; Limberger, R. P. *J. Am. Soc. Quest. Doc. Exam. Inc.*, **2019**, pp 19–35.
15. Ortiz-Herrero, L.; Bartolomé, L.; Durán, I.; Velasco, I.; Alonso, M. L.; Maguregui, M. I.; Ezcurra, M. *Microchem. J.,* **2018**, *140*, pp 158–166 (https://doi.org/10.1016/j.microc.2018.04.019).
16. Sharma, V.; Kumar, R. *Microchem. J.,* **2017**, *134*, pp 104–113 (https://doi.org/10.1016/J.MICROC.2017.05.014).
17. Sauzier, G.; McGann, J.; Lewis, S. W.; Van Bronswijk, W. *Anal. Methods*, **2018**, *10*, pp 5613–5621 (https://doi.org/10.1039/c8ay01418c).
18. Senior, S.; Hamed, E.; Masoud, M.; Shehata, E. *J. Forensic Sci.*, **2012**, *7*, pp 1087–1093 (https://doi.org/10.1111/j.1556-4029.2012.02091.x).
19. Gorziza, R. P.; Carvalho, C. M. B.; González, M.; Ortiz, R. S.; Helfer, G. A.; Ferrão, M. F.; Limberger, R. P. *Brazilian Journal of Forensic Sciences / Medical Law and Bioethics*, **2020**, *9*, pp 331–355 (https://doi.org/10.17063/bjfs9(3)y2020331).
20. Teixeira, C. A.; Poppi, R. J. *Microchem. J.,* **2019**, *144*, pp 411–418 (https://doi.org/10.1016/j.microc.2018.10.002).
21. de Carvalho, C. M. B.; Ortiz, R. S.; do Reis, M.; Zamboni, A.; Limberger, R. P.; Ferrão, M. F.; Gontijo, B. V. *Forensic Sci. Addict. Res*., **2018**, *2*, pp 1–8 (https://doi.org/10.31031/fsar.2018.02.000537).
22. Kamil, M.; Asri, M.; Desa, W.; Ismail, D. *Malaysian J. Forensic Sci.*, **2015**, *6*, pp 48–53.
23. Amador, V. S.; Pereira, H. V.; Sena, M.; Augusti, M.; Piccin, R. E. *J. Am. Soc. Mass Spectrom*., **2017**, *28*, pp 1965–1976 (https://doi.org/10.1007/s13361-017-1686-z).
24. Sharma, V.; Kumar, R. *Vib. Spectrosc.*, **2017**, *92*, pp 96–104 (https://doi.org/10.1016/J.VIBSPEC.2017.05.006).
25. Asri, M. N. M.; Desa, W. N. S. M.; Ismail, D. *Aust. J. Forensic Sci.,* **2017**, *49*, pp 175–185 (https://doi.org/10.1080/00450618.2016.1153712).

26. Lee, L. C.; Othman, M. R.; Pua, H.; Ishak, A. A. *Malaysian J. Anal. Sci.*, **2012**, *3*, pp 5-10.
27. Lee, L. C.; Othman, M. R.; Pua, H. *Malaysian J. Anal. Sci.*, **2012**, *16*, pp 262–272.
28. Kumar, R.; Sharma, V. *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.,* **2017**, *175*, pp 67–75 (https://doi.org/10.1016/j.saa.2016.12.008).
29. Halim, M. I. A.; Aziz, N. S. A.; Saim, N.; Osman, R.; Jasmani, H. *Malaysian J. Fundam. Appl. Sci.,* **2014**, *8*, pp 159–165 (https://doi.org/10.11113/mjfas.v8n3.141).
30. Borba, F. S. L.; Honorato, R. S.; de Juan, A. *Forensic Sci. Int*., **2015**, *49*, pp 73–82 (https://doi.org/10.1016/j.forsciint.2015.01.027).
31. Alamilla, F.; Calcerrada, M.; García-Ruiz, C.; Torre, M. *Forensic Sci. Int.*, **2013**, *228*, pp 1–7 (https://doi.org/10.1016/j.forsciint.2013.01.034).
32. Lee, L. C. *Forensic Sci. J.*, **2014**, *13* (1), pp 15–22. Available at: fsjournal.cpu.edu.tw
33. Sharma, V.; Kumar, R.; Devgan, K.; Mishra, P. K.; Ekielski, A.; Kumar, V.; Kumar, V. *Spectrosc. Lett.*, **2018**, *51*, pp 205–215 (https://doi.org/10.1080/00387010.2018.1452265).
34. Asri, M. N. M.; Noor, N. A. M.; Desa, W. N. S. M.; Ismail, D. *Forensic Sci. Forensic Med*., **2019**, *1*, pp 1174–1184 (https://doi.org/10.26735/16586794.2019.004).
35. Al Balah, O. F.; Nassef, O. A. T. *Arab J. Nucl. Sci. Appl*., **2019**, 52 (2), pp 72–78 (https://doi.org/10.21608/ajnsa.2019.4231.1097).
36. Chlebda, D. K.; Majda, A.; Łojewski, T.; Łojewska, J. *Appl. Phys. A Mater. Sci. Process.,* **2016**, *122*, pp 1–13 (https://doi.org/10.1007/s00339-016-0494-9).
37. Yadav, P. K.; Sharma, R. M. *Vib. Spectrosc.,* **2020**, *108*, 103054 (https://doi.org/10.1016/j.vibspec.2020.103054).
38. da Silva, V. A. G.; Talhavini, M.; Zacca, J. J.; Trindade, B. R.; Braga, J. W. B. *J. Braz. Chem. Soc.*, **2014**, *25*, pp 1552–1564 (https://doi.org/10.5935/0103-5053.20140140).
39. da Silva, V. O. G.; Talhavini, M.; Peixoto, I. C. F.; Zacca, J. J.; Maldaner, A. O.; Braga, J. W. B. *Microchem. J.*, **2014**, *116*, pp 235–243 (https://doi.org/10.1016/j.microc.2014.05.013).
40. Valderrama, L.; Valderrama, P. *Chemom. Intell. Lab. Syst.*, **2016**, *156*, pp 188–195 (https://doi.org/10.1016/j.chemolab.2016.06.009).
41. Pereira, J. F. Q.; Silva, C. S.; Braz, A.; Pimentel, M. F.; Honorato, R. S.; Pasquini, C.; Wentzell, P. D. *Microchem. J.,* **2017**, *130*, pp 412–419 (https://doi.org/10.1016/j.microc.2016.10.024).
42. Goacher, R. E.; Difonzo, L. G.; Lesko, K. C. *Anal. Chem*., **2017**, *89*, pp 759–766 (https://doi.org/10.1021/acs.analchem.6b03411).
43. Martins, A. R.; Dourado, C. S.; Talhavini, M.; Braz, A.; Braga, J. W. B. *Forensic Sci. Int.*, **2019**, *296*, pp 91–100 (https://doi.org/10.1016/j.forsciint.2019.01.021).
44. Rodrigues e Brito, L.; Chaves, A. B.; Braz, A.; Pimentel, M. F. *Spectrochim. Acta, Part A*, **2019**, *223*, 117287 (https://doi.org/10.1016/j.saa.2019.117287).
45. Rodrigues e Brito, L.; Braz, A.; Honorato, R. S.; Pimentel, M. F.; Pasquini, C. *Forensic Sci. Int.*, **2019**, *298*, pp 169–176 (https://doi.org/10.1016/j.forsciint.2019.02.043).
46. Ramli, S.; Talib, R. A.; Rahman, R. A.; Zainuddin, N.; Othman, S. H.; Rashid, N. M. *J. Spectrosc.*, **2015** (https://doi.org/10.1155/2015/502340).
47. Haase, E.; Arroyo, L.; Trejos, T. *Spectrochim. Acta, Part B.*, **2020**, *172* (https://doi.org/10.1016/j.sab.2020.105963).
48. Melendez-Perez, J. J.; Correa, D. N.; Hernandes, V. V.; De Morais, D. R.; De Oliveira, R. B.; De Souza, W. J.; Santos, M.; Eberlin, M. N. *Appl. Spectrosc.*, **2016**, *16*, pp 1910–1915 (https://doi.org/10.1177/0003702816645352).
49. Hoehse, M.; Paul, A.; Gornushkin, I.; Panne, U. *Anal. Bioanal. Chem.*, **2012**, *402*, pp 1443–1450 (https://doi.org/10.1007/s00216-011-5287-6).
50. Trejos, T.; Torrione, P.; Corzo, R.; Raeva, A.; Subedi, K.; Williamson, R.; Yoo, J.; Almirall, J. *J. Forensic Sci.*, **2016**, *61*, pp 715–724 (https://doi.org/10.1111/1556-4029.13109).
51. Kumar, R.; Samkaria, A.; Sharma, V. *Sci. Justice,* **2020**, *60*, pp 347–357 (https://doi.org/10.1016/j.scijus.2020.01.004).

52. Oravec, M.; Beganović, A.; Gál, L.; Čeppan, M.; Huck, C. W. *Forensic Sci. Int.,* **2019**, *299*, pp 128–134 (https://doi.org/10.1016/j.forsciint.2019.03.041).
53. Materazzi, S.; Risoluti, R.; Pinci, S.; Romolo, F. S. *Talanta*, **2017**, *174*, pp 673–678 (https://doi.org/10.1016/j.talanta.2017.06.044).
54. Verma, N.; Sharma, V.; Kumar, R.; Sharma, R.; Joshi, M. C.; Umapathy, G. R.; Ohja, S.; Chopra, S. *Anal. Bioanal. Chem.*, **2019**, *411*, pp 3477–3495 (https://doi.org/10.1007/s00216-019-01824-z).
55. Verma, N.; Kumar, R.; Sharma, V. *Spectrochim. Acta - Part A*, **2018**, *196*, pp 40–48 (https://doi.org/10.1016/j.saa.2018.02.001).
56. Gál, L.; Oravec, M.; Kiššová, M.; Gemeiner, P.; Čeppan, M. *Chem. Pap.*, **2020**, *74*, pp 3269–3277 (https://doi.org/10.1007/s11696-020-01145-x).
57. Farid, S.; Kasem, M. A.; Zedan, A. F.; Mohamed, G. G.; El-Hussein, A. *Opt. Laser Technol.*, **2021**, *135* (https://doi.org/10.1016/j.optlastec.2020.106704).
58. Sugawara, S.; Huck, C. W. *Infrared Phys. Technol.*, **2020**, *105* (https://doi.org/10.1016/j.infrared.2020.103212).
59. Valderrama, L.; Março, P. H.; Valderrama, P. *J. Chemom.*, **2020**, *34* (12), e3265 (https://doi.org/10.1002/cem.3265).
60. Xia, J.; Zhang, J.; Zhao, Y.; Huang, Y.; Xiong, Y.; Min, S. *Spectrochim. Acta - Part A*, **2019**, *219*, pp 8–14 (https://doi.org/10.1016/j.saa.2018.09.059).
61. Kumar, R.; Sharma, V.; Verma, N.; Diwan, P. K.; Kumar, V.; Kumar, V. *Aust. J. Forensic Sci.*, **2019**, *51*, pp 22–39 (https://doi.org/10.1080/00450618.2017.1310921).
62. Kumar, R.; Kumar, V.; Sharma, V. *Spectrochim. Acta - Part A*, **2017**, *170*, pp 19–28 (https://doi.org/10.1016/j.saa.2016.06.042).
63. Xia, J.; Huang, Y.; Zhang, J.; Du, X.; Yan, H.; Li, Q.; Li, Y.; Xiong, Y.; Min, S. *Cellulose*, **2020**, *27*, pp 5323–5335 (https://doi.org/10.1007/s10570-019-02892-1).
64. Ortiz-Herrero, L.; Blanco, M. E.; García-Ruiz, C.; Bartolomé, L. *J. Anal. Appl. Pyrolysis*, **2018**, *131*, pp 9–16 (https://doi.org/10.1016/j.jaap.2018.02.018).
65. Silva, C. S.; Pimentel, M. F.; Amigo, J. M.; García-Ruiz, C.; Ortega-Ojeda, F. *Anal. Chim. Acta*, **2018**, *1031*, pp 28–37 (https://doi.org/10.1016/j.aca.2018.06.031).
66. Sharma, V.; Kaur, J.; Kumar, R. *Aust. J. Forensic Sci.*, **2020** (https://doi.org/10.1080/00450618.2020.1781254).
67. Trafela, T.; Mizuno, M.; Fukunaga, K.; Strlič, M. *THz spectroscopy and chemometrics for quantitative determination of chemical properties and dating of historic paper*. 35th International Conference on Infrared, Millimeter, and Terahertz Waves, **2010** (https://doi.org/10.1109/ICIMW.2010.5612350).
68. Vittorazzi, B. V.; Costa, R. A.; Coelho, L. M.; Isidoro, M. M.; Lima, K. M. G.; Filgueiras, P. R.; Romão, W. *Quim. Nova*, **2020**, *43*, pp 447–454 (https://doi.org/10.21577/0100-4042.20170508).
69. Rodrigues, A. R. N.; Melquiades, F. L.; Appoloni, C. R.; Marques, E. N. *Forensic Sci. Int.*, **2019**, *302*, 109872 (https://doi.org/10.1016/j.forsciint.2019.06.030).
70. Appoloni, C. R.; Melquiades, F. L. *Appl. Radiat. Isot.*, **2014**, *85*, pp 92–95 (https://doi.org/10.1016/j.apradiso.2013.12.004).
71. de Almeida, M. R.; Correa, D. N.; Rocha, W. F. C.; Scafi, F. J. O.; Poppi, R. J. *Microchem. J.*, **2013**, *109*, pp 170–177 (https://doi.org/10.1016/j.microc.2012.03.006).
72. Gong, F.; Wang, B. T.; Chau, F. T.; Liang, Y. Z. *Anal. Lett.*, **2005**, *38*, pp 2475–2492 (https://doi.org/10.1080/00032710500318338).
73. Geladi, P. *Spectrochim. Acta - Part B*, **2003**, *58*, pp 767–782 (https://doi.org/10.1016/S0584-8547(03)00037-5).
74. Gadžurić, S. B.; Podunavac, S. O.; Kuzmanović, M. B.; Vraneš, M.; Petrin, T.; Bugarski, S. Z.; Kovačević, S. Z. *Iran. J. Pharm. Res.*, **2016**, *15*, pp 725–734 (https://doi.org/10.22037/ijpr.2016.1905).
75. Sarker, S. D.; Nahar, L. Applications of High Performance Liquid Chromatography in the Analysis of Herbal Products. In: Mukherjee, P. K. (Ed.) *Evidence-Based Validation of Herbal Medicine.* Elsevier Inc., **2015**, Chapter 19, pp 405–425 (https://doi.org/10.1016/B978-0-12-800874-4.00019-2).

76. Santana, F. B.; Souza, A. M.; Almeida, M. R.; Breitkreitz, M. C.; Filgueiras, P. R.; Sena, M. M.; Poppi, R. J. *Quim. Nova*, **2020**, *43*, pp 371-381 (https://doi.org/10.21577/0100-4042.20170480).
77. Silva, C. S.; Braz, A.; Pimentel, M. F. *J. Braz. Chem. Soc.*, **2019**, *30*, pp 2259–2290 (https://doi.org/10.21577/0103-5053.20190140).
78. Duarte, A. C.; Capelo, S. *J. Liq. Chromatogr. Relat. Technol.*, **2006**, *29*, pp 1143–1176 (https://doi.org/10.1080/10826070600574929).
79. De Lathauwer, L.; De Moor, B.; Vandewalle, J. *J. Chemom*., **2000**, *14*, pp 123–149 (https://doi.org/10.1002/1099-128X(200005/06)14:3<123::AID-CEM589>3.0.CO;2-1).
80. Noble, W. S. *Nat. Biotechnol.*, **2006**, *24*, pp 1565–1567 (https://doi.org/10.1038/nbt1206-1565).
81. Março, P. H.; Valderrama, P.; Alexandrino, G. L.; Poppi, R. J.; Tauler, R. *Quim. Nova*., **2014**, *37*, pp 1525–1532 (https://doi.org/10.5935/0100-4042.20140205).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 52-78
doi: 10.30744/brjac.2179-3425.RV-41-2021

BrJAC

REVIEW

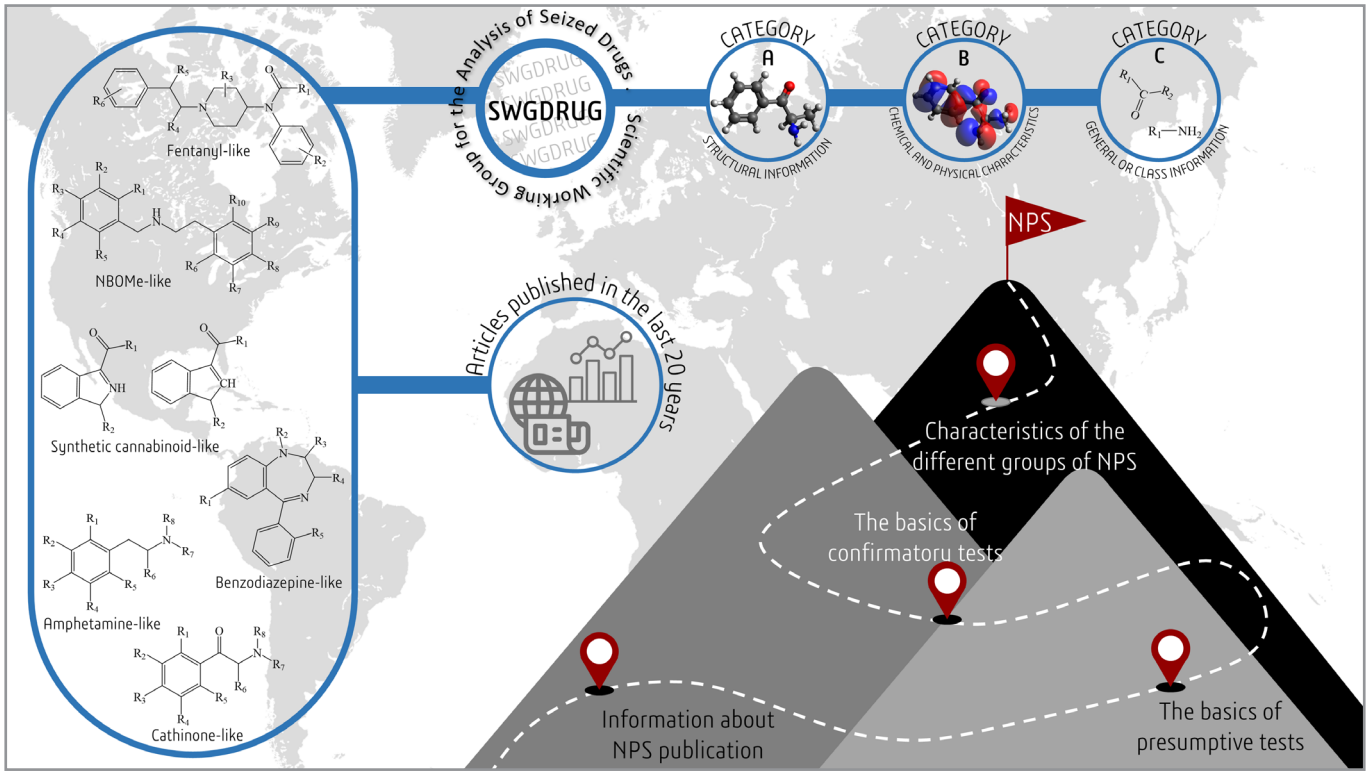
Analytical Challenges for Identification of New Psychoactive Substances

A Literature-Based Study for Seized Drugs

Aline Thais Bruni[1,2*], Caio Henrique Pinke Rodrigues[1], Christiano dos Santos[1], Jade Simões de Castro[1], Livia Salviano Mariotto[1], Luiz Felipe Colli Sinhorini[1]

[1]*Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP. Avenida Bandeirantes, 3900, Monte Alegre, Ribeirão Preto, SP, Brazil*

[2]*Instituto Nacional de Ciência e Tecnologia Forense (INCT-Forense)*



Correct identification of substances is essential to understand drug use and trafficking trends and guide measures for harm reduction and treatment. Two steps are needed to verify the nature of a substance properly: a presumptive test and a confirmatory test. There are presumptive tests which presents deficiencies, such as providing false-positive and false-negative results. Confirmatory tests are more reliable,

Cite: Bruni, A. T.; Rodrigues, C. H. P.; dos Santos, C.; de Castro, J. S.; Mariotto, L. S.; Sinhorini, L. F. C. Analytical Challenges for Identification of New Psychoactive Substances: A Literature-Based Study for Seized Drugs. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 52-78. doi: http://dx.doi.org/10.30744/brjac.2179-3425.RV-41-2021

Submitted 23 March 2021, Resubmitted 09 July 2021, 2nd time Resubmitted 30 July 2021, 3rd time Resubmitted 06 August 2021, Accepted 16 August 2021, Available online 29 September 2021.

but they are more expensive. With the appearance of New Psychoactive Substances (NPS), identifying and characterizing illicit substances has become more challenging. This paper focuses on presenting information about NPS characteristics and analysis. For this purpose, we have reviewed the literature to address the main aspects of five groups of NPS: amphetamine-type stimulants, synthetic cannabinoids, N-methoxybenzyl-methoxyphenylethylamine (NBOMe), synthetic opioids, and benzodiazepines. We present the main characteristics of each group and certain aspects of presumptive and confirmatory tests regarding these groups. Our findings show obstacles in developing methodologies that can correctly identify these substances, and problems can increase as new structures appear. This information can be helpful to drive research into identifying NPS and inform law enforcement and law practitioners about the main characteristics of each group and the main questions involving their identification.

Keywords: New Psychoactive Substances, presumptive tests, confirmatory tests, amphetamine-type stimulants, synthetic cannabinoids, NBOMes, synthetic opioids, synthetic benzodiazepines.

Abbreviations

4-ANPP: N-phenyl-1-(2-phenylethyl)-4-piperidinamine	GC-MS: Gas chromatography with mass detector	NPS: New Psychoactive Substances
ATR: Attenuated Total Reflection	GC-MS-EI: Gas chromatography with cold electron ionization	NSO: New Synthetic Opioids
ATS: Amphetamine-Type Stimulants	HPLC: High-performance liquid chromatography	MS: Mass spectrometry
CBD: Cannabidiol	HPLC-AD: High-performance liquid chromatography coupled to amperometric detector	PP: Protein precipitation
CBN: Cannabinol	HPLC-UV: High-performance liquid chromatography coupled to ultraviolet detector	Rf: Retention factors
DAPPI: Desorption atmospheric pressure photoionization	HRMS: High-resolution mass spectrometry	SERT: Serotonin transporter
DART-MS: Direct analysis in real-time mass spectrometry	IMS: Ion mobility spectrometry	SPE: Solid-phase extraction
DAT: Dopamine transporter	IR: Infrared spectroscopy	SWGDRUG: Scientific Working Group for the Analysis of Seized Drugs
DESI-MS: Desorption electrospray ionization with mass spectrometry	LC-HRMS: Liquid chromatography coupled to high-resolution mass spectrometry	TD-DART-MS: Thermal desorption in real-time mass spectrometry
DOA: Drugs of abuse	LC-MS: Liquid chromatography coupled to mass spectrometry	THC: Δ9-tetrahydrocannabinol
EU: European Union	LLE: Liquid-liquid extraction	TLC: Thin-layer chromatography
FBB: Fast Blue B	LSD: Lysergic acid diethylamide	UHPLC: Ultra-high performance liquid chromatography
FBBB: Fast Blue BB	MALDI-TOF-MS: Matrix-assisted laser desorption/ionization mass spectrometry	UHPLC-MS/MS: Ultra-high performance liquid chromatography coupled to mass spectrometry
FITC: Fluorescein isothiocyanate	MDMA: Methylenedioxymethamphetamine	UK: United Kingdom
FTIR: Fourier transform infrared	NET: Norepinephrine transporter	UNODC: United Nations Office on Drugs and Crime
GC-FID: Gas chromatography with flame ionization detector	NMR: Nuclear magnetic resonance	UPLC-MS: Ultra-performance liquid chromatography coupled to mass spectrometry
GC-IRD: Gas chromatography with infrared detector	NPF: Nonpharmaceutical Fentanyl	USA: United States of America

INTRODUCTION

The use of recreational drugs of abuse (DOA) has always been present in different historical moments and societies and has increased over the years. Excessive prohibition of substance use in the drug war model has produced and consolidated illegal markets controlled by criminals [1]. Drug war prohibitions and policies have created a phenomenon that has given rise to New Psychoactive Substances (NPS) [2,3], which bear structural modifications of well-known substances such as cocaine, lysergic acid diethylamide (LSD), and cannabis, among others. The idea behind NPS is to provide the consumer with an alternative that is both recreational and non-illegal. According to the United Nations Office on Drugs and Crime (UNODC), 899 NPS were reported between 2009 and 2018 worldwide, and that number continues to grow [4,5].

Drug regulation mainly deals with conventional substances, and the prohibition is based on specific structural information. Because of this, most NPS are under-regulated: as the legislation starts to target newly discovered structures, new analogous substances or derivatives continue to emerge to circumvent the regulations, in a faster movement than the legal system and regulatory agencies can cope with [6]. Countries are using a total ban on structural classes as an alternative procedure [7–9]. Nevertheless, this strategy has disadvantages; for example, access to more detailed research into NPS may be unavailable, not to mention that an unregulated and unrestricted market can cause as much damage as the prohibitive model [2,10,11].

The rise of NPS represents a challenge for both legal control and health. From the legal aspect, scientific knowledge about these substances is lacking. Classifications for controlling psychoactive substances consider factors related to culture, production, manufacturing, consumption, market, and factors that can be characteristic of the country. However, prohibitions lack a scientific basis given that there are not enough studies to prove the harmful effects of these substances on the user or the society. Science is rarely part of the decision-making process and, when it can offer recommendations, they are seldom considered. If the undesirable effects of a specific substance cannot be proven, showing its possible benefits is beyond the scope of the evaluation. Without a scientific basis for establishing harm or benefits, the repression associated with a particular substance can be disproportionate and require a great deal of effort in law enforcement. Thus, NPS monitoring demands forensic, toxicological, and clinical data [12–14].

The conventional drug monitoring model considers only a small number of well-known and controlled substances. Forensic and toxicological analytical approaches can detect, identify, and, depending on the technique, quantify substances in seized or biological samples. It is essential to understand that the traditional way of responding to illicit drugs may not suit NPS [14]. In forensic terms, proper identification of substances is crucial when assessing suspect seized samples and evaluating cases of possible intoxication and requires reliable and accurate identification methods [6].

Among other factors, knowing the effects of using a particular drug can be necessary to elucidate post-mortem aspects and to assist in cases involving drug-facilitated crimes. Drugs can have consequences concerning human behavior, leading to consequences within the legal system [15]. Drug detection in biological samples can present problems. The lack of pharmacokinetic and pharmacodynamic knowledge can compromise the identification of metabolites [14,16].

From the analytical point of view, the correct identification of NPS is a significant challenge. The difficulties inherent in NPS evaluation include the diversity of changes to molecules and the speed with which they appear in the market. New molecules emerge faster than the development of analytical protocols. One of the main problems is the lack of certified standards for reference [14].

Analytical protocols involve presumptive detection, which is usually qualitative, and later confirmation [16]. Presumptive detection methods are called presumptive or field tests. These tests allow rapid and low-cost identification in both the clinical and forensic fields and indicate the presence or absence of a substance of interest and drug abuse. They can identify a particular group of chemicals, but they are not selective enough to indicate the substance within the group. They have advantages such as not requiring specific training, equipment, or sample preparation and being highly sensitive and portable, facilitating

substance identification on site. These characteristics and advantages have enabled their extensive use for law enforcement despite their low discretion performance [16–20]. With the increase in drug-facilitated crimes and addiction, these tests may be useful in point-of-care for harm reduction actions [21,22].

For law enforcement, a confirmatory test is required after presumptive identification to establish the chemical nature of the substance(s). Confirmatory drug identification is more trustful than presumptive tests. However, confirmatory tests demand sophisticated analytical equipment, specialized knowledge, and complex sample preparation, which can be destructive [14,23,24].

The appearance of new drugs has been increasing. Legal actions to try to control these substances have not been enough to stop their production. It is necessary to understand their diversity and to develop analytical methods to identify them. Analytical methods developed for classic drugs may not be able to identify these substances correctly [26,27].

Enhancing the use of accepted and validated scientific practices involving accurate and reliable analytical methods requires time. It may not keep pace with the appearance of these substances in real-time. Analytical techniques and methodologies shall be able to help to understand how the drug market evolves. Trustful methods shall assure that the new compounds can be correctly determined. Furthermore, producing or acquiring reference standards is essential [6,15,25]. All these situations share challenges: correctly identifying substances, obtaining standards for comparison, assessing the toxicological potential associated with them, and establishing harm reduction mechanisms.

This paper aims to provide an overview of five groups of synthetic classes of NPS: amphetamine-type stimulants (amphetamines and cathinones), cannabinoids, N-methoxybenzyl-methoxyphenylethylamine (NBOMe), opioids, and benzodiazepines. These sets comprise stimulants, hallucinogens, and depressants [28]. The overall idea is to present the characteristics of each group and particularities regarding their testing. This paper does not aim to exhaust the subject, but the intention is to show that there is a lot to learn about these substances. The information presented here can help the reader interested in drug analysis to understand the issue of NPS diversity and how it affects the identification of these substances. It can be helpful to increase the knowledge of researchers, forensic scientists, and law practitioners about these substances.

METHOD

We conducted a literature-based review to collect information about NPS. We delimited our research into four sections:

- Section I: Information about NPS publication. This section shows the interest in publishing research about NPS. We performed a search of both the SCOPUS and Web of Science databases. First, we focused on the broad publications by using the search keyword “New Psychoactive Substances”, and the keywords regarding the groups of synthetic substances amphetamines, cathinones, cannabinoids, NBOMes, opioids, and benzodiazepines. We also collected information about presumptive and confirmatory tests for the previous keywords. This search was delimited in a twenty-year interval.
- Section II: The basics of presumptive tests. This section provides an overview of presumptive tests for drugs.
- Section III: The basics of confirmatory tests. This section presents information about the main confirmatory tests for use in drug identification.
- Section IV: Characteristics of the different groups of NPS. This section presents the main characteristics of each group of NPS and comments on presumptive and confirmatory tests for them.

For Sections II-IV, we collected information from academic and scientific works, social data, grey literature, institutional websites, and news published on the Internet. Most of the cited references are current ones. Around 65% of them were published in the last five years (2017–2021); 29.5% were published after 2008 because, according to the literature, this date is related to the appearance of NPS [7,29]. The rest of the references (around 5.5%) were necessary for background information.

Section I: Information about publication on NPS

Figure 1 shows the SCOPUS database results. Figure 1(a) presents the overall publications for the terms “New Psychoactive Substances” and those regarding the groups of synthetic substances: amphetamines, cathinones, cannabinoids, NBOMes, opioids, and benzodiazepines. Figures 1(b) and 1(c) show information about presumptive and confirmatory tests for each case. Figure 2 shows information from the Web of Science database. Figures 2(a)-(c) follow the same organization presented in Figure 2.

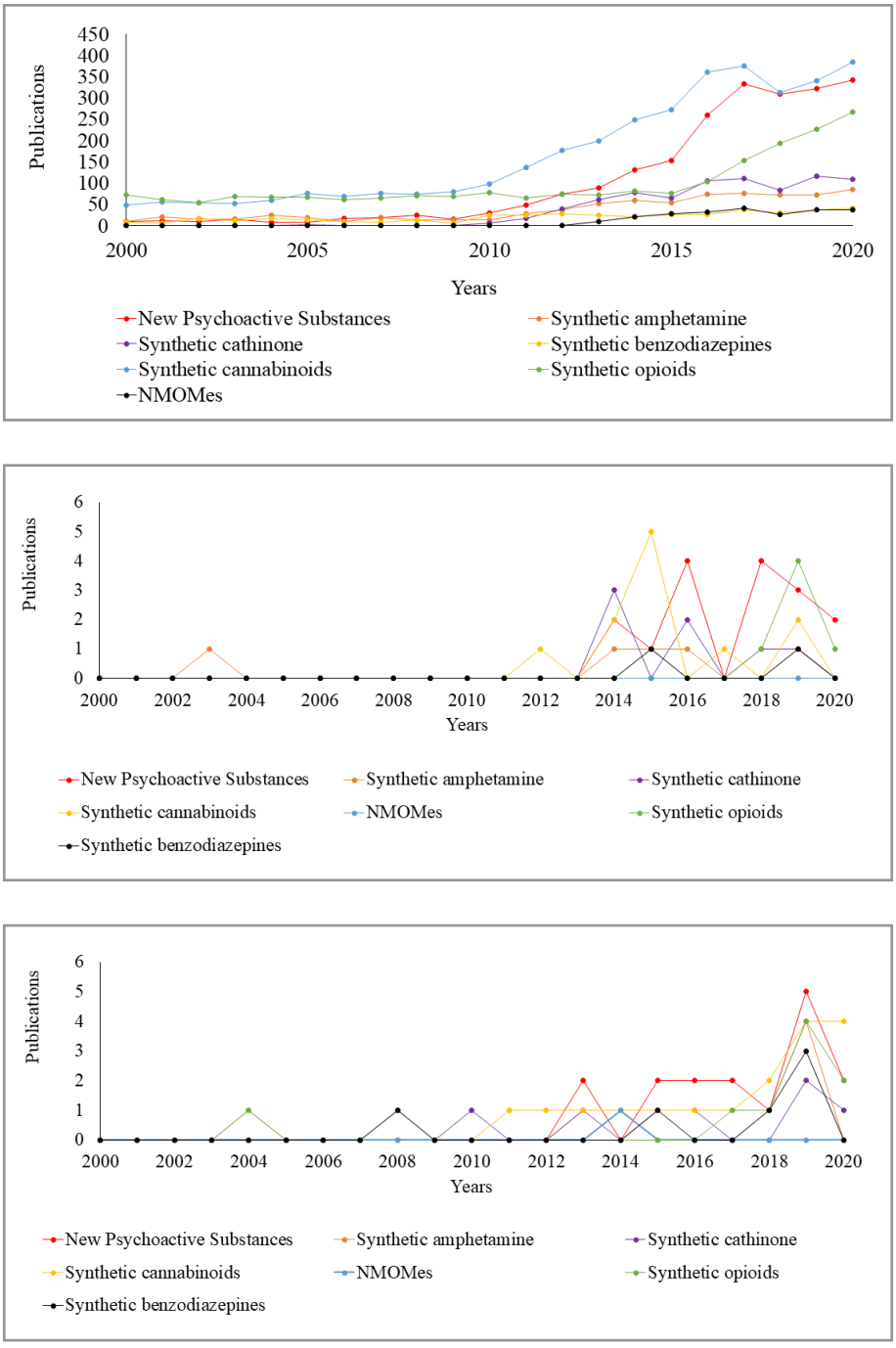


Figure 1. Articles published about NPS in the last 20 years in the SCOPUS database: (a) results for the terms “New Psychoactive Substance” and for each group of NPS; (b) results for presumptive tests; and (c) results for confirmatory tests.

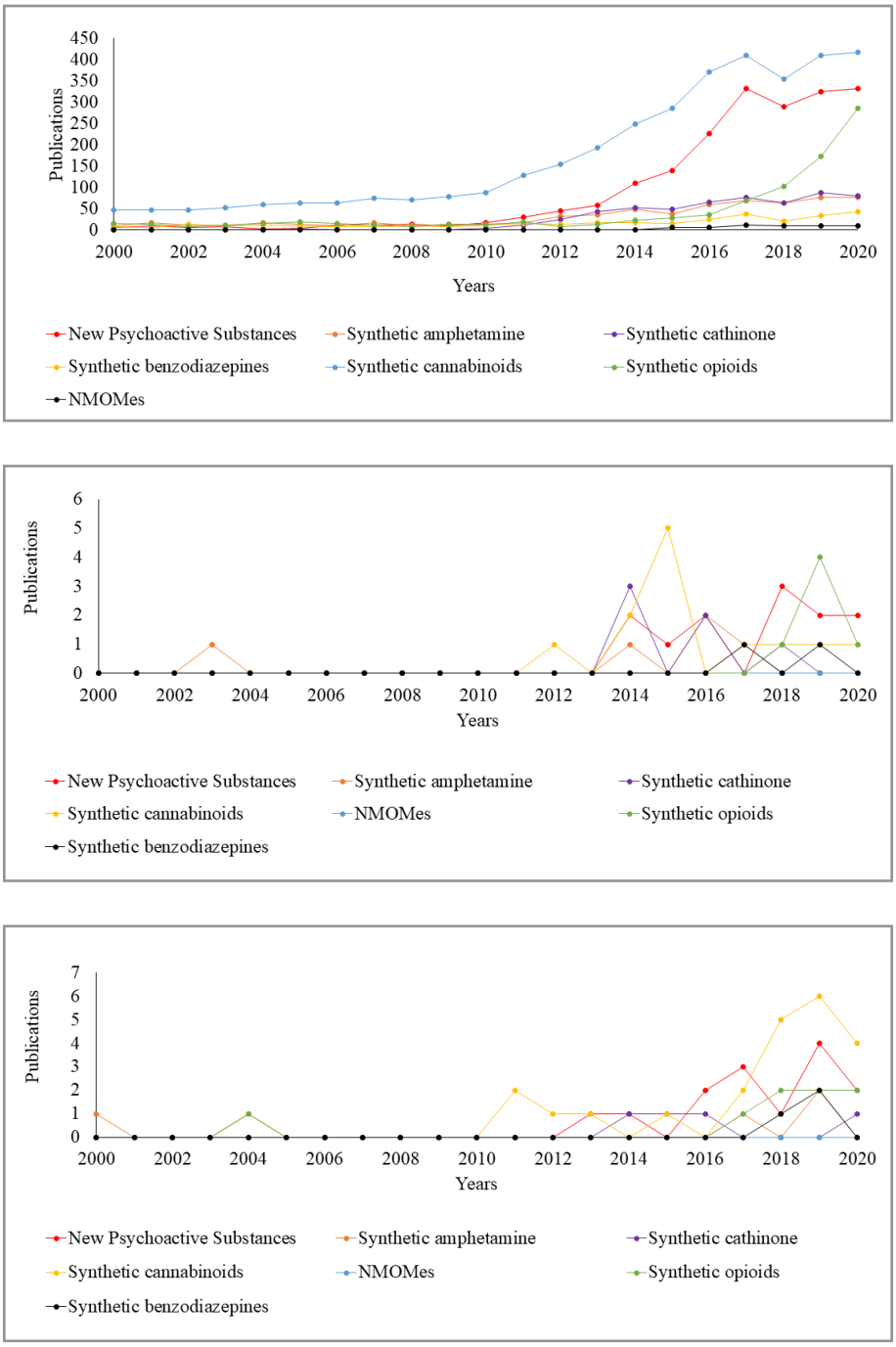


Figure 2. Articles published about NPS in the last 20 years in the Web of Science database: (a) results for the terms "New Psychoactive Substances" and for each group of NPS; (b) results for presumptive tests; and (c) results for confirmatory tests.

In both databases, we observed an overall increase in the studies for all groups of substances. Synthetic cannabinoids and synthetic opioids have received greater general interest.

Synthetic amphetamines were the first group to be studied regarding presumptive tests. We can observe similarities in the patterns for these publications in the two databases. There is no constant research production for these issues. Still concerning presumptive tests, synthetic cannabinoids are the most studied group of NPS.

For confirmatory tests, both databases show that there have been more publications on synthetic cannabinoids. The SCOPUS database showed growing interest for most drugs, except for NBOMes. The same trend could be observed in the Web of Science dataset, where there are no publications on NBOMes. These results strengthen the importance of studying these issues for seized NPS.

Section II: The basics of presumptive tests

When a suspicious substance is found, a presumptive test is usually the first step in the analysis. This test is generally run at the place of arrest and aims to indicate the presence or absence of a suspected illicit substance. There are new technologies to run this test on-site, and portable equipment has been developed for this purpose, such as infrared and Raman spectroscopy, and mass spectrometry, which is easy to use and enables fast identification within just a few minutes. However, portable advanced analytical techniques are expensive, and the team is not expected to have this equipment available during apprehension [20,30]. Electrochemical, voltammetric, and piezoelectric devices have also been extensively tested for presumptive drug tests to allow the presence of a broader range of drugs to be determined [20,30–32].

There are several presumptive tests. Among them, colorimetric and immunoassay tests are worthy of note. Colorimetric tests are generally the most common. In this case, reagents are added to a small amount of the suspected substance, and the appearance of a specific color may indicate the presence of the illicit substance. In immunoassay tests, the alleged substance reacts with a particular antibody. This reaction is also characteristic of chemical classes [20,33]. For immunoassay tests, it is necessary to have antibodies that specifically bind to the structures. It can be a problem for NPS because of their fast emergence in the market [34].

Because colorimetric tests are low-cost, portable, and sensitive, they are widely used. Color variation occurs due to specific reactions within a functional group within a class of drugs. The color test must be selective for its class of drugs [6,17,20,26,30]. Despite the apparent advantage of colorimetric tests, there are concerns about their application, and agents must be aware of the possible results. A presumptive test can provide accurate results, *i.e.*, it can correctly indicate the substance. However, false results; that is, false-positive and false-negative results, are also possible. It is undesirable because they can prevent the law from being correctly applied. For example, a false negative means that the test cannot detect the substance even if it is present in the sample. Consequently, the law cannot be enforced, and the banned substance can freely circulate, endangering society. On the other hand, false positives indicate the presence of an illegal substance that is not present in the sample. They happen because colors can react with impurities or similar compounds to reveal the presence of a specific class of substances, which may be mistaken for the illicit substance [20,30]. It is worrisome because a person carrying a lawful product can suffer the legal consequences related to the banned substance. A typical example is cocaine, which can be confused with other substances, such as caffeine, lidocaine, and procaine. In contrast, cocaine identification by spot tests can return false positives because it is not frequently sold in the pure form. Given that drug screening aims to identify the substance correctly and to assist in future confirmation, studies are being published to avoid misidentification [35].

Colorimetric tests are primarily used because a chemical reaction yields a color change [21,36]. These tests have gained prominence and have been widely applied for traditional substances, as shown in Figure 3, which illustrates an example of a forensic analysis route for an unknown seized substance.

Colorimetric tests do not demonstrate the same effectiveness for NPS: responses can vary according to the NPS concentration in the sample [37]. Besides, a broad spectrum of color responses is possible [38,39] because of the absence of control in NPS production, which can provide reaction residues, which affect the color results [38,40,41]. Bearing in mind that instabilities in presumptive test results occur even for well-known traditional substances, NPS analysis poses additional challenges, and uncertainties in NPS color tests require a better presumptive tool. This situation calls for more frequent application of advanced analytical equipment at the apprehension sites.

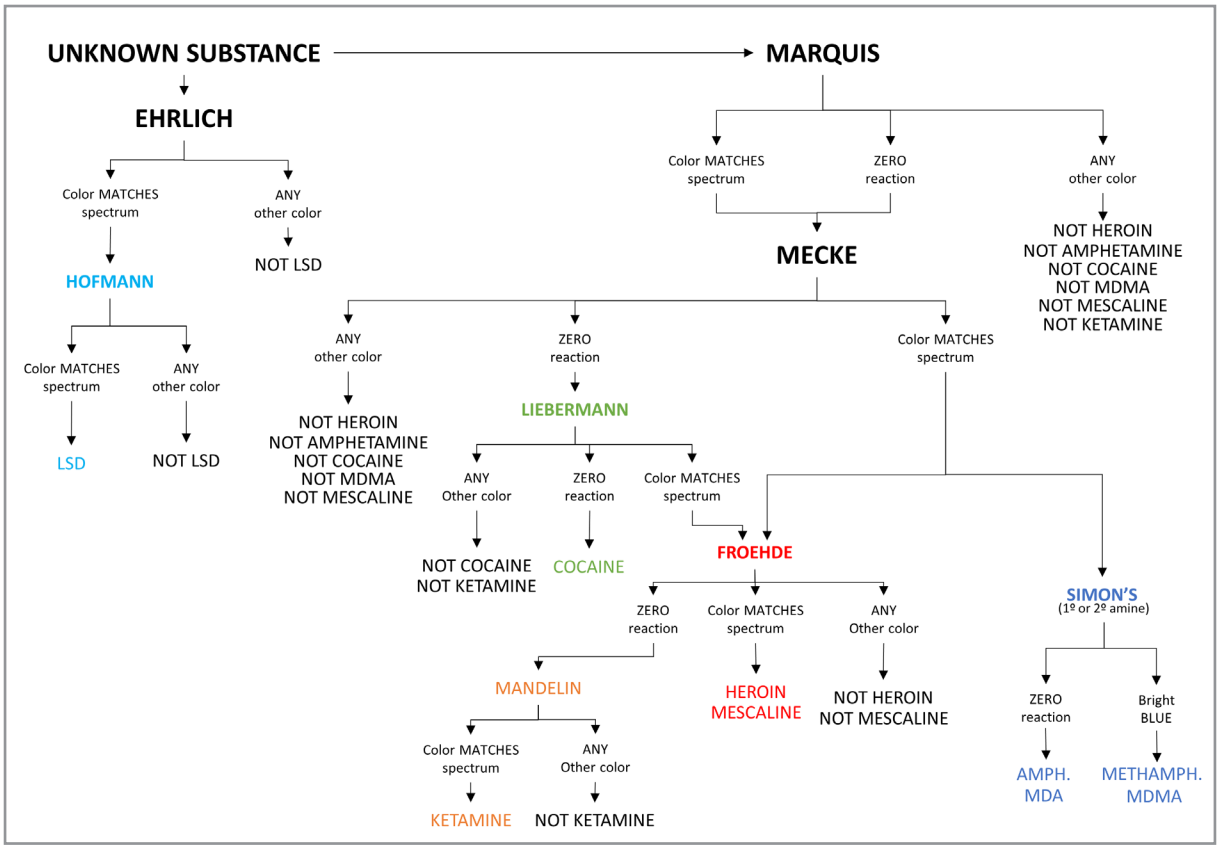


Figure 3. Schematic representation of the use of colorimetric tests as a filter to indicate possible substances [17,21,38,40,42].

Section III: The basics of confirmatory tests

After a positive presumptive test, the substance structure must be confirmed. In this step, the use of reliable analytical equipment is mandatory.

The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) recommendations [43] establishes three categories of techniques grouped according to their highest level of selectivity (see Table I). In each identification category, the techniques are grouped according to the selectivity. For Category A, selectivity is based on structural information. For Category B, selectivity is based on chemical and physical characteristics. For Category C, selectivity is based on general or class information. Additionally, the SWGDRUG recommendations state that:

- When a Category A technique is incorporated into an analytical scheme, at least one other technique from Category A, B, or C that explores different chemical or physical properties of the analyte must be used to support identification.
- When a Category A technique is not used, at least three different techniques must be employed; two must be Category B techniques, whose combination should provide a high degree of selectivity. The third technique (Category B or C) is required to support the identification.

For the analyte to be successfully identified, the test results must be positive, meet all quality control requirements, and achieve the required selectivity. The UNODC's Manual for use by National Drug Analysis Laboratories [44] recommends that at least three entirely different analytical techniques (*e.g.,* color tests, chromatography, and spectroscopy) be used.

Table I. SWGDRUG recommended techniques [43]

Category	Technique
A Selectivity based on Structural Information	Infrared Spectroscopy Mass Spectrometry Nuclear Magnetic Resonance Raman Spectroscopy X-Ray Diffractometry
B Selectivity based on Chemical and Physical Characteristics	Capillary electrophoresis Gas chromatography Ion mobility spectrometry Liquid chromatography Microcrystalline tests Pharmaceutical identifiers Thin layer chromatography
C Selectivity based on General or Class Information	Color tests Fluorescence spectroscopy Immunoassay Melting point Ultraviolet spectroscopy

There are disadvantages associated with confirmatory tests. Sample preparation can be significant and time-consuming. Besides that, the sample can be destroyed during the testing process, and consumable materials could be required [45–47].

Amphetamine-type stimulants – ATS (amphetamines and cathinones)

Phenylethylamines (phenylethan-2-amine) correspond to a group of small alkaloids with a basic structure containing a benzene ring and an ethyleneamine carbon chain (Figure 4(a)) [48]. Amphetamines and cathinones are among the molecules that belong to this group. Both structures are known as substituted phenylethylamines. α-Methylphenylethylamine is the simplest amphetamine known to date (Figure 4(b)) [49]; α-aminopropiophenone is a cathinone (Figure 4(c)) [50,51]. Along with synthetic cannabinoids, these two groups are the main classes of substances that have fostered the rapid growth of NPS's worldwide phenomenon [52–55]. Cathinones and amphetamines have similar structures and hence similar stimulating effects [56]. Their effects are comparable to the effects of cocaine on the body [57–59]. Specifically, they act by inhibiting the return of the monoamine transporter proteins from the synaptic cleft to the pre-synaptic neuron [60–62]. Although there are three monoamine transporters — serotonin transporter (SERT), dopamine transporter (DAT), and norepinephrine transporter (NET) [63–65] — nonspecific transport between serotonin, dopamine, or norepinephrine and SERT, DAT, or NET has been reported [66,67]. Cathinones and amphetamines are divided into classes depending on the carrier they preferentially act [68,69]. The stimulating effects are complex but similar to the effects of cocaine [61,69], and they include paranoia reflexes, delirium, tachycardia, hypertension, aggressive behavior, and pulmonary edema [24,68,70–72].

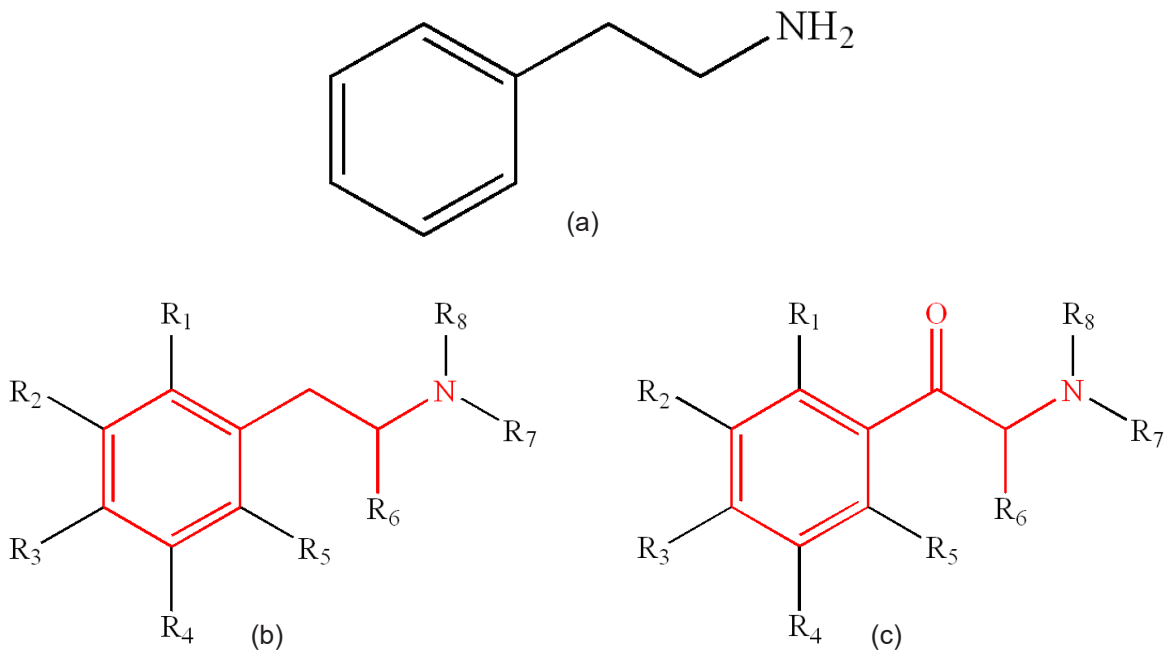


Figure 4. Schematic representation of the molecular structures of (a) Phenylethylamine, (b) Amphetamine-like substances, and (c) Cathinone-like substances.

Amphetamines and cathinones have a similar chemical structure [73], which is challenging for their characterization and differentiation. When colorimetric tests are used for initial evaluation, detection is not based on structures but on a chemical reaction characteristic of specific functional groups. This reaction causes a certain color to appear when the result is positive. However, false positives and false negatives can occur because there are tests which lack specificity [74,75]. For example, the Marquis Test (Color test - Category C) [38] provides a positive response for methylenedioxymethamphetamine (MDMA), fentanyl, and tramadol [40], whose structures belong to different classes of drugs. It is necessary to have specific detectors aligned with NPS demands, minimizing errors in presumptive tests. Possibilities include new molecules for detection [76,77], nanomaterials [78–80], and macromolecules in sensors [81,82].

For amphetamines and cathinones, Category B techniques can chemically differentiate between them due to differences in lipophilicity. The most straightforward structures may not have groups that alter lipophilicity. Differentiation is possible due to the presence of β-ketone in the cathinone derivatives, making them less lipophilic than amphetamines [4].

Different laboratory techniques can be applied to confirm presumptive tests. Mass spectrometry (MS) [83–86] provides characteristic fragmentations for amphetamines and cathinones. Due to the presence of the β-keto group in cathinones, chromatographic techniques can identify them [87–91]. Techniques such as nuclear magnetic resonance (NMR) [92–96], spectroscopy in the infrared region (IR) [21,97–99], and X-ray diffraction [100,101], among others [88,102–104], can accurately distinguish between amphetamines and cathinones. From a legal perspective, the technical professional that carries out the analyses must be able to distinguish between these substances because there is no consensus on banning amphetamines and cathinones.

Although validation methodologies are available, detection techniques pose challenges. One of them is that a condition of polysubstance may exist during apprehension. Substances such as caffeine, paracetamol, and methaclopramide, among others, may structurally resemble amphetamines and cathinones. Besides that, degradation products or residues from the synthesis of these substances must be considered [105]. Preparing samples from simple biological material, such as urine and saliva, does not require extensive and complex procedures [83,89,106,107]. For more complex biological samples, like blood, meconium,

or other unconventional matrixes [108–111], preparation requires steps that include the use of liquid or solid extractions [112,113]. When the substances are still in their commercial form, in powders or tablets, analysis can be directly performed by spectroscopic techniques or through dissolution for further analysis [114–116]. Despite the constant challenges in analyzing amphetamines and cathinones, techniques have been reported for their identification and characterization, including molecular imprinting extraction [117], analysis of stable isotopes [118], methods based on electrochemical techniques [119–122], nanoparticles or macroparticles [123,124], and miniaturization [125], all of which can be combined with chemometrics [126–129].

Synthetic cannabinoids

Δ9-tetrahydrocannabinol (THC) and other cannabinoids are distributed differently in the brain, with high concentrations in the neocortical, limbic, sensory, and motor areas. Cannabis affects almost every system in the body; acts as anxiolytic, sedative, analgesic, and psychedelic agent; stimulates appetite; and has systemic effects [130,131].

THC and other CB1 cannabinoid receptor agonists react to the responses of the central nervous, providing beneficial analgesia, attenuating nausea, and vomiting in cancer chemotherapy, reducing intraocular pressure, stimulating appetite in stressful syndromes, relieving muscle spasms/spasticity in multiple sclerosis, and decreasing intestinal motility. However, undesirable side effects accompany these therapeutic responses, such as changes in cognition and memory, dysphoria/euphoria, and sedation [132–135].

Cannabis impairs cognitive and psychomotor performance. Its effects resemble the effects of alcohol and benzodiazepines and include reaction deceleration, motor incoordination, specific defects in short-term memory, difficulty concentrating, and impairment in complex tasks requiring divided attention. Cannabinoids produce dose-related tachycardia that can reach rates of up to 160 beats/minute or more, but tolerance is developed with chronic use. Chronic marijuana smoking is associated with bronchitis and emphysema. At high doses, the effects can change and (i) lead to recent memory loss and difficulty performing tasks that require mental performance, (ii) cause anxiety, and (iii) trigger or aggravate a psychotic condition [130]. Synthetic cannabinoids comprise different products with chemical structures that resemble the structure of THC (Figure 5), the primary psychoactive principle of natural cannabis. The structural characteristics of synthetic cannabinoids allow them to bind to one of the known cannabinoid receptors, namely CB1 or CB2, present in human cells. The emergence of synthetic cannabinoids such as NPS, sold under names like “Spice” and “K2”, was first reported in 2004. Since then, new drugs have been increasingly reported in different parts of the world [136,137]. Synthetic cannabinoids encompass various structurally different substances, with the possibility of structural changes, potentially modifying affinity for cannabinoid receptors. In general, they represent a diverse group of potent psychoactive substances that can result in agonistic, inverse agonistic, or antagonistic effects [136].

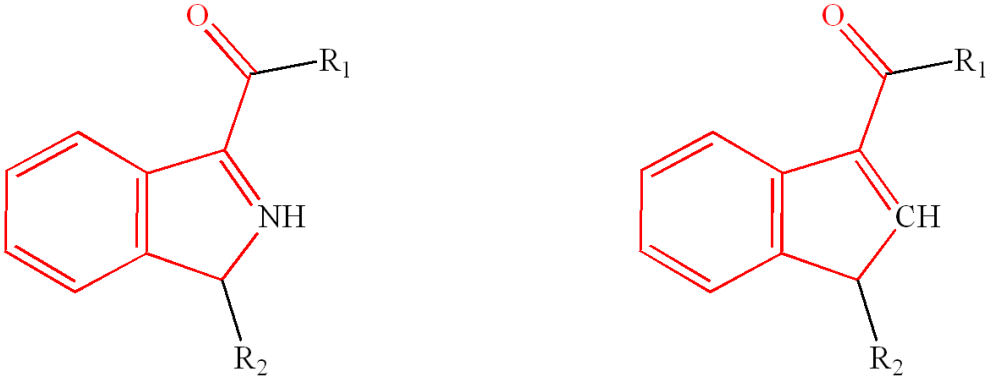


Figure 5. Schematic representation of the molecular structure of synthetic cannabinoid-like substances.

The large variety of molecular structures poses a challenge for forensic analysis and the identification of synthetic cannabinoids [138]. The most common presumptive tests for *Cannabis sativa* L. are Fast Blue B (FBB) and Duquenóis-Levine, which are based on the reaction of the analyte with the phenolic groups present in the chemical structure of the FBB and Duquenóis-Levine reagents [139]. The emergence of synthetic cannabinoids has posed significant problems for their presumptive identification by traditional color tests. Despite being marketed and sold as "legal high" products, these substances do not contain the active constituent present in marijuana, THC, so that Duquenóis-Levine test could be inappropriate. Synthetic cannabinoids are a diverse class of NPS that contain different sub-class structures [17]. A specific test for these substances is challenging because analogous compounds present in vegetables can behave similarly [139]. Literature shows studies on tests with synthetic cannabinoids. There have been reported tests for the cannabinoid JWH-019 which provide false positives results [140]. The Fast Blue BB reagent (FBBB) has been studied to test three cannabinoids: THC, cannabidiol (CBD), and cannabinol (CBN). There are commercial teas which have been used for comparison and proven to interfere in the test when extracted with polar solvent [141]. Microcrystal testing is not an alternative for analyzing synthetic cannabinoids, either, because herbal mixtures have low concentrations of analytes. Commercially available tests do not provide satisfactory results for all synthetic cannabinoids [136,142]. There are examples of experimental conditions to determine specific synthetic cannabinoids by thin-layer chromatography (TLC) [143].

Color and microcrystal tests are unsuitable for analyzing herbal products due to the low concentration of analytes and possible interferents. In this case, ion mobility spectrometry (IMS) can be considered a sensitive screening method for use as a presumptive test. IMS is a fast and sensitive technique that can detect traces of organic compounds, does not require extraction, and allows easy sampling and handling. The technique can be used as a rapid field detection technique [30,136].

The analytical approach for obtaining information about the chemical structure of synthetic cannabinoids differs from the classic analysis of phytocannabinoids. An essential aspect to consider is sampling: although each product is sold under a specific commercial name, the same group or lot might have different contents. Sensitive methods are necessary to analyze low concentrations of synthetic cannabinoids (usually 1–30 mg g^{-1}), and matrix interference may be possible. Several methods can assist in the analysis: gas chromatography with flame ionization detector (GC-FID), gas chromatography with infrared detector (GC-IRD), gas chromatography with mass detector (GC-MS), TLC, Fourier transform infrared (FTIR), and attenuated total reflectance FTIR (ATR-FTIR) [136].

Simple extraction procedures are crucial for chromatographic analysis because active substances usually adhere to the surface of the plant material. GC-MS analysis can be considered the gold standard because it provides excellent chromatographic resolution. Furthermore, it allows active ingredients to be identified by their spectra with cold electron ionization (GC-MS-EI). However, this technique can be limited when position isomers are analyzed. The analyst should consider performing additional measurements with other IR or GC-IRD techniques to distinguish between them and to provide unambiguous identification [136,144].

Different synthetic cannabinoids exist, so GC-IRD is a valuable tool to identify similar molecules, such as regioisomers, diastereomers, and other isobaric molecules that exhibit almost identical MS spectra.

TLC is a cheap and fast technique that allows large numbers of samples to be processed. When coupled with ambient mass spectrometry techniques, such as Desorption Electrospray Ionization-Mass Spectroscopy (DESI-MS), a wide range of analytes can be identified [145].

In general, an extraction step allows a good IR spectrum to be obtained by evaporating the extract directly into the ATR diamond cell. Mobile FTIR systems are also helpful for fast sorting of materials seized in the field [146].

For quantitative analyses, techniques such as GC-FID, ultra-high performance liquid chromatography (UHPLC), and liquid chromatography coupled to mass spectrometry (LC-MS/MS) can be used [136]. GC-FID can be employed for both qualitative and quantitative determinations. For samples with very

low concentrations of the analyte, a more sensitive technique, such as liquid chromatographic methods, should be used. This is because there are fatty acid derivatives can interfere in gas chromatography methods. LC-MS/MS is suitable for analyses of low concentrations of synthetic cannabinoids in complex herbal mixtures. Its low detection limits allow tracking and analysis of biological specimens, such as blood and hair [147,148].

Other techniques and approaches can be applied to analyze synthetic cannabinoids in herbal products [136]. Direct analysis in real-time mass spectrometry (DART-MS) or desorption atmospheric pressure photoionization (DAPPI) can be directly used in plant material, without the need for extraction or sample preparation [149,150]. High-resolution mass spectrometry (HRMS) can be used to determine the precise elementary compositions of new synthetic molecules, double bonds, and precise mass of ions/fragment [151]. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) enables direct qualitative analysis of herbal mixtures, offers fast and straightforward operation, provides high analysis performance, and can be used for initial "screening" of confiscated material [152]. As for NMR, it allows the structure of new unknown synthetic cannabinoids to be identified and elucidated [153].

NBOMes

NBOMes appeared in 2013 and were initially sold on the Internet as a legal alternative to LSD. They are known as N-Bomb, Smiles, Pandora, and Dime. Increased consumption of these substances can be attributed to their low price and wide availability, and they can be sold as powder, pills, ampoules, and stamps. They are usually associated with intoxication and even death and have no reported therapeutic use or adverse effects. This group consists of class 2C hallucinogens; more specifically, phenethylamines (Figure 6). Despite their structural diversity, one of the most common NBOMes belong to the 25C-NBOMe group [154–157].

The "2C" indication describes the chemical structure in which the phenylamino group is separated by two carbon atoms. The appearance of NBOMes in the underground drug market was favored by the fact that 2C substances contain substituents such as N-(2-methoxybenzyl) phenethylamines. NBOMes are classified according to their substitution in the 4th position of the dimethoxy phenyl ring, to give substances like 25I-NBOMe (Iodine), 25B-NBOMe (Bromine), 25C-NBOMe (Chlorine), and 25H-NBOMe (Hydrogen) and organic groups such as 25D-NBOMe (Methyl), 25E-NBOMe (Ethyl), and 25N-NBOMe (nitro). A "complexant" called hydroxypropyl-b-cyclodextrin can be added to NBOMes during manufacture to make these substances cross membranes more easily, which potentializes their effects. Studies on the activity of NBOMe structures have indicated that the 5-HT2A receptor significantly increases the activity of these substances and hence their pharmacological action. Their hallucinogenic effect stems from activation of these receptors (which imply the same pathophysiology of depression and schizophrenia) given that N-benzyl derivatives have a greater affinity for receptors than analogous "2C" substances. Like lysergic acid, NBOMes are active in minimal doses, so they are often sold as an alternative to LSD [4,156,158–160]. Because NBOMes are new, there is a lack of information about their toxicological properties. They were prohibited only a short time ago.

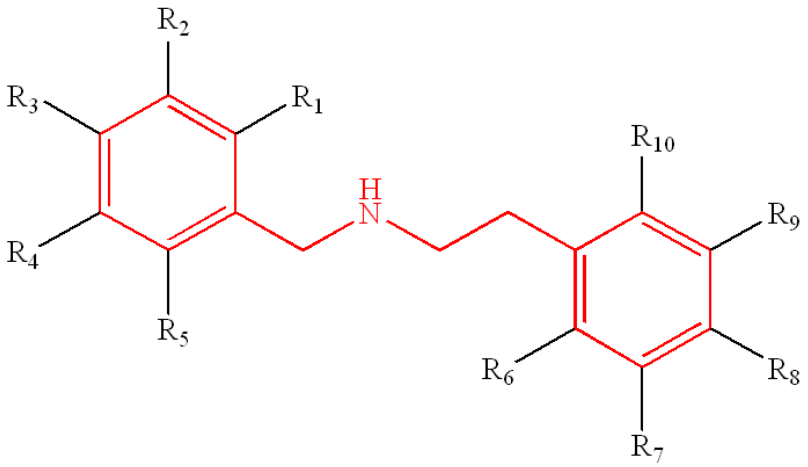


Figure 6. Schematic representation of the molecular structure of NBOMe-like substances.

One of the presumptive tests for detection of NBOMes is the Marquis reagent. This reagent consists of a mixture of 37% formaldehyde and glacial acetic acid, and drops of sulfuric acid [161]. In contact with NBOMe samples, the reagent may reveal different colors. According to the PRO Test 2021 color chart from Chemical Safety, the colors are as follows: orange for 25C-NBOMe, 25D-NBOMe, 25G-NBOMe, and 25T-NBOMe; greenish for 25B-NBOMe; pink for 25E-NBOMe; reddish-brown for 25IP-NBOMe and 25N-NBOMe; and brown for 25H-NBOMe and 25I-NBOMe. The Mecke test, which consists of adding selenous acid to sulfuric acid, can also be used [161]. According to the same color table mentioned previously, the revealed colors are green for 25E-NBOMe and 25IP-NBOMe, dark brown for 25I-NBOMe and 25N-NBOMe, greenish yellow for 25D-NBOMe, dark green for 25H-NBOMe, and lilac for 25T-NBOMe. Bearing in mind that the Marquis test is also used to detect MDMA and amphetamines and can react with common substances such as sugars, false positives can occur because the same colors that would correspond to a specific NBOMe can be revealed when in fact other substances are present in the sample.

The most recommended confirmatory tests to detect and to quantify NBOMe derivatives are liquid and gas mass spectrometry, high-performance liquid chromatography (HPLC), and FTIR. Analysis of NBOMes on blotter papers usually does not require sample preparation when infrared methods are employed. This analysis is non-destructive and preserves the characteristics of the sample, being interesting for forensic objectives. As for liquid chromatography, electroanalytical, and gas chromatography methods, they demand organic solvent extraction, usually methanol. Analysis of biological samples, like blood, urine, serum, vitreous humor, liver, and gastric content, requires extraction with organic solvent for most techniques [162–166]. In samples seized on stamps, GC-MS or LC-MS/MS are generally used. Spectrometric techniques are advantageous: they are not destructive, so the samples are preserved after analysis. Another advantage of these confirmatory tests is the possibility of quantifying and verifying possible impurities. Despite the reliability of these tests, special attention is still needed in the case of GC-MS. Depending on sample preparation, it can be confusing for 2C molecules. These confirmatory tests are mainly applied for seized and biological samples such as fluids and tissues [167–169].

Synthetic opioids

Opioids and opiates are different. Opiates are alkaloids directly isolated from opium, an extract from poppy (*Papaver somniferum*), a plant with medicinal properties. Morphine, codeine, and thebaine are prominent examples of opiates. Chemical modifications to these opiates afford semi-synthetic opioids. Heroin is the most remarkable example of this class – it is obtained by deacetylation of morphine. In turn, synthetic opioids are entirely produced in the laboratory, and they simulate pharmacophoric groups of morphine. All these substances have agonist activity at opioid receptors and present similar mechanisms of action, symptoms, and effects, but potency, time, and duration of action are different [170].

New Synthetic Opioids (NSO) started to appear in 2010. In 2013, they became a serious public health issue, starting the so-called opioid epidemic in the United States of America (USA) and the European Union (EU). NSO can be divided into fentanyl analogous substances and non-fentanyl-related substances. Fentanyl analogous substances (Figure 7(a)) are known as Nonpharmaceutical Fentanyl (NPF) and include substances of the class 4-anilidopiperidine, which have been synthesized clandestinely or have not been approved for medical use. In addition to fentanyl itself, the main fentanyl analogous substances include carfentanyl, 3-methylfentanyl, alfentanil, acetylfentanyl, butyrfentanyl, furanylfentanyl, and *p*-fluorofentanyl, among others. Non-fentanyl-related substances are less varied and do not belong to a single structural class. Their terminology is linked to the pharmaceutical industry or to the researcher who synthesized it. The main examples of these substances are U-47700, U-50488, AH-7921, MT-45, and fenampromide (Figure 7(b)–(e)) [171,172].

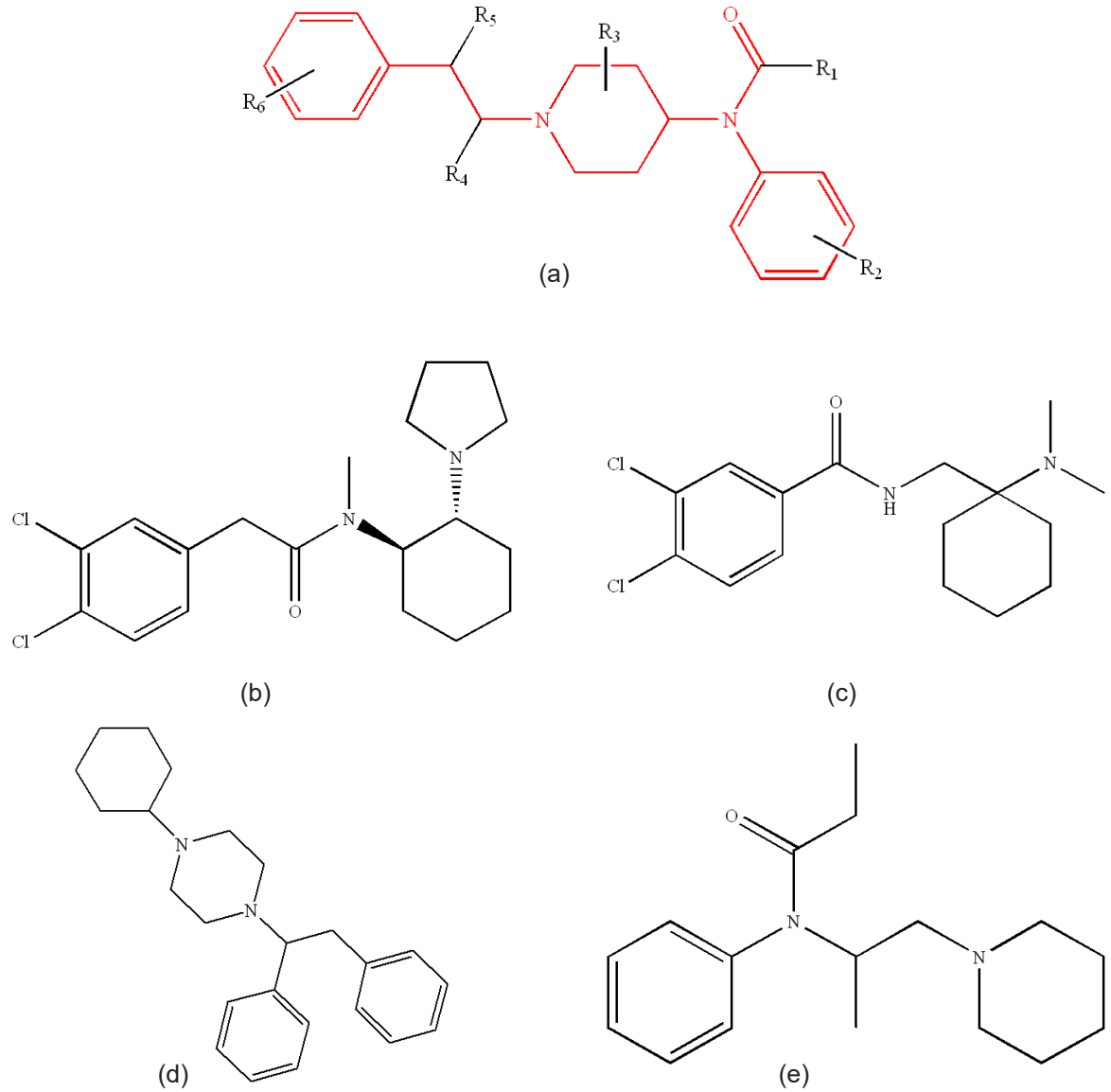


Figure 7. Schematic representation of the molecular structure of (a) Fentanyl-like substances, (b) U-50488, (c) AH-7921, (d) MT-45, and (e) Fenampromide.

The most used technique for detection of synthetic opioids is chromatography; more specifically, GC-MS and LC-MS/MS and liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) [173–175]. High-performance liquid chromatography coupled to ultraviolet detector (HPLC-UV) or amperometric detector (HPLC-AD) [176], ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) [177], and ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS /MS) are also used [178]. Other techniques include IMS and direct analysis of thermal desorption in real-time mass spectrometry (TD-DART-MS) [179], Raman and Infrared spectroscopy [180], and NMR [181].

Analyzed matrixes include blood (whole blood, serum, and plasma), urine, liver, bile, vitreous humor, brain, gastric tissue, kidney, seized samples, and sewage. As for extraction techniques, dilution, liquid-liquid extraction (LLE), solid-phase extraction (SPE), and protein precipitation (PP) are generally used [88,173].

The presumptive tests that are most used to detect synthetic opioids are colorimetric tests, thin-layer chromatography, strip tests, and immunoassays [21]. Among the colorimetric tests, the commercially available Marquis test and Scott test stand out, as well as the Eosin Y test, which is not yet commercially available. These three tests provide adequate results for the presumptive detection of 18 fentanyl analogs. The fentanyl analogs react with the Marquis reagent, to form an orange color immediately and a dark brown color after five minutes. MDMA, heroin, codeine, and morphine also respond to the Marquis reagent, albeit with different colors. The Scott reagent reacts with the fentanyl analogs, benzocaine, and cocaine, to form a blue tint. The Eosin Y reagent reacts with the fentanyl analogs, to give a dark pink color, and with benzocaine, procaine, caffeine, acetaminophen, cocaine, codeine, morphine, and heroin, to result in a light pink color. The discriminating ability of TLC (SiO_2, chloroform-benzene-methanol (10:2:1v/v/v)) has been tested for fentanyl, 4-ANPP, and heroin analogs. TLC can detect the analytes with the modified Dragendorff-Ludy-Tenger reagent, and their retention factors (Rf) have been recorded. Although discrimination of all the 18 fentanyl analogs is not entirely possible, discriminating the fentanyl analogs from N-phenyl-1-(2-phenylethyl)-4-piperidinamine (4-ANPP) and heroin is feasible under these conditions [88].

Immunoassay-based strip tests have been tested for 28 fentanyl analogs. The selectivity and sensitivity of the tests allow between 21 and 24 analogs to be detected. The tests depend entirely on the concentration of the drug solution [182]. Evaluation of immunoassay-based tests has shown that they can detect 11 fentanyl analogs [183]. Immunoassays for opioids use mainly morphine to indicate the result, which can make detection difficult when various drugs are present in the seized sample, making identification impossible [33].

Benzodiazepines

Benzodiazepines, or benzos, are central nervous system depressants with a fused benzene and a diazepine ring system [184]. Figure 8 shows their basic chemical structure. They are classified as sedative-hypnotic medications. They have been prescribed since the late 1960s in the United States because they are highly effective for several medical disorders and short-term treatment of mental health disorders such as anxiety, insomnia, and panic disorder, with the advantage that they are safer than other drugs, especially barbiturates [184–189]. The most common benzodiazepines are diazepam (Valium), lorazepam, and alprazolam, and their abuse has been discussed by Cole and Chiarello [185].

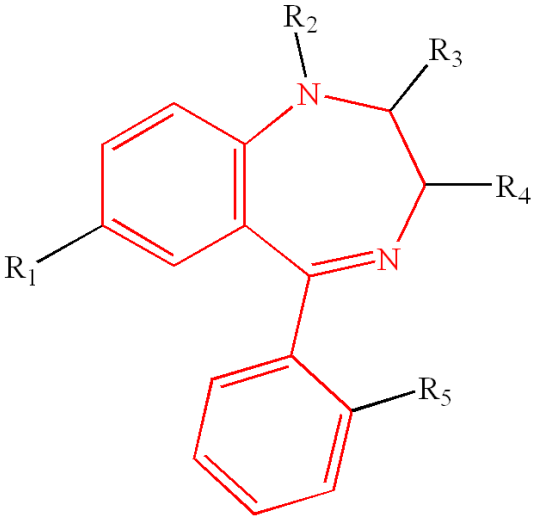


Figure 8. The classical structure of benzodiazepines is based on a 5-aryl-1,4-benzodiazepine structure. “R” labels denote common locations of side chains, which give different benzodiazepines their unique properties [44].

The abuse potential of benzodiazepines was recognized as soon as they started being prescribed, which led the United Nations Convention on Psychotropic Substances 1971 to place 38 benzodiazepines under control [54,190]. Benzodiazepines are one of the most prescribed drugs in the world. They have been linked to a relevant number of deaths. Indeed, many people who were prescribed these drugs for legitimate medical treatment became addicted to them and developed abusive behavior. In the United Kingdom (UK), 21 million benzodiazepine prescriptions take place every year, and 1.5 million people are estimated to be addicted to them [188,191,192].

The abuse of benzodiazepines is a global issue, and an increasing number of NPS-benzodiazepines have appeared in various countries [186,187,193,194]. Elliot and Evans [186] reported that benzodiazepines were present in 38 of 203 cases related to NPS between 2010 and 2012. By 2013, new benzodiazepines such as etizolam and flubromazepam had emerged. Benzodiazepine abuse was reported to have contributed to 45% of the opioid-induced deaths in Australia in 2016. Furthermore, in 2017, sedative hypnotic substances represented 33% of the overall NPS reported to UNODC for the first time at global level [195].

Manchester *et al.* [190] mentioned the emergence of NPS-benzodiazepines in many countries all over Europe. Several of these substances have never undergone the clinical testing that is required of licensed medicines, so their increasing availability poses serious health risks to polydrug users and benzodiazepine-dependent patients who can no longer obtain their prescription and may turn to other means of obtaining benzodiazepines.

In the case of benzodiazepines, which are usually supplied as tablets, capsules, or liquids to be injected, the presumptive test is the Zimmerman test [18,44]. This test is not specific for benzodiazepines, so analysts are advised to combine TLC and cooler development after spraying with selected reagents as a presumptive test. In seized samples, benzodiazepines are commonly present as the free base, hydrochloride, mesylate, or salt.

TLC is a technique that gives good separations for several benzodiazepines. Different preparation methods and visualization are prescribed, and results have been reported for three solvent systems [44].

A colorimetric test based on cobalt thiocyanate has been developed for presumptive identification of benzodiazepines. Such test can be used to quantify these substances and has the advantage of being rapid, highly specific, and low-cost. This color test produces green color for eight benzodiazepines – nitrazepam, temazepam, diazepam, bromazepam, clonazepam, estazolam, lormetazolam, and alprazolam, whereas developed color was absent in other controlled or pharmaceutical substances tested during the study.

Therefore, this test can be a helpful presumptive screening tool for benzodiazepines in suspected illicit samples and pharmaceuticals [196].

GC-FID can analyze most benzodiazepines. However, many of these substances may undergo thermal degradation. GC-MS provides precise spectral data for individual analytes in complex mixtures, often without prior separation. HPLC presents limitations regarding compound separation with benzodiazepines. However, there are recommended methods for qualitative and quantitative analysis of the substances under international control. LC-MS/MS represents a rapid, simple, and overly sensitive procedure for simultaneous analysis of fourteen benzodiazepines. IR usually provides unequivocal identification of benzodiazepines. Still, there are cases of poor solubility in chloroform samples, so the drug cannot be separated in its pure form, which is a limitation of this method and requires other preparation methods [44].

Analytical methods for determining benzodiazepines in human biological specimens have also been reported [190,197,198].

CONCLUSION

Correct identification of illicit substances is essential for law enforcement and health care and driving justice and public policies. Although drug testing is routine in Forensic Laboratories, new analytical challenges have emerged with the rise of new psychoactive substances. Even though various methodologies can be used for drug identification, there is no specific testing methodology for NPS.

In the case of presumptive tests, joint problems and pitfalls include the fact that these tests cannot differentiate numerous compounds that are prohibited. The alternative would be using portable spectroscopic equipment to increase reliability. However, such equipment is expensive and inaccessible to investigative bodies. On the other hand, reliable confirmatory tests are costly and require special conditions for analysis. They can require extensive sample preparation and may be destructive. One of the significant challenges is obtaining analytical standards that can serve as a reference. Even though these tests are reliable, the lack of analytical standards does not allow correct definitive identification. Furthermore, analysis is subject to the analyst's interpretations, which can be subjective. Even when resources for confirmatory tests are available, they may not be sufficient to provide accurate information, thus limiting correct identification [199].

In this paper, we have provided an overview of five classes of compounds. We have presented characteristics and made comments regarding their testing. Information about these drugs is widespread in the literature. It is essential to highlight that there is no ideal method for NPS identification. Combining different analytical tools might be necessary when evaluating these substances. Besides that, new analytical approaches can be constantly searched to improve detection and identification [28,138,200].

There is a constant demand for new information about the chemical characteristics and effects of these substances. Concrete data on seizures, quantity, and types of substances are essential to feed collaborative actions in law enforcement, regulation control, and harm reduction and help set drug policies.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgment

The authors thank the Brazilian Agencies: *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq, Grant 465450/2014-8), *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil* (CAPES) - Finance Code 001. We also thank Cynthia Maria de Campos Prado Manso for language editing and proofreading.

REFERENCES

1. https://www.globalcommissionondrugs.org/wp-content/uploads/2020/06/2020report_EN_web_100620.pdf [Accessed 9 July 2021].
2. https://www.marshallcenter.org/en/publications/occasional-papers/new-psychoactive-substances-challenges-law-enforcement-agencies-and-law [Accessed 9 July 2021].
3. Gomes-Medeiros, D.; Faria, P. H.; Campos, G. W. S.; Tófoli, L. F.; Tófoli, C. L. *Cad. Saúde Pública*, **2019**, *35* (7), 242618 (https://doi.org/10.1590/0102-311X00242618).
4. Zawilska, J. B.; Kacela, M.; Adamowicz, P. *Front. Neurosci.*, **2020**, *14*, p 78 (https://doi.org/10.3389/fnins.2020.00078).
5. Korf, D.; Benschop, A.; Werse, B.; Kamphausen, G.; Felvinczi, K.; Dabrowska, K.; Hernriques, S.; Nabben, T.; Wieczorek, L.; Bujalski, M.; et al. *Int. J. Ment. Health Addict*., **2021**, *19*, pp 873-890 (https://doi.org/10.1007/s11469-019-0052-8).
6. Liu, L.; Wheeler, S. E.; Venkataramanan, R.; Rymer, J. A.; Pizon, A. F.; Lynch, M. J.; Tamama, K. *Am. J. Clin. Pathol*., **2018**, *149* (2), pp 105-116 (https://doi.org/10.1093/AJCP/AQX138).
7. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/368583/NPSexpertReviewPanelReport.pdf [Accessed 22 March 2021].
8. https://www.gov.br/anvisa/pt-br/assuntos/medicamentos/controlados/lista-substancias [Accessed 09 September 2021].
9. https://www.in.gov.br/en/web/dou/-/resolucao-rdc-n-372-de-15-de-abril-de-2020-252726528 [Accessed 09 September 2021].
10. http://www.globalcommissionondrugs.org/wp-content/uploads/2018/09/ENG-2018_Regulation_Report_WEB-FINAL.pdf [Accessed 9 July 2021].
11. Kurcevič, E.; Lines, R. *Harm Reduct. J*., **2020**, *17*, Article number 94 (https://doi.org/10.1186/s12954-020-00448-2).
12. http://www.globalcommissionondrugs.org/wp-content/uploads/2019/06/2019Report_POR_web.pdf [Accessed 09 September 2021].
13. Nutt, D. J.; King, L. A.; Phillips, L. D. *Lancet*, **2010**, *376* (9752), pp 1558-1565 (https://doi.org/10.1016/S0140-6736(10)61462-6).
14. Peacock, A.; Bruno, R.; Gisev, N.; Degenhardt, L.; Hall, W.; Sedefov, R.; White, J.; Thomas, K. V; Farrell, M. *Lancet*, **2019**, *394* (10209), pp 1668-1684 (https://doi.org/10.1016/S0140-6736(19)32231-7).
15. Steuer, A. E.; Brockbals, L.; Kraemer, T. *Front. Chem*., **2019**, *7*, p 319 (https://doi.org/10.3389/fchem.2019.00319).
16. Gerostamoulos, D.; Elliott, S.; Walls, H. C.; Peters, F. T.; Lynch, M.; Drummer, O. H. *J. Anal. Toxicol*., **2016**, *40* (4), pp 318-320 (https://doi.org/10.1093/jat/bkw013).
17. Philp, M.; Fu, S. *Drug Test. Anal*., **2018**, *10* (1), pp 95-108 (https://doi.org/10.1002/dta.2300).
18. Wolstenholme, R.; Jickells, S.; Forbes, S. (Eds.) *Analytical Techniques in Forensic Science*, 1st ed., Wiley, Chichester, UK, **2021**.
19. Clancy, L.; Philp, M.; Shimmon, R.; Fu, S. *Drug Test. Anal*., **2020**, *13* (5), pp 929-943 (https://doi.org/10.1002/dta.2905).
20. Maskell, P. D.; Jackson, G. *WIREs Forensic Sci*., **2020** (https://doi.org/10.1002/wfs2.1371).
21. Harper, L.; Powell, J.; Pijl, E. M. *Harm Reduct. J*., **2017**, *14*, Article number 52 (https://doi.org/10.1186/s12954-017-0179-5).
22. Zhao, L.; Wang, P. *Arch. Pathol. Lab. Med*., **2020**, *144* (11), pp 1325-1334 (https://doi.org/10.5858/arpa.2020-0055-RA).
23. Penido, C. A. F. O.; Pacheco, M. T. T.; Lednev, I. K.; Silveira, L. *J. Raman Spectrosc*., **2016**, *47*, pp 28-38 (https://doi.org/10.1002/jrs.4864).
24. Hondebrink, L.; Nugteren-van Lonkhuyzen, J. J.; Van Der Gouwe, D.; Brunt, T. M. *Drug Alcohol Depend*., **2015**, *147*, pp 109-115 (https://doi.org/10.1016/j.drugalcdep.2014.11.033).

25. United Nations Office on Drugs and Crime. *Recommended methods for the Identification and Analysis of Synthetic Cathinones in Seized Materials* (Revised and updated). United Nations Publication, Vienna, **2020**.
26. Bulska, E.; Bachliński, R.; Cyrański, M. K.; Michalska-Kacymirow, M.; Kośnik, W.; Małecki, P.; Grela, K.; Dobrowolski, M. A. *Front. Chem*., **2020**, *8*, p 693 (https://doi.org/10.3389/fchem.2020.00693).
27. dos Santos, P. F.; Souza, L. M.; Merlo, B. B.; Costa, H. B.; Tose, L. V.; Santos, H.; Vanini, G.; Machado, L. F.; Ortiz, R. S.; Limberger, R. P.; et al. *Quim. Nova*, **2015**, *39* (2), pp 229-237 (https://doi.org/10.5935/0100-4042.20150178).
28. Shafi, A.; Berry, A. J.; Sumnall, H.; Wood, D. M.; Tracy, D. K. *Ther. Adv. Psychopharmacol*., **2020**, *10* (https://doi.org/10.1177/2045125320967197).
29. United Nations Office on Drugs and Crime. *World Drug Report 2013*. United Nations Publication, Sales No. E.13.XI.6: Vienna, **2013**.
30. Snyder, C. *Developing a Presumptive Test for Select Synthetic Cannabinoids*. Master thesis, **2013**, University of Arkansas, Fayetteville, United States.
31. Tadini, M. C.; Balbino, M. A.; Eleoterio, I. C.; De Oliveira, L. S.; Dias, L. G.; Jean-franc, G.; De Oliveira, M. F. *Electrochimica Acta*, **2014**, *121*, pp 188-193 (https://doi.org/10.1016/j.electacta.2013.12.107).
32. Freitas, J. M.; Ramos, D. L. O.; Sousa, R. M. F.; Paixão, T. R. L. C.; Santana, M. H. P.; Muñoz, R. A. A.; Richter, E. M. *Sensors Actuators B. Chem*., **2017**, *243*, pp 557-565 (https://doi.org/10.1016/j.snb.2016.12.024).
33. Milone, M. C. *J. Med. Toxicol*., **2012**, *8*, pp 408-416 (https://doi.org/10.1007/s13181-012-0274-7).
34. Grafinger, K. E.; Liechti, M. E.; Liakoni, E. *Br. J. Clin. Pharmacol*., **2020**, *86* (3), pp 429–436 (https://doi.org/10.1111/bcp.14115).
35. Marcelo, M. C. A.; Mariotti, K. C.; Ortiz, R. S.; Ferrão, M. F.; Anzanello, M. J. *Microchem. J*., **2016**, *127*, pp 87-93 (https://doi.org/10.1016/j.microc.2016.02.012).
36. Toole, K.; Philp, M.; Krayem, N.; Fu, S.; Shimmon, R.; Taflaga, S. In: Musah, R. A., Ed. *Analysis of Drugs of Abuse*. Humana Press, New York, NY, **2018**, pp 1–11.
37. Philp, M.; Shimmon, R.; Stojanovska, N.; Tahtouh, M.; Fu, S. *Anal. Methods*, **2013**, *5*, pp 5402-5410 (https://doi.org/10.1039/c3ay40511g).
38. Darsigny, C.; Couture, M. L.; Desgagné-Penix, I. *Austin J. Forensic Sci. Criminol*., **2018**, *5* (1), id1074.
39. Toole, K. E.; Fu, S.; Shimmon, R. G.; Kraymen, N.; Taflaga, S. *Microgram J*., **2012**, *9*, pp 27-32.
40. Hafer, K. E.; Brettell, T. A. In: *Encyclopedia of Analytical Chemistry*, John Wiley & Sons, Ltd. Chichester, UK, **2018**, pp 1–18.
41. Tsumura, Y.; Mitome, T.; Kimoto, S. *Forensic Sci. Int*., **2005**, *155* (2-3), pp 158-164 (https://doi.org/10.1016/j.forsciint.2004.11.011).
42. https://bunkpolice.com/ [Accessed 22 March 2021].
43. https://www.swgdrug.org/Documents/Comment%20Adjudication%20for%20IIIB.pdf [Accessed 09 September 2021].
44. Gray, A.; Tettey, J.; Lillisunde, P.; Naidia, I. R*ecommended methods for the Identification and Analysis of Barbiturates and Benzodiazepines under International Control*. United Nations of Drugs and Crime, New York, **2012**, pp 1–78.
45. Shergill, A.; Zahid, S.; Bauman, I. In: Clayton, M.; Abbas, N. (Eds.) *Voices of Forensic Science: Are We There Yet? The Golden Standards of Forensic Science*. Forensic Science Program, University of Toronto Mississauga, Mississauga, ON, Canada, **2021**, pp 213–230.
46. Hulme, M. C. *New Psychoactive Substances – New Analytical Challenges and Approaches*. Doctoral thesis, **2018**, Department of Natural Sciences, Manchester Metropolitan University.
47. Klingberg, J. C. *Detection and Profiling of Synthetic Opioids Certificate of authorship and originality*. Doctoral thesis, **2021**, University of Technology Sydney, Australia.
48. Kelly, J. P. *Drug Test. Anal*., **2011**, *3* (7-8), pp 439-453 (https://doi.org/10.1002/dta.313).

49. Cipriano, P. A. D. *Fenetilaminas: De Drogas de Abuso ao Uso Medicinal – Síntese, Propriedades Farmacológicas e Toxicológicas*, Monography (Chemistry), **2018**, University Federal of São João del-Rei, São João del-Rei, MG, Brazil.
50. Berrang, B. D.; Lewin, A. H.; Carroll, F. I. *J. Org. Chem*., **1982**, *47* (13), pp 2643-2647 (https://doi.org/10.1021/jo00134a026).
51. Gussow, L. *Emerg. Med. News*., **2015**, *37* (11), p 24 (https://doi.org/10.1097/01.EEM.0000473176.23346.fc).
52. United Nations Office on Drugs and Crime. *Market Analysis of Synthetic Drugs. Amphetamine-type stimulants, new psychoactive substances.* World Drug Report, United Nations Publication, Sales No. E.17.XI.6: Vienna, Austria, **2017**.
53. Feng, L.-Y.; Battulga, A.; Han, E.; Chung, H.; Li, J.-H. *J. Food Drug Anal*., **2017**, *25* (3), pp 461-471 (https://doi.org/10.1016/j.jfda.2017.04.001).
54. United Nations Office on Drugs and Crime. *Current NPS Threats,* Volume III, 1st ed. United Nations Publication. Vienna, **2020**, pp 1-6.
55. United Nations Office on Drugs and Crime. *Cross-cutting issues: evolving trends and new challenges*. World Drug Report. United Nations Publication, Sales No. E.20.XI.6. Vienna, **2020**.
56. Tortajada, R. E.; San Miguel, J. P.; Doménech, M. G.; Oltra-Cucarella, J.; Costa, M. A. *Rev. Esp. Drog*., **2015**, *40*, 56.
57. Coppola, M.; Mondola, R. *Toxicol. Lett*., **2012**, *211* (2), pp 144-149 (https://doi.org/10.1016/j.toxlet.2012.03.009).
58. Aarde, S. M.; Creehan, K. M.; Vandewater, S. A.; Dickerson, T. J.; Taffe, M. A. *Psychopharmacology (Berl)*, **2015**, *232*, pp 3045-3055 (https://doi.org/10.1007/s00213-015-3944-8).
59. Carboni, E.; Spielewoy, C.; Vacca, C.; Nosten-Bertrand, M.; Giros, B.; Di Chiara, G. *J. Neurosci*., **2001**, *21* (9), RC141 (https://doi.org/10.1523/JNEUROSCI.21-09-j0001.2001).
60. Iversen, L.; White, M.; Treble, R. *Neuropharmacology*, **2014**, *87*, pp 59-65 (https://doi.org/10.1016/j.neuropharm.2014.01.015).
61. Rickli, A.; Hoener, M. C.; Liechti, M. E. *Eur. Neuropsychopharmacology*, **2015**, *25* (3), pp 365-376 (https://doi.org/10.1016/j.euroneuro.2014.12.012).
62. Wang, K. H.; Penmatsa, A.; Gouaux, E. *Nature*, **2015**, *521*, pp 322-327 (https://doi.org/10.1038/nature14431).
63. O'Brien, C. P. In: Brunton, L. L.; Lazo, J. S.; Parker, K. L. (Eds.) *Goodman & Gilman's: The Pharmacological Basis of Therapeutics*, McGraw-Hill Professional, **2009**, pp 607–627.
64. Simmler, L. D.; Buser, T. A.; Donzelli, M.; Schramm, Y.; Dieu, L.-H. H.; Huwyler, J.; Chaboz, S.; Hoener, M. C.; Liechti, M. E. *Br. J. Pharmacol*., **2013**, *168* (2), pp 458-470 (https://doi.org/10.1111/j.1476-5381.2012.02145.x).
65. Schmitt, K. C.; Reith, M. E. A. *Ann. N. Y. Acad. Sci*., **2010**, *1187* (1), pp 316-340 (https://doi.org/10.1111/j.1749-6632.2009.05148.x).
66. Mayer, F. P.; Wimmer, L.; Dillon-Carter, O.; Partilla, J. S.; Burchardt, N. V.; Mihovilovic, M. D.; Baumann, M. H.; Sitte, H. H. *Br. J. Pharmacol*., **2016**, *173* (17), pp 2657-2668 (https://doi.org/10.1111/bph.13547).
67. DeLarge, A. F.; Erwin, L. L.; Winsauer, P. J. *Neuropharmacology*, **2017**, *119*, pp 62-75 (https://doi.org/10.1016/j.neuropharm.2017.04.006).
68. Potocka-Banaś, B.; Janus, T.; Majdanik, S.; Banaś, T.; Dembińska, T.; Borowiak, K. *J. Forensic Sci*., **2017**, *62* (2), pp 553-556 (https://doi.org/10.1111/1556-4029.13326).
69. Lopez-Rodriguez, A. B.; Viveros, M. P. *Psychopharmacology (Berl)*, **2019**, *236*, pp 1001-1014 (https://doi.org/10.1007/s00213-019-05213-3).
70. Castellanos, D.; Menendez, B.; Logan, B. K.; Mohr, A. L. A.; Ayer, D.; Thomas, M.; Foster, A. *J. Drug Abus*., **2018**, *4* (1-4) (https://doi.org/10.21767/2471-853x.100071).

71. Katz, D. P.; Bhattacharya, D.; Bhattacharya, S.; Deruiter, J.; Clark, C. R.; Suppiramaniam, V.; Dhanasekaran, M. *Toxicol. Lett*., **2014**, *229* (2), pp 349-356 (http://dx.doi.org/10.1016/j.toxlet.2014.06.020).
72. Banks, M. L.; Worst, T. J.; Sprague, J. E. *J. Emerg. Med*., **2014**, *46* (5), pp 632-642 (https://doi.org/10.1016/j.jemermed.2013.11.104).
73. Luethi, D.; Liechti, M. E. *Arch. Toxicol*., **2020**, *94*, pp 1085-1133 (https://doi.org/10.1007/s00204-020-02693-7).
74. Anderson, C. *J. Chem. Educ*., **2005**, *82* (12), 1809 (https://doi.org/10.1021/ed082p1809).
75. Smith, D. E.; Marzilli, L.; Davidson, L. D. In: *Textbook of Addiction Treatment*. Springer International Publishing, Cham, **2021**, pp 733–756 (https://doi.org/10.1007/978-3-030-36391-8_51).
76. Lloyd, A.; Russell, M.; Blanes, L.; Somerville, R.; Doble, P.; Roux, C. *Forensic Sci. Int*., **2014**, *242*, pp 16-23 (https://doi.org/10.1016/j.forsciint.2014.06.013).
77. Rodrigues, C. H. P.; Bruni, A. T. *Estudos in silico do comportamento de catinonas sintéticas com interesse forense.* Master's thesis, **2018**, Universidade de São Paulo, Ribeirão Preto, SP, Brazil (https://doi.org/10.11606/D.59.2019.tde-23102018-112244).
78. Zargar, T.; Khayamian, T.; Jafari, M. T. *Microchim. Acta*, **2018**, *185*, 103 (https://doi.org/10.1007/s00604-017-2623-3).
79. Sabzehzari, M.; Ajamgard, M.; Shamlouei, H. R. *Struct. Chem*., **2019**, *30*, pp 1853-1857 (https://doi.org/10.1007/s11224-019-01316-x).
80. Kumar, V.; Kumar, P.; Pournara, A.; Vellingiri, K.; Kim, K.-H. *TrAC Trends Anal. Chem*., **2018**, *106*, pp 84-115 (https://doi.org/10.1016/j.trac.2018.07.003).
81. Jang, Y.; Jang, M.; Kim, H.; Lee, S. J.; Jin, E.; Koo, J. Y.; Hwang, I.-C.; Kim, Y.; Ko, Y. H.; Hwang, I.; Oh, J. H.; Kim, K. *Chem*, **2017**, *3*, pp 641-651 (https://doi.org/10.1016/j.chempr.2017.08.015).
82. Horáček, M.; Gyepes, R.; Císařová, I.; Kubišta, J.; Pinkas, J.; Mach, K. *J. Organomet. Chem*., **2010**, *695*, (21), pp 2338-2344 (https://doi.org/10.1016/j.jorganchem.2010.06.025).
83. Mercieca, G.; Odoardi, S.; Cassar, M.; Rossi, S. S. *J. Pharm. Biomed. Anal*., **2018**, *149*, pp 494-501 (https://doi.org/10.1016/j.jpba.2017.11.024).
84. Dhabbah, A. M. *Forensic Sci. Int*., **2020**, *307*, 110105 (https://doi.org/10.1016/j.forsciint.2019.110105).
85. Alsenedi, K. A.; Morrison, C. *Anal. Methods*, **2018**, *10*, pp 1431-1440 (https://doi.org/10.1039/C8AY00041G).
86. Woźniak, M. K.; Banaszkiewicz, L.; Wiergowski, M.; Tomczak, E.; Kata, M.; Szpiech, B.; Namieśnik, J.; Biziuk, M. *Forensic Toxicol*., **2020**, *38*, pp 42-58 (https://doi.org/10.1007/s11419-019-00485-y).
87. Alsenedi, K. A.; Morrison, C. *J. Chromatogr. B*, **2018**, *1076*, pp 91-102 (https://doi.org/10.1016/j.jchromb.2018.01.027).
88. Gilbert, N.; Antonides, L. H.; Schofield, C. J.; Costello, A.; Kilkelly, B.; Cain, A. R.; Dalziel, P. R. V.; Horner, K.; Mewis, R. E.; Sutcliffe, O. B. *Drug Test. Anal*., **2020**, *12*, 798 (6), pp 798-811 (https://doi.org/10.1002/dta.2771).
89. Gerace, E.; Caneparo, D.; Borio, F.; Salomone, A.; Vincenti, M. *J. Sep. Sci*., **2019**, *42* (8), pp 1577-1584 (https://doi.org/10.1002/jssc.201801249).
90. Loganathan, D.; Yi, R.; Patel, B.; Zhang, J.; Kong, N. *Anal. Bioanal. Chem*., **2021**, *413*, pp 2147-2161 (https://doi.org/10.1007/s00216-021-03182-1).
91. Pérez-Alcaraz, A.; Borrull, F.; Calull, M.; Aguilar, C. *TrAC Trends Anal. Chem*., **2021**, *143*, 116347 (https://doi.org/10.1016/j.trac.2021.116347).
92. Hulme, M. C.; Hayatbakhsh, A.; Brignall, R. M.; Gilbert, N.; Costello, A.; Schofield, C. J.; Williamson, D. C.; Kemsley, E. K.; Sutcliffe, O. B.; Mewis, R. E. *Magn. Reson. Chem*., **2021**, mrc. 5156 (https://doi.org/10.1002/mrc.5156).
93. Trinklein, T. J.; Thapa, M.; Lanphere, L. A.; Frost, J. A.; Koresch, S. M.; Aldstadt, J. H. *Talanta*, **2021**, *231*, 122355 (https://doi.org/10.1016/j.talanta.2021.122355).

94. Zhao, Y.; Wu, B.; Hua, Z.; Xu, P.; Xu, H.; Shen, W.; Di, B.; Wang, Y.; Su, M. *Anal. Sci*., **2021** (https://doi.org/10.2116/analsci.21P048).
95. Westphal, F.; Girreser, U.; Waldmüller, D. *Drug Test. Anal*., **2016**, *8* (9), pp 910-919 (https://doi.org/10.1002/dta.1889).
96. Araújo, A. M.; Valente, M. J.; Carvalho, M.; da Silva, D. D.; Gaspar, H.; Carvalho, F.; Bastos, M. L.; de Pinho, P. G. *Arch. Toxicol*., **2015**, *89*, pp 757-771 (https://doi.org/10.1007/s00204-014-1278-7).
97. Sharma, V.; Kumar, R. *Microchem. J*., **2017**, *134*, pp 104-113 (https://doi.org/10.1016/j.microc.2017.05.014).
98. Gastegger, M.; Behler, J.; Marquetand, P. *Chem. Sci*., **2017**, *8*, pp 6924-6935 (https://doi.org/10.1039/C7SC02267K).
99. Ferus, M.; Cassone, G.; Táborský, V.; Heays, A.; Petera, L.; Knížek, A.; Kalvoda, T.; Bouša, M.; Šponer, J.; Šponer, J. E.; et al. *ACS Omega*, **2021**, *6* (22), pp 14447-14457 (https://doi.org/10.1021/acsomega.1c01325).
100. Zancajo, V. M. R. R.; Brito, J.; Carrasco, M. P.; Bronze, M. R.; Moreira, R.; Lopes, A. *Forensic Sci. Int*., **2014**, *244*, pp 102-110 (https://doi.org/10.1016/j.forsciint.2014.08.010).
101. Spálovská, D.; Paškan, M.; Jurásek, B.; Kuchař, M.; Kohout, M.; Setnička, V. *New J. Chem*., **2021**, *45*, pp 850-860 (https://doi.org/10.1039/D0NJ05065B).
102. Milhazes, N.; Martins, P.; Uriarte, E.; Garrido, J.; Calheiros, R.; Marques, M. P. M.; Borges, F. *Anal. Chim. Acta*, **2007**, *596* (2), pp 231-241 (https://doi.org/10.1016/j.aca.2007.06.027).
103. Christian, J. D. R. *Forensic Investigation of Clandestine Laboratories*. CRC Press, **2003** (https://doi.org/10.1201/9780203484548).
104. Braz, A.; Silva, C. S.; Peixoto, A. C.; Pimentel, M. F.; Pereira, G.; Braga, P. C. C. S.; Martini, A. L.; Alcântara, T. L. F. *J. Raman Spectrosc*., **2021**, *52* (4), pp 901-913 (https://doi.org/10.1002/jrs.6074).
105. Martins, D.; Valente, H.; Pires, C. *Saúde Soc. São Paulo*, **2015**, *24* (2), pp 646-660 (https://doi.org/10.1590/S0104-12902015000200020).
106. Pendl, E.; Pauritsch, U.; Kollroser, M.; Schmid, M. G. *Forensic Sci. Int*., **2021**, *319*, 110658 (https://doi.org/10.1016/j.forsciint.2020.110658).
107. Pascual-Caro, S.; Borrull, F.; Aguilar, C.; Calull, M. *Separations*, **2020**, *7*, 53 (https://doi.org/10.3390/separations7040053).
108. Wang, Y.; Shi, Y.; Yu, Y.; Chen, L.; Jiang, J.; Long, J.; Xiang, P.; Duan, G. *J. Anal. Toxicol*., **2021**, *45* (7), pp 633-643 (https://doi.org/10.1093/jat/bkaa106).
109. Adamowicz, P. *Clin. Toxicol*., **2021**, *59* (7), pp 648-654 (https://doi.org/10.1080/15563650.2020.1848100).
110. Carlier, J.; La Maida, N.; Di Trana, A.; Huestis, M. A.; Pichini, S.; Busardò, F. P. *Therapeutic Drug Monitoring*, **2020**, *42* (2), pp 205-221 (https://doi.org/10.1097/FTD.0000000000000719).
111. Santos Jr, W. J. R.; De Martinis, B. S. *Bioanalysis*, **2020**, *12* (17) (https://doi.org/10.4155/bio-2020-0155).
112. Esteve-Turrillas, F. A.; Armenta, S.; de la Guardia, M. *J. Chromatogr. A*, **2020**, *1633*, 461615 (https://doi.org/10.1016/j.chroma.2020.461615).
113. Daryanavard, S. M.; Zolfaghari, H.; Abdel-Rehim, A.; Abdel-Rehim, M. *Biomed. Chromatogr*., **2021**, *35* (7), e5105 (https://doi.org/10.1002/bmc.5105).
114. Aldubayyan, A. A.; Castrignanò, E.; Elliott, S.; Abbate, V. *Drug Test. Anal*., **2021**, *13* (1), pp 44-68 (https://doi.org/10.1002/dta.2990).
115. Piorunska-Sedlak, K.; Stypulkowska, K. *Forensic Sci. Int*., **2020**, *312*, 110262 (https://doi.org/10.1016/j.forsciint.2020.110262).
116. Rojkiewicz, M.; Kuś, P.; Kusz, J.; Książek, M.; Sochanik, A. *Forensic Toxicol*., **2020**, *38*, pp 481-489 (https://doi.org/10.1007/s11419-020-00525-y).
117. Sorribes-Soriano, A.; Esteve-Turrillas, F. A.; Armenta, S.; Amorós, P.; Herrero-Martínez, J. M. *Anal. Chim. Acta*, **2019**, *1052*, pp 73-83 (https://doi.org/10.1016/j.aca.2018.11.046).

118. Tai, S.; Morrison, C. *TrAC - Trends Anal. Chem.*, **2017**, *86*, pp 251-262 (https://doi.org/10.1016/j.trac.2016.11.008).
119. De Rycke, E.; Stove, C.; Dubruel, P.; De Saeger, S.; Beloglazova, N. *Biosens. Bioelectron*., **2020**, *169*, 112579 (https://doi.org/10.1016/j.bios.2020.112579).
120. Gallardo-González, J.; Baraket, A.; Bonhomme, A.; Zine, N.; Sigaud, M.; Bausells, J.; Errachid, A. *Anal. Lett*., **2018**, *51* (3), pp 348-358 (https://doi.org/10.1080/00032719.2017.1326053).
121. Takahashi, F.; Nitta, S.; Shimizu, R.; Jin, J. *Forensic Toxicol*., **2018**, *36*, pp 185-191 (https://doi.org/10.1007/s11419-017-0388-3).
122. Teófilo, K. R.; Arantes, L. C.; Marinho, P. A.; Macedo, A. A.; Pimentel, D. M.; Rocha, D. P.; de Oliveira, A. C.; Richter, E. M.; Munoz, R. A. A.; dos Santos, W. T. P. *Microchem. J*., **2020**, *157*, 105088 (https://doi.org/10.1016/j.microc.2020.105088).
123. Li, H.; Hu, X.; Zhao, J.; Koh, K.; Chen, H. *Electrochem. Commun*., **2019**, *100*, pp 126-133 (https://doi.org/10.1016/j.elecom.2019.02.002).
124. Masemola, D. P.; Mafa, P. J.; Nyoni, H.; Mamba, B. B.; Msagati, T. A. *J. Environ. Sci. Heal. Part B*, **2020**, *55* (5), pp 455-461 (https://doi.org/10.1080/03601234.2020.1713670).
125. Risoluti, R.; Gullifa, G.; Battistini, A.; Materazzi, S. *Anal. Chem*., **2019**, *91* (10), pp 6435-6439 (https://doi.org/10.1021/acs.analchem.9b00197).
126. Risoluti, R.; Gullifa, G.; Buiarelli, F.; Materazzi, S. *Talanta*, **2020**, *208*, 120456 (https://doi.org/10.1016/j.talanta.2019.120456).
127. Bovens, M.; Ahrens, B.; Alberink, I.; Nordgaard, A.; Salonen, T.; Huhtala, S. *Forensic Sci. Int*., **2019**, *301*, pp 82-90 (https://doi.org/10.1016/j.forsciint.2019.05.030).
128. Salonen, T.; Ahrens, B.; Bovens, M.; Eliaerts, J.; Huhtala, S.; Nordgaard, A.; Alberink, I. *Forensic Sci. Int*., **2020**, *307*, 110138 (https://doi.org/10.1016/j.forsciint.2019.110138).
129. Popovic, A.; Morelato, M.; Roux, C.; Beavis, A. *Forensic Sci. Int*., **2019**, *302*, 109911 (https://doi.org/10.1016/j.forsciint.2019.109911).
130. Ashton, C. H. *Br. J. Psychiatry*, **2001**, *178* (2), pp 101-106 (https://doi.org/10.1192/bjp.178.2.101).
131. Amin, M. R.; Ali, D. W. Pharmacology of Medical Cannabis. In: Bukiya, A. (Ed.) *Recent Advances in Cannabinoid Physiology and Pathology.* Part of the Advances in Experimental Medicine and Biology book series, vol 1162. Springer, Cham., **2019**, pp 151-165 (https://doi.org/10.1007/978-3-030-21737-2_8).
132. Howlett, A. C.; Barth, F.; Bonner, T. I.; Cabral, G.; Casellas, P.; Devane, W. A.; Felder, C. C.; Herkenham, M.; Mackie, K.; Martin, B. R.; et al. *Pharmacol Rev.*, **2002**, *54* (2), pp 161-202 (https://doi.org/10.1124/pr.54.2.161).
133. Howlett A. C.; Abood, M. E. *Adv. Pharm.,* **2017**, *80*, pp 169-206 (https://doi.org/10.1016/bs.apha.2017.03.007).
134. Gonçalves, G. A. M.; Schlichting, L. C. R. *Rev. Bras. Psiquiatr*., **2014**, *20* (1), pp 92-97.
135. Sarne, Y. *Am. J. Drug Alcohol Abuse*, **2019**, *45* (6), pp 551-562 (https://doi.org/10.1080/00952990.2019.1578366).
136. United Nations Office on Drugs and Crime. *Recommended Methods for the Identification and Analysis of Synthetic Cannabinoid Receptor Agonists in Seized Materials (Revised and updated).* United Nations Publication, Vienna, **2020**.
137. United Nations Office on Drugs and Crime (UNODC) *World Drug Report - Executive Summary: Policy Implications*, United Nations Publication, Sales No. E.21.XI.8, Vienna, **2021** (https://doi.org/10.18356/a6fa3135-en).
138. Mercieca, G.; Odoardi, S.; Mestria, S.; Cassar, M.; Strano-Rossi, S. *J. Sep. Sci*., **2020**, *43* (14), pp 2858-2868 (https://doi.org/10.1002/jssc.202000181).
139. Bordin, D. C.; Messias, M.; Lanaro, R.; Cazenave, S. O. S.; Costa, J. L. *Quim. Nova*, **2012**, *35* (10), pp 2040-2043 (https://doi.org/10.1590/S0100-40422012001000025).

140. França, H. S.; Acosta, A.; Jamal, A.; Romao, W.; Mulloor, J.; Almirall, J. R. *Forensic Chem.*, **2020**, *17*, 100212 (https://doi.org/10.1016/j.forc.2019.100212).
141. United Nations Office on Drugs and Crime *Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products*. United Nations Publication, Vienna, **2013.**
142. Sherma, J.; Rabel, F. *J. Liq. Chromatogr. Relat. Technol.*, **2019**, *42* (19-20), pp 613-628 (https://doi.org/10.1080/10826076.2019.1663529).
143. Dresen, S.; Ferreirós, N.; Pütz, M.; Westphal, F.; Zimmermann, R.; Auwärter, V. *J. Mass Spectrom.*, **2010**, *45* (10), pp 1186-1194 (https://doi.org/10.1002/jms.1811).
144. Andres, J.-N.; Siddiqui, H.; Fong, M. The Chemists' Gold: An Analysis of Gas Chromatography-Mass Spectrometry and Future Directions. In: Clayton, M.; Abbas, N. (Eds). *Voices of Forensic Science: Are We There Yet? The Golden Standards of Forensic Science*. Forensic Science Program, University of Toronto Mississauga, Mississauga, ON, Canada, **2021**, pp 231–249.
145. Kneisel, S.; Westphal, F.; Moosmann, B.; Brecht, V.; Bisel, P.; Vidal, C.; Jacobsen-Bauer, A.; Bork, W.-R.; Auwärter, V. *Toxichem Krimtech*, **2011**, *78*, 465.
146. Knittel, J. L.; Holler, J. M.; Chmiel, J. D.; Vorce, S. P.; Magluilo, J.; Levine, B.; Ramos, G.; Bosy, T. Z. *J. Anal. Toxicol.*, **2016**, *40* (3), pp 173-186 (https://doi.org/10.1093/jat/bkv137).
147. Dresen, S.; Kneisel, S.; Weinmann, W.; Zimmermann, R.; Auẅarter, V. *J. Mass Spectrom.*, **2011**, *46* (2), pp 163-171 (https://doi.org/10.1002/jms.1877).
148. Musah, R. A.; Domin, M. A.; Walling, M. A.; Shepard, J. R. E. *Rapid Commun. Mass Spectrom.*, **2012**, *26* (9), pp 1109-1114 (https://doi.org/10.1002/rcm.6205).
149. Kauppila, T. J.; Flink, A.; Haapala, M.; Laakkonen, U. M.; Aalberg, L.; Ketola, R. A.; Kostiainen, R. *Forensic Sci. Int.*, **2011**, *210* (1-3), pp 206-212 (https://doi.org/10.1016/j.forsciint.2011.03.018).
150. Grabenauer, M.; Krol, W. L.; Wiley, J. L.; Thomas, B. F. *Anal Chem.*, **2012**, *84* (13), pp 5574-5581 (https://doi.org/10.1021/ac300509h).
151. Gottardo, R.; Chiarini, A.; Dal Prà, I.; Seri, C.; Rimondo, C.; Serpelloni, G.; Armato, U.; Tagliaro, F. *J. Mass Spectrom.*, **2012**, *47* (1), pp 141-146 (https://doi.org/10.1002/jms.2036).
152. Ernst, L.; Schiebel, H. M.; Theuring, C.; Lindigkeit, R.; Beuerle, T. *Forensic Sci. Int.*, **2011**, *208* (1-3), pp e31-e35 (https://doi.org/10.1016/j.forsciint.2011.03.020).
153. Bersani, F. S.; Corazza, O.; Albano, G.; Valeriani, G.; Santacroce, R.; Posocco, F. B. M.; Cinosi, E.; Simonato, P.; Martinotti, G.; Bersani, G.; Schifano, F. *Biomed Res. Int.*, **2014**, *2014*, Article ID 734749, (https://doi.org/10.1155/2014/734749).
154. de Morais, D. R.; da Cunha, K. F.; Rodrigues, T. B.; Lanaro, R.; Barbosa, L. M.; Zacca, J. J.; Eberlin, M. N.; Costa, J. L. *Forensic Sci. Int.*, **2020**, *309*, 110184 (https://doi.org/10.1016/j.forsciint.2020.110184).
155. Moreira, A. M. S.; de Oliveira, H. L.; Allochio Filho, J. F.; Florez, D. H. Â.; Borges, M. M. C.; Lacerda, V.; Romão, W.; Borges, K. B.; et al. *TrAC Trends Anal. Chem.*, **2019**, *114*, pp 260-277 (https://doi.org/10.1016/j.trac.2019.02.034).
156. Andreasen, M. F.; Telving, R.; Rosendal, I.; Eg, M. B.; Hasselstrøm, J. B.; Andersen, L. V. *Forensic Sci. Int.*, **2015**, *251*, pp e1-e8 (https://doi.org/10.1016/j.forsciint.2015.03.012).
157. Kristofic, J. J.; Chmiel, J. D.; Jackson, G. F.; Vorce, S. P.; Holler, J. M.; Robinson, S. L.; Bosy, T. Z. *J. Anal. Toxicol.*, **2016**, *40* (6), pp 466-472 (https://doi.org/10.1093/jat/bkw035).
158. Zuba, D.; Sekuła, K.; Buczek, A. *Forensic Sci. Int.*, **2013**, *227* (1-3), pp 7-14 (https://doi.org/10.1016/j.forsciint.2012.08.027).
159. Seo, J.-Y.; Hur, K.-H.; Ko, Y.-H.; Kim, K.; Lee, B.-R.; Kim, Y.-J.; Kim, S.-K.; Kim, S.-E.; Lee, Y.-S.; Kim, H.-C.; Lee, S.-Y.; Jang, C.-G. *Brain Res. Bull.*, **2019**, *152*, pp 19-26 (https://doi.org/10.1016/j.brainresbull.2019.07.002).
160. United Nations Office on Drugs and Crime. *Rapid Testing Methods of Drugs of Abuse*. United Nations publication, Vienna, **1994.**

161. Souza, G. A. *Identificação e determinação de novas substâncias psicoativas em amostras de selos por técnicas voltamétricas usando eletrodo de diamante dopado com boro*. Master's dissertation, **2018**, University Federal of Vales do Jequitinhonha e Mucuri, Diamantina, MG, Brazil.
162. Davidson, J. T.; Jackson, G. P. *Forensic Chem*., **2019**, *14*, 100160 (https://doi.org/10.1016/j.forc.2019.100160).
163. Morini, L.; Bernini, M.; Vezzoli, S.; Restori, M.; Moretti, M.; Crenna, S.; Papa, P.; Locatelli, C.; Osculati, A. M. M.; Vignali, C.; Groppi, A. *Forensic Sci. Int*., **2017**, *279*, pp e1-e6 (https://doi.org/10.1016/j.forsciint.2017.08.028).
164. Magalhães, L. de O. *Desenvolvimento de métodos quimiométricos para triagem de novas substâncias psicoativas em selos utilizando técnicas espectroscópicas na região do infravermelho*. Doctoral thesis, **2019**, Institute of Chemistry, University of Brasília, Brasília, DF, Brazil.
165. Magalhães, L. O.; Arantes, L. C.; Braga, J. W. B. *Microchem. J*., **2019**, *144*, pp 151-158 (https://doi.org/10.1016/j.microc.2018.08.051).
166. Custódio, M. F.; Magalhães, L. O.; Arantes, L. C.; Braga, J. W. B. *J. Braz. Chem. Soc*., **2021**, *32* (3), pp 513-522 (https://doi.org/10.21577/0103-5053.20200205).
167. Nikolaou, P.; Papoutsis, I.; Stefanidou, M.; Spiliopoulou, C.; Athanaselis, S. *Drug Chem. Toxicol*., **2015**, *38*, pp 113–119 (https://doi.org/10.3109/01480545.2014.911882).
168. Poklis, J. L.; Raso, S. A.; Alford, K. N.; Poklis, A.; Peace, M. R. *J. Anal. Toxicol*., **2015**, *39* (8), pp 617-623 (https://doi.org/10.1093/jat/bkv073).
169. Suzuki, J.; El-Haddad, S. *Drug Alcohol Depend*., **2017**, *171*, pp 107-116 (https://doi.org/10.1016/j.drugalcdep.2016.11.033).
170. Zawilska, J. B. *Front. Psychiatry*, **2017**, *8*, p 110 (https://doi.org/10.3389/fpsyt.2017.00110).
171. Baumann, M. H.; Tocco, G.; Papsun, D. M.; Mohr, A. L.; Fogarty, M. F.; Krotulski, A. J. *Brain Sci*., **2020**, *10* (11) (https://doi.org/10.3390/brainsci10110895).
172. Marchei, E.; Pacifici, R.; Mannocchi, G.; Marinelli, E.; Busardò, F.; Pichini, S. *Trends Anal. Chem*., **2018**, *102*, pp 1-15 (https://doi.org/10.1016/j.trac.2018.01.007).
173. Armenian, P.; Vo, K. T.; Barr-Walker, J.; Lynch, K. L. *Neuropharmacology*, **2018**, *134*, pp 121-132 (https://doi.org/10.1016/j.neuropharm.2017.10.016).
174. Jannetto, P. J.; Helander, A.; Garg, U.; Janis, G. C.; Goldberger, B.; Ketha, H. *Clin. Chem*., **2019**, *65* (2), pp 242-253 (https://doi.org/10.1373/clinchem.2017.281626).
175. Elbardisy, H.; Foster, C.; Cumba, L.; Antonides, L.; Gilbert, N.; Schofield, C. *Anal. Methods*, **2019**, *11*, pp 1053-1063 (https://doi.org/10.1039/C9AY00009G).
176. Fernández, M.; Wille, S.; Jankowski, D.; Hill, V.; Samyn, N. *Forensic Sci. Int*., **2020**, *307*, 110137 (https://doi.org/10.1016/j.forsciint.2019.110137).
177. Nan, Q.; Ping, X.; Baohua, S.; Xianyi, Z.; Yan, S.; Fenyun, S. *J. Chromatogr. B*, **2019**, *1124*, pp 82-99 (https://doi.org/10.1016/j.jchromb.2019.05.025).
178. Sisco, E.; Verkouteren, J.; Staymates, J.; Lawrence, J. *Forensic Chem*., **2017**, *4*, pp 108-115 (https://doi.org/10.1016/j.forc.2017.04.001).
179. Green, T. C.; Park, J. N.; Gilbert, M.; McKenzie, M.; Struth, E.; Lucas, R.; Clarke, W.; Sherman, S. G. *Int. J. Drug Policy*, **2020**, *77*, 102661 (https://doi.org/10.1016/j.drugpo.2020.102661).
180. Prekupec, M. P.; Mansky, P. A.; Baumann, M. H. *J. Addict. Med*., **2017**, *11* (4), pp 256-265 (https://doi.org/10.1097/ADM.0000000000000324).
181. Bergh, M.; Oiestad, A.; Baumann, M.; Bogen, I. *Int. J. Drug Policy*, **2021**, *90*, 103065 (https://doi.org/10.1016/j.drugpo.2020.103065).
182. Angelini, D.; Biggs, T.; Maughan, M.; Feasel, M.; Sisco, E.; Sekowski, J. *Forensic Sci. Int*., **2019**, *300*, pp 75-81 (https://doi.org/10.1016/j.forsciint.2019.04.019).
183. Moosmann, B.; Auwärter, V. Designer Benzodiazepines: Another Class of New Psychoactive Substances. In: Maurer, H.; Brandt, S. (Eds). *New Psychoactive Substances.* Handbook of Experimental Pharmacology, vol. 252. Springer, Cham, **2018**, pp 383-410 (https://doi.org/10.1007/164_2018_154).

184. Cole, J. O.; Chiarello, R. J. *J. Psychiatr. Res*., **1990**, *24* (S2), pp 135-144 (https://doi.org/10.1016/0022-3956(90)90045-R).
185. Elliott, S.; Evans, J. *Forensic Sci. Int*., **2014**, *243*, pp 55-60 (https://doi.org/10.1016/j.forsciint.2014.04.017).
186. United Nations Office on Drugs and Crime. *World Drug Report 2014*. United Nations Publication, Sales No. E.14.XI.7, New York, **2014.**
187. Zawilska, J. B.; Wojcieszak, J. *Neurotoxicology*, **2019**, *73*, pp 8-16 (https://doi.org/10.1016/j.neuro.2019.02.015).
188. Fudin, H. R.; Babin, J. L.; Hong, L. T.; Ku, J.; May, A. L.; Wisner, A.; Scott Hall, F.; Ray, S. D., **2018**, pp 29–89 (https://doi.org/10.1016/bs.seda.2018.08.015).
189. Manchester, K. R.; Lomas, E. C.; Waters, L.; Dempsey, F. C.; Maskell, P. D. *Drug Test. Anal*., **2018**, *10* (1), pp 37-53 (https://doi.org/10.1002/dta.2211).
190. https://www.ukat.co.uk/benzodiazepines/ [Accessed 6 September 2021].
191. Schmitz, A. *Ment. Heal. Clin*., **2016**, *6* (3), pp 120-126 (https://doi.org/10.9740/mhc.2016.05.120).
192. Beharry, S.; Gibbons, S. *Forensic Sci. Int*., **2016**, *267*, pp 25-34 (https://doi.org/10.1016/j.forsciint.2016.08.013).
193. Høiseth, G.; Tuv, S. S.; Karinen, R. *Forensic Sci. Int*., **2016**, *268*, pp 35-38 (https://doi.org/10.1016/j.forsciint.2016.09.006).
194. United Nations Office on Drugs and Crime. *World drug report 2017: Global overview of drug demand and supply*. United Nations publication, Sales No. E.17.XI.6, Vienna, **2017**.
195. Mahmood, Z.; Muhammad, S.; Arshad, N.; Ma, T.; Mz, Q.; Usman, M. *J. Appl. Pharm*., **2018**, *10*, 3. Available at: https://www.longdom.org/open-access/a-new-colorimetric-identification-of-benzodiazepines-using-cobalt-thiocyanate-as-reagent-1920-4159-1000265.pdf [Accessed September 2021].
196. Qriouet, Z.; Qmichou, Z.; Bouchoutrouch, N.; Mahi, H.; Cherrah, Y.; Sefrioui, H. *J. Anal. Methods Chem.,* **2019**, *2019*, Article ID 2035492 (https://doi.org/10.1155/2019/2035492).
197. Szatkowska, P.; Koba, M.; Kośliński, P.; Wandas, J.; Bączek, T. *Cent. Eur. J. Chem*., **2014**, *12* (10), pp 994-1007 (https://doi.org/10.2478/s11532-014-0551-1).
198. Dror, I. E. *Anal. Chem*., **2020**, *92* (12), pp 7998-8004 (https://doi.org/10.1021/acs.analchem.0c00704).
199. Ahmad, S. M.; Nogueira, J. M. F. Talanta, **2019**, *199*, pp 195-202 (https://doi.org/10.1016/j.talanta.2019.02.004).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 79-105
doi: 10.30744/brjac.2179-3425.RV-50-2021

BrJAC

REVIEW

Promising Applications of Additive-Manufactured (3D-printed) Electrochemical Sensors for Forensic Chemistry

Sílvia V. F. Castro[1], Raquel G. Rocha[1], Afonso F. João[1,2], Eduardo M. Richter[1], Rodrigo A. A. Munoz[1]*

[1]Núcleo de Pesquisa em Eletroanalítica (NuPE), Instituto de Química, Universidade Federal de Uberlândia, 38400-902, Uberlândia, MG, Brazil
[2]Department of Math and Natural Science, Púnguè University, Chimoio, Mozambique

Additive-manufacturing is one of the major pillars of the new industrial revolution and the three-dimensional (3D) printing technology has been highlighted in this scenario. Among the many areas benefited by 3D-printing, the development of electrochemical sensors has appeared in evidence in the last years. One potential application of 3D-printed electrochemical sensors is devoted to forensic chemistry, which demands for portable analytical methods that can provide on-site measurements and thus bring a relevant information *in loco*. In this context, this review highlights the recent contribution of 3D-printing technology on the development of electrochemical sensors with great promises for on-site analysis in "real-world" forensic scenarios. From the detection of trace explosives, gunshot residues, illicit drugs and chemical threats, to the measurement of adulterants in food and fuels, we show the wide range of applications that 3D-printed electrochemical sensors have been proposed and future demands that can be addressed by such a powerful, affordable, and accessible tool.

Keywords: Forensics, Illicit drugs, Crime Scene, Electrodes, Composite.

Abbreviations: 3D, three dimensional; AAS, atomic absorption spectroscopy; ABS, acrylonitrile butadiene styrene; ASTM, American Society for Testing and Materials; BIA, batch injection analysis; CB/PLA, carbon black/polylactic acid; DMF, dimethylformamide; DNT, 2,4-dinitrotoluene; DPV, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; FDM, fused deposition modeling; G/PLA,

Cite: Castro, S. V. F.; Rocha, R. G.; João, A. F.; Richter, E. M.; Munoz, R. A. A. Promising Applications of Additive-Manufactured (3D-printed) Electrochemical Sensors for Forensic Chemistry: A Review. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 79-105. doi: http://dx.doi.org/10.30744/brjac.2179-3425.RV-50-2021

Submitted 27 March 2021, Resubmitted 08 July 2021, Accepted 13 July 2021, Available online 31 August 2021.

graphene/polylactic acid; GSR, gunshot residue; HMTD, hexamethylene triperoxide diamine; HPLC, High performance liquid chromatography; ICP OES, inductively coupled plasma with optical emission spectrometry; ICP-MS, inductively coupled plasma with mass spectrometry; IMS, ion mobility spectrometry; LIBS, laser-induced breakdown spectroscopy; NAA, neutron activation analysis; NPS, novel psychoactive substances; PB, Prussian blue; PETN, pentaerythritol tetranitrate; PGE, pencil graphite electrode; RDX, cyclo-1,3,5-trimethylene-2,4,6-trinitramine; SEM/EDX, scanning electron microscopy with energy dispersive X-ray analysis; SLA, stereolithography; SLM, selective laser melting; SPE, screen-printed electrode; SWASV, square-wave anodic stripping voltammetry; SWV, square-wave voltammetry; TATP, triacetone triperoxide; TNT, 2,4,6-trinitrotoluene;

INTRODUCTION

Three-dimensional (3D) printing, which is considered an additive manufacturing technology, has been considered one of the pillars of the fourth industrial revolution due to the enormous benefits brought to several areas, including dentistry, medicine, electronics, aerospace, engineering, civil construction, food and many others [1]. The chemistry area has also been greatly impacted by 3D printers, as they can provide fast and low-cost fabrication of geometrically complex 3D structures and many parts or objects to replace broken pieces of instruments inside research and teaching laboratories [2]. Moreover, 3D printing can be used to fabricate versatile reaction ware for chemical synthesis [3] as well as electrodes for rechargeable batteries [4]. Probably, electrochemistry and analytical chemistry are two major traditional areas that have been taken several advantages from this technology for several applications as reported in recent revisions for electrochemical energy application [5], electrochemical sensors [6], analytical detectors [7] and microfluidic devices [8].

One field of investigation that can be greatly benefited by 3D printing is the forensic science, specifically forensic chemistry. A recent review highlighted the applications of 3D printing in forensic science, including crime scene reconstruction, ballistic reconstruction, pattern and impression evidence, forensic archeology, medicine, anthropology, taphonomy, odontology and engineering; however, there are no mentions of forensic chemistry [9]. Aiming to inspire forensic chemistry researchers, this review shows potential applications of 3D printing for electrochemical sensing of different molecules of forensic interest. The focus on electrochemistry is explained by the inherent advantages provided by electrochemical methods for portable analysis, which is an Achilles tendon in forensic chemistry, for instance most analytical methods for onsite monitoring of chemical evidences are based on colorimetric assays. Hence, this review aims to shed light on electrochemical sensors for forensic analyses as well as on the introduction of 3D printing in the development of affordable and large-scale electrochemical devices aiming at obtaining chemical evidences to aid police forces or regulatory agencies to solve criminal issues. The review is divided into three sections (a: explosive and gunshot residue; b: illicit drugs; and c: food and fuel) in which 3D-printed electrodes were successfully applied. Special attention is given to the fused deposition modelling (FDM) which has been demonstrated a powerful tool for the fabrication of low-cost electrochemical devices. As conclusions, many other possibilities of 3D printing for forensic electrochemistry are envisaged showing great perspectives of such a powerful tool.

EXPLOSIVES AND GUNSHOT RESIDUE

The development of new analytical methods for the detection of explosives is one of the fields of forensic analysis that has attracted great interest by different research groups [10,11]. This interest is a consequence of the need for finding alternatives to improve public security, since explosives are commonly used in terrorist attacks [12]. In this context, the application of chemical knowledge combined with portable instrumentation has made possible the development of analytical devices that enable the detection of traces of explosives at the crime scene to provide information to assist in the identification of suspects. Some of the widely used explosive compounds are 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (DNT), cyclo-1,3,5-trimethylene-2,4,6-trinitramine (RDX), pentaerythritol tetranitrate (PETN), triacetone triperoxide (TATP), and hexamethylene triperoxide diamine (HMTD) [10,13].

The literature presents some methods commonly used in the identification and determination of these explosives, with great emphasis on ion mobility spectrometry (IMS), Raman and colorimetric detection methods, which have been widely used over the past few years [11]. Additionally, electrochemical methods have also been widely explored due to some special features, such as sensitivity, low cost, easy miniaturization, and portable instrumentation, which can be combined with chemically-modified electrodes to generate electrochemical sensors with improved selectivity and stability [10,14]. Among the main conductive materials used for the development of electrochemical sensors, we highlight the large number of studies involving carbon-based electrodes [15–19].

With the increase of interest in 3D printing, a variety of electrochemical devices have emerged, and the analysis of explosives has also become the object of study by several research groups, as showed in Table I. Tan *et al*. demonstrated the utilization of a gold plated-3D printed stainless steel electrode for the determination of TNT and DNT, as summarized in Table I – Line A [20]. The 3D-printed metallic sensor was built using the SLM printing technique, and subjected to a gold electroplating step. Differential pulse voltammetry (DPV) was the selected electrochemical technique for the analysis of explosives. To evaluate the performance of the proposed sensor, the authors performed comparative studies with a glassy carbon electrode (GCE). Importantly, on bare 3D-printed electrode, no electrochemical signs of DNT and TNT were observed, which highlights the need for modifications of the electrode surface to improve the electrochemical performance of such electrodes. This improvement was attributed to the increase in surface area and electrocatalytic properties of the sensor. The gold-plated 3D-printed metallic sensor showed better sensitivity, 3.6 times greater than GCE in the determination of DNT, while for TNT, the sensitivity was 1.4 times greater than GCE. The results also evidenced a good linear range for DNT using the 3D-printed electrode (60 to 220 μmol L^{-1}) in comparison with GCE (1 to 200 μmol L^{-1}). For TNT, the obtained linear range was set between 220 and 400 μmol L^{-1}.

In addition to explosives, the authors also demonstrated the application of the sensor for the determination of fenitrothion, a pesticide that may be found in contaminated natural waters [20]. Considering that metallic electrodes can be used for several applications in electroanalysis, including forensic electrochemistry, the selective laser melting (SLM) technique would provide great promises for electrochemical sensing; however, an SLM 3D-printer presents a very high cost compared with other 3D printers, such as FDM, and thus is less accessible to many laboratories.

Table I. 3D printed electrochemical sensors applied for the determination of explosives and gunshot residue

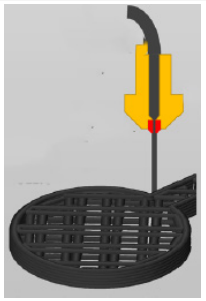
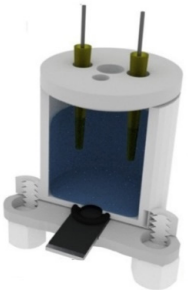
Electrochemical device	Treatment/ Activation	3D printing technique	Design	Analyte	Class	Analytical technique	Ref.
Gold plated-3D printed stainless steel (Line A)	—	SLM		TNT and DNT	Explosives	DPV	[20]
G/PLA (Line B)	Chemical activation	FDM		Picric Acid	Explosives	CV	[21]
G/PLA (Line C)	Thermal annealing	FDM		Picric acid	Explosives	CV	[30]
G/PLA (Line D)	Mechanical polishing	FDM		TNT	Explosives	SWV	[31]

Table I. 3D printed electrochemical sensors applied for the determination of explosives and gunshot residue (Continuation)

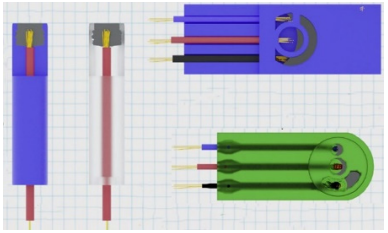
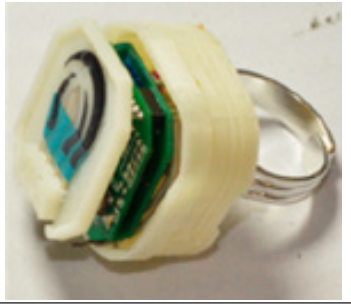
Electrochemical device	Treatment/ Activation	3D printing technique	Design	Analyte	Class	Analytical technique	Ref.
CB/PLA (Line E)	Electrochemical activation	3D printing pen		TNT	Explosives	SWV	[35]
Dual bioink printed nose (Line F)	—	-		TNT	Explosives	EIS	[36]
Ring-based screen-printed sensor (Line G)	—	FDM		DNT	Explosives	SWV	[38]

Table I. 3D printed electrochemical sensors applied for the determination of explosives and gunshot residue (Continuation)

Electrochemical device	Treatment/ Activation	3D printing technique	Design	Analyte	Class	Analytical technique	Ref.
G/PLA (Line H)	Mechanical polishing and chemical activation	FDM		Pb^{2+} and Sb^{3+}	Gunshot residue	SWASV	[62]

Electrochemical device: G/PLA: graphene/polylactic acid; CB/PLA: carbon black/polylactic acid.
3D printing technique: SLM: selective laser melting; FDM: fused deposition modelling.
Analytical technique: DPV: differential pulse voltammetry; CV: cyclic voltammetry; SWV: square-wave voltammetry; EIS: electrochemical impedance spectroscopy.
Images reprinted with permission from American Chemical Society:
● Tan, C.; Nasir, M.Z.M.; Ambrosi, A.; Pumera, M. 3D Printed Electrodes for Detection of Nitroaromatic Explosives and Nerve Agents. Anal. Chem., 2017, 89 (17) 8995–9001 (https://doi.org/10.1021/acs.analchem.7b01614). Copyright © 2017 American Chemical Society. [20]
● Palenzuela, C.L M.; Novotný, F.; Krupička, P.; Sofer, Z.; Pumera, M. 3D-Printed Graphene/Polylactic Acid Electrodes Promise High Sensitivity in Electroanalysis. Anal. Chem., 2018, 90, 5753–5757 (https://doi.org/10.1021/acs.analchem.8b00083). Copyright © 2018 American Chemical Society. [21]
● Novotny, F.; Urbanova, V.; Plutnar, J.; Pumera, M. Preserving Fine Structure Details and Dramatically Enhancing Electron Transfer Rates in Graphene 3D-Printed Electrodes via Thermal Annealing: Toward Nitroaromatic Explosives Sensing. Applied Materials, 2019, 11, pp 35371−35375 (https://doi.org/10.1021/acsami.9b06683). Copyright © 2019 American Chemical Society. [30]
● Sempionatto, J. R.; Mishra, R. K.; Martín, A.; Tang, G.; Nakagawa, T.; Lu, X.; Campbell, A. S.; Lyu, K. M.; Wang, J. Wearable Ring-Based Sensing Platform for Detecting Chemical Threats. ACS Sensors, 2017, 2 (10), pp 1531–1538 (https://doi.org/10.1021/acssensors.7b00603). Copyright © 2017 American Chemical Society. [38]
Images reprinted with permission from Elsevier:
● Sensors and Actuators B: Chemical, Vol. 292, Cardoso, R.M.; Castro, S.V.F.; Silva, M.NT.; Lima, A.P.; Santana, M.H.P.; Nossol, E.; Silva, R.A.B.; Richter, E.M.; Paixão, T.R.L.C.; Muñoz, R.A.A.,3D-printed flexible device combining sampling and detection of explosives, Pages No. 308-313, Copyright 2019, with permission from Elsevier. [31]
● Analytica Chimica Acta, Vol. 1132, Cardoso, R.M.; Rocha, D.P.; Rocha, R.G.; Stefano, J.S.; Silva, R.A.B.; Richter, E.M.; Muñoz, R.A.A., 3D-printing pen versus desktop 3D-printers: Fabrication of carbon black/polylactic acid electrodes for single-drop detection of 2,4,6-trinitrotoluene, Pages No. 10-19, Copyright 2020, with permission from Elsevier. [35]
● Analytica Chimica Acta, Vol. 1130, Castro, S.V.F.; Lima, A.P.; Rocha, R.G.; Montes, R.H.O.; Santana, M.H.P.; Richter, E.M.; Muñoz, R.A.A., Simultaneous determination of lead and antimony in gunshot residue using a 3D-printed platform working as sampler and sensor, Pages No. 126-136, Copyright 2020, with permission from Elsevier. [62]
Images reprinted with permission from Wiley:
● Jodat, Y. A.; Kiaee, K.; Jarquin, D. V.; Hernández, R. L. D. la G.; Wang, T.; Joshi, S.; Rezaei, Z.; Melo, B. A. G. de; Ge, D.; Mannoor, M. S.; Shin, S. R. Adv. Sci., 2019, 7, 1901878 (https://doi.org/10.1002/advs.201901878). Creative Commons Attribution License 2020, with permission from Wiley. [36]

Palenzuela *et al*. performed the determination of picric acid using as electrochemical sensor a 3D-printed electrode fabricated by FDM and using a thermoplastic filament composed of graphene and polylactic acid (G/PLA) [21]. The commercialization of conductive filaments has strengthened the development of devices for electrochemical application, including sensing and biosensing [6]. FDM is the most affordable 3D-printing technology and for this reason has become very popular, which has encouraged new users of such 3D-printers. However, generally the FDM 3D-printed carbon-based PLA electrodes requires a surface treatment to improve their electrochemical activity as evidenced by cyclic voltammetric experiments of redox probes before and after treatment [22–29]. The work by Palenzuela *et al*. proposed a simple treatment with dimethylformamide (DMF) (immersion of the 3D-printed electrode for 10 min) [21]. The electrodes were printed in two different designs: disc and ring-shaped electrodes (Table I – Line B).

For these two designs, the calculated electroactive area was about 2.6 and 1.9 times greater than the geometric area of the electrodes in ring and disc-shaped, respectively, which is directly related to the higher roughness of the 3D-printed electrodes. Due the larger active area, the authors chose to use the ring-shaped electrode for the determination of picric acid. For this, the cyclic voltammetry was used to study the electrochemical behavior of the analyte, which was similar to that obtained on a GCE, in addition to allow the determination of the analyte in a wide linear range at low concentrations (5 to 350 mg L^{-1}). Moreover, the determination of ascorbic acid was also demonstrated to expand the application of the sensor in different fields [21].

The determination of picric acid was again performed on a 3D-printed G/PLA sensor developed by Novotný *et al*., as showed in Table I – Line C. The main novelty of this work was a new chemical-free pre-treatment of the FDM 3D-printed electrode surface [30]. This treatment consisted of a thermal annealing in a vacuum oven, which promoted a great improvement in the electrochemical activity of the material that was evidenced by cyclic voltammetric scans before and after treatment. Compared to a chemically activated electrode with DMF, the results were also superior, with lower values of capacitance and resistance to charge transfer (Rct). This new type of treatment also provided lower values of ΔEp (difference between oxidation and reduction peaks) and RSD in inter-electrode tests (n = 3) when compared with an electrode without any treatment and treated with DMF. The durability of the 3D-printed electrode was evaluated by carrying out analyses after 8 weeks of manufacture and the results showed the conservation of the electrochemical performance. With promising initial studies, the determination of picric acid was successfully performed in a range from 5 to 50 mg L^{-1}, obtaining a LOD of 0.1 mg L^{-1}, results similar to those obtained using the chemically-treated electrode and GCE, which demonstrates that this form of chemical-free treatment emerges as a viable alternative of treatment.

Important for forensic applications, a novel application of 3D-printed G/PLA electrodes was demonstrated as sampler of explosive residues. The sensor was 3D-printed using the FDM technique and treated by a simple mechanical polishing. After this, the device was used for the collection of TNT residues by swiping the sensor over suspected surfaces (working as a swab), and next placed in an electrochemical cell (Table I – Line D), filled with supporting electrode, and the presence of TNT residues was detected by square-wave voltammetry (SWV) [31]. This strategy can be considered as a voltammetric approach of immobilized particles [32], because the voltammetric scan occurs immediately after immobilization of TNT particles that likely continues adhered on the electrode surface during the voltammetric scan. The adherence of the TNT particles is evident because the first scan presents a much higher current response than the following scans for the same 3D-printed electrode used for TNT sampling. Considering experiments is solution, TNT can be quantified within a linear range of 1 to 870 μmol L^{-1} and LOD of 0.4 μmol L^{-1}. Moreover, the proposed sensor has a satisfactory intra and inter-electrode precisions, with RSD values lower than 10%. For the samples, the amount of TNT collected was estimated using Faraday's Law and the results showed that the 3D-printed sensor acted as a good collector, as it was able to collect 3.2, 20 and 15 ng of TNT on granite, metal and glove surfaces, respectively [31].

Another possibility for obtaining 3D printed sensors was presented by Cardoso and coauthors who performed a comparative study between electrodes obtained by a desktop FDM 3D-printer and a 3D

printing pen [33]. The 3D-printed electrodes were constructed using a carbon black/polylactic acid (CB/PLA) filament and were evaluated in two designs: 1) single disc-shaped working electrode placed in an electrochemical with conventional counter and references electrodes and 2) three-electrode system on a planar substrate similar to a commercially-available screen-printed electrode (Table I – Line E). In both cases, the 3D-printed electrodes were electrochemically activated in 0.5 mol L^{-1} NaOH solution [27,34]. The three-electrode planar system presented better inter-electrode reproducibility (RSD = 4%) and electrochemical performance for the sensing of TNT in a single drop. These results demonstrated the great potential of 3D printing pens in the construction of new devices for the determination of explosives, with a linear range of 5 to 500 µmol L^{-1} and LOD of 1.5 µmol L^{-1}, with high precision between measurements [35].

Although the analytical parameters are not superior in comparison to other works reported in the literature, for example the work by Cardoso and collaborators that also proposes the determination of TNT using 3D-printed electrodes [31], this work brings as main advantages the lower cost, the portability of the three-electrode platform and the possibility of analysis in a single drop, which considerably minimizes the consumption of reagents. Moreover, the authors highlight the possibility of reuse of the sensor up to three times through a mechanical polishing step that promotes the renewal of the surface [35].

In addition to the sensors described above, some research groups have used the advantages of 3D printing for the development of more sophisticated electronic devices combined with electrochemical techniques for the determination of explosives. Jodat *et al*. developed a dual bioink-printed nose constructed with an integrated biosensing system (Table I – Line F) and demonstrated the potential of the device for detection of explosive odor (TNT vapor) [36]. For this, the authors used electrochemical impedance spectroscopy (EIS) associated with a gold-based biosensor functionalized with a TNT-specific peptide. The system was capable to mimic the mechanism of odor detection carried out in the olfactory epithelium and allowed the detection of explosives with high sensitivity, with a wide linear range between 1 and 1000 pg mL^{-1} and LOD of 0.38 pg mL^{-1} of TNT. These results are superior to another bioelectronic nose developed by Gao and collaborators for the TNT detection [37]. Due to the characteristics of the device developed by Jodat *et al.* [36], they also evaluated the degradation of the peptide after the biosensor functionalization process and observed a stability of use for up to 5 days, which indicates a new functionalization step of the substrate every 5 days [36]. Then, this strategy appears as a very interesting alternative for the detection of the explosives in vapor phase using a 3D printed system with high sensitivity, since the vast majority of works, such as those described above, are focused on performing analysis in solution [20,31,35] and residual solid particles [31].

Sempionatto *et al*. presented a wireless wearable ring-based multiplexed chemical sensor platform that was applied in the determination of DNT in liquid and vapor phase, as showed in Table I – Line G. The device consists of a 3D-printed ring containing electronic microstructures integrated with a screen-printed carbon electrode. For explosive detection, the authors used a semisolid agarose hydrogel to cover the electrode surface and SWV was selected as electrochemical technique for DNT determination [38]. To evaluate the stability and the performance of the proposed sensor, the authors performed experiments in 0.1 mol L^{-1} PBS solution, in which it was possible to observe the occurrence of two clear and well-defined peaks referred to DNT reduction. The linear range was obtained between 0 and 100 µg L^{-1}, with clear signals from 10 µg L^{-1} and an LOD estimated to be 4 µg L^{-1}. The excellent performance of the system enabled the detection of DNT vapor. The studies were performed with the presence of 5, 50 and 100 mg of DNT in the system constructed by the authors and which contained the ring as a detector, and the SWV scans showed the increase in the current proportional to the mass of DNT. It is important to emphasize that the increase in the incubation time of explosive in the container also results in an increase in the electrochemical signal, which indicates an accumulation effect. Analytical parameters such as reproducibility and selectivity were studied and again the results are promising.

This method appears as an excellent alternative, especially due to the low cost of screen-printed electrodes, which can be easily replaced to ensure the continuous operation of the device. However, the wearable ring has a limitation related to the stability of the agarose gel, which is susceptible to water

loss and can influence currents acquired by SWV. Stability tests showed that, in a sealed container and in the presence of the analyte, the gel remains stable for a period of 24 h. In addition to this application, the authors also demonstrated the possibility of using the 3D-printed ring for the determination of H_2O_2 and organophosphate, demonstrating the great potential of this device for different voltammetric and amperometric analyses [38].

The papers discussed above demonstrate a crescent interest by research groups in applying the advantages of 3D printing technology in the construction of devices with portable characteristics. Taking that into account, the main highlight is the use of the FDM technique that has been widely explored in the development of devices with the most varied designs. Moreover, most of these works showed the need for a surface pre-treatment of the FDM 3D-printed electrode surfaces to improve their electrochemical activity, with a great emphasis on electrochemical treatments and thermal annealing, since they are more ecological alternatives. Along with this, the use the 3D pen offers great promises since they can also be used construct electrochemical devices with similar performance to desktop 3D-printers. Additionally, the use of 3D pens presents as advantages the lower acquisition cost and the use of small amounts of conductive filament to manufacture the sensor [39,40].

Regarding the analytical performance, it is observed that the sensors showed excellent sensitivity, evidenced by the low values of LOD and wide linear ranges at low concentrations. The results are comparable to other sensors already reported in the literature for the determination of picric acid [41,42], DNT [43–45] and TNT [17,46]. However, especially for TNT, it was observed that the analytical characteristics of sensors obtained by 3D printing are still inferior to several studies involving the use of modified electrodes [43,47–49]. At the same time, this type of electrode is commonly associated with previous steps of modification that increase the time and cost of analysis. On the other hand, 3D printed electrodes, in the most of the time, only require simple surface treatments that bring great improvements in their electrochemical performance.

It is important to highlight that the vast majority of the works described here bring low cost as one of the main characteristics of 3D printing, making the sensors disposable. The exception observed is in the work of Cardoso *et al*. [35], which demonstrated the potential for the reuse of the sensor through surface renewal carried out by mechanical polishing. However, although the sensors are considered disposable, the authors did not present proposals or ways to correctly dispose these devices, which could be an environmental problem. TNT, for example, is considered an environmental pollutant and can cause problems for human health, such as discoloration of hair and skin, aplastic anaemia and liver function disturbances [50]. Therefore, procedures to correctly dispose these sensors aimed at forensic analysis still need to be further explored.

Also related with public security, another area that has attracted great interest in recent years is the examination for the presence or absence of gunshot residue (GSR). These residues are generated during the shot and contain organic and inorganic components, for instance, lead, barium and antimony, which are the main inorganic components [10]. These particles can often adhere in hands and clothes of individuals who fired and can also be found on surfaces closes to the location where the shot was fired [51]. The search for these residues on different surfaces is an important step in the investigation process and can provide information to help elucidate the dynamics of crime events, such as the identification of suspects or the collection of elements forward the identification. As a result of it, highly sensitive and fast methods are necessary, and several researchers have been working to solve these demands.

For the identification of inorganic gunshot residue, the main method used during investigations is scanning electron microscopy with energy dispersive X-ray analysis (SEM/EDX). This technique allows the identification of microparticles formed between metals and the collection of this microparticles is performed by devices called as stubs, which favor the adhesion of the residues through abrasive contact with the contaminated surface [52]. Although SEM/EDX is a reliable and reproductive method, a limitation is the lack of portability of the system, which requires the sample to be stored and transported to the laboratory for analysis. In addition, other methods that have also been reported in the literature for this purpose, such

as using neutron activation analysis (NAA), inductively coupled plasma optical emission spectrometry (ICP OES), inductively coupled plasma mass spectrometry (ICP-MS), and laser-induced breakdown spectroscopy (LIBS), among other techniques [53]; however, they also make use of bulky instrumentation.

In the field of electrochemistry, several sensors have been explored for the identification of GSR, mainly associated with voltammetric stripping methods. The main sensors used for this aim are mercury-based electrodes [54–56] and several types of screen-printed electrodes [57–61].

The use of 3D printing for the development of an electrochemical sensor aimed at the identification of GSR also began to arouse the interest of researchers and was recently reported for the first time by Castro *et al*. In this work, the authors used a G/PLA electrode obtained by FDM 3D printer to identify the presence of GSR on clothes and hands of shooters; the device and electrochemical cell are summarized in Table I – Line H. The dual device (printed rectangular piece), used as a sampler/sensor, was previously subjected to mechanical polishing, followed by chemical treatment (immersion in DMF for 10 min), and later used as a residue collector through direct contact with the studied surfaces. After this, the rectangular piece was coupled to a 3D-printed cell and, through a square-wave anodic stripping voltammetry (SWASV) scan, the detection of nanograms of Pb^{2+} and Sb^{3+} was possible, two of the main components present in this type of sample [62]. Prior to the sample analysis steps, the method was optimized in solution and the analytical parameters were evaluated. The authors obtained a wide linear range for the simultaneous determination of Pb^{2+} and Sb^{3+} (from 50 to 1500 μg L^{-1}), and LODs estimated to be 0.5 and 1.8 μg L^{-1}, respectively.

The sensor was also evaluated through intra and inter-electrode studies, with proper RSD values obtained for both analytes, indicating the precision of the sensor during measurements and also the reproducibility of 3D printing and previous treatments performed. Due to complexity of GSR samples, the sensor was also evaluated as a function of its selectivity in the presence of other metals and, again, satisfactory results were obtained. Additionally, the authors also demonstrated the possibility of reusing the sensor up to 3 times without considerable loss of performance, performing simple steps of mechanical polishing between each reuse, which is enough to promote cleaning and renewal of the electrode surface. Then, the obtained analytical parameters as well as the results achieved with the samples are comparable and even superior to other works reported in the literature [63,64].

One of the challenges related to electrochemical analysis of GSR is the determination of barium. However, due to the need for application of extreme potentials [65], new strategies such as surface modification with bismuth (incorporation of bismuth in the polymeric matrix) or other material to reduce the hydrogen overvoltage can be investigated. Anyway, this work demonstrates how 3D printing can bring new alternatives for the analysis of GSR in portable systems and opens up opportunities to study new materials and develop new methods to try to solve this limitation and make possible the simultaneous determination of barium, lead and antimony through electrochemical scans.

ILLICIT DRUGS

Over the recent years, it has been increasing the inquires towards the forensic traces of drugs at crime scenes [66]. Generally, these drugs can be used for recreational purposes due to its sedative, hallucinogenic and/or stimulant effects [67,68]. The attractive properties of 3D-printing technology (ease construction of device, low cost and large-scale production, rapid prototyping of complex structures) have allowed its use for the development of portable systems for forensic applications [6,69,70]. Moreover, 3D-printing technology could help the scientific police in the control of drugs, as well as, in the development of portable devices to obtain crucial information and chemical evidence on site, avoiding the laborious steps in conventional analysis procedures [31,62]. Currently, colorimetric tests are widely available for qualitative and semi-quantitative preliminary tests of illicit drugs in seized samples [10,68,71,72]. These types of tests are known to have desirable characteristics such as rapidity, simplicity, portability and low cost [73–75]. However, the presence of contaminants/adulterants in the sample matrices may induce to false positive or negative results if colorimetric tests are used [76]. The chemical profiling is also carried out in forensic laboratories of many countries using spectroscopic and chromatographic techniques [77,78].

Nevertheless, they require laborious sample pre-treatment steps, high-cost instrumentation, laboratories with good infrastructure and time-consuming. In this context, electroanalytical methods provide portability, low-cost instrumentation, and adequate limit of detection. The combination of such advantages with large-scale and low-cost production of 3D printed electrodes has great potential to increase the popularity of electroanalysis for forensics applications [6,13,68]. Table II highlights the main applications devoted to the determination illicit drugs.

Cocaine is one of the most illegal drugs used in the world and can be found with diluents and/or adulterants to increase the bulk and pharmacological effects [79,80]. In addition, the determination of adulteration patterns in seized drug samples is important for forensic analysis in which allows to understand the traffic route since the compounds or patterns used to adulterate the cocaine [71,77].

In this sense, Rocha *et al*. showed for the first time a potential application of 3D-printed electrodes for the identification and quantification of cocaine and adulterants in a forensic scenario [28]. For this purpose, a 3D-printed three-electrode electrochemical cell was used for all electrochemical measurements, which was manufactured using acrylonitrile butadiene styrene (ABS) filament. The 3D-printed working electrode was printed using the commercially-available G/PLA filament. It is worth highlighting that initially the electrochemical response of cocaine was evaluated using non-treated electrodes; however, no cocaine oxidation peak was obtained. Thus, different surface activation procedures of 3D printed electrodes (DMF immersion or electrochemical activation) were explored based on the literature [26,29,34]. The best results were achieved by the application of +1.76 V (*vs*. Ag|AgCl|$KCl_{(sat.)}$) for 900 s followed by application of -1.76 V (*vs*. Ag|AgCl|$KCl_{(sat.)}$) for 50 s [29] using 0.1 mol L^{-1} phosphate buffer solution (pH = 7.4) as the supporting electrolyte. Interestingly, the CB/PLA filament was also evaluated to print electrodes for cocaine detection; however, their voltammetric response was not satisfactory even after the same surface treatment applied to G/PLA electrodes, which indicates that graphene or surface functional groups formed after treatment contributed to the improvement electrochemical activity of G/PLA. Using the proposed 3D-printed portable system, the electrochemical sensing of cocaine was described with an acceptable limit of detection (7 µmol L^{-1}) and good linear response (from 20 to 100 mol L^{-1}). The on-site screening of cocaine and its most common contaminants (phenacetin, caffeine, levamisole, lidocaine, paracetamol, procaine and benzocaine) in spiked samples using the proposed system was successfully demonstrated. Hence, the proposed 3D-printed sensor can be used as a quicky test to determine cocaine in the presence of most common adulterants [28].

Rocha and coworkers [81] proposed a new surface treatment of CB/PLA 3D-printed electrodes based on a Photo-Thermal approach with a CO_2 laser to improve their electrochemical performance; such electrodes were applied for the detection of the contaminant paracetamol in a real seized cocaine sample. A scheme of the 3D-printed electrode and cell is showed in Table II – Line J. The authors highlighted that the as-printed electrode provided ill-defined peaks and low conductivity for paracetamol using cyclic voltammetric measurements. However, after the surface post-treatment, a significant enhancement in the electrochemical response of paracetamol was achieved and an increase in peak current of about 3-fold and anticipation of potential peak were acquired. Thus, using the proposed surface treatment of 3D-printed electrodes, the authors estimated an LOD of 0.154 µmol L^{-1}. Moreover, a standard addition method was used to determine paracetamol in spiked seized cocaine sample and a recovery value of 97.8% was obtained.

Similar analytical results were acquired using high-cost commercial carbon-based electrodes [79,82–84]. It is important to mention that most of the examples found in the literature for detection of cocaine or adulterants include screen-printed electrodes in which can be used for portable analyses [13]. However, 3D-printing technology enables lower cost of production and customized electrodes, including the manufacture of three electrodes with a reduced cost.

Table II. 3D printed electrochemical sensors applied for the determination of illicit drugs and contaminants in food and fuel samples

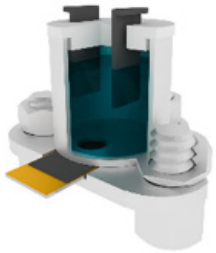
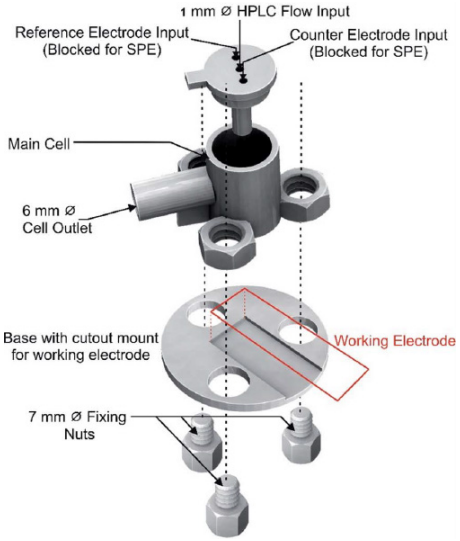
Electrochemical device	Treatment/ Activation	3D printing technique	Design	Analyte	Class	Analytical technique	Ref.
G/PLA (Line I)	Electrochemical activation	FDM	—	Cocaine	Illicit drugs	SWV	[28]
CB/PLA (Line J)	Reagentless and sub-minute laser-scribing treatment	FDM		Paracetamol in cocaine sample	Illicit drugs	SWV	[81]
PGE (Line K)	Electrochemical activation	Body of device: SLA	—	Clozapine	Illicit Drugs	FIA-AD	[86]
CB/PLA G/PLA NG/PLA GS SPE (Line L)	CB/PLA and G/PLA: Electrochemical activation NG/PLA: no treatment	3D printed flow cell: SLA 3D printed electrodes: FDM		NBOMes	Illicit drugs	HPLC-AD	[92]

Table II. 3D printed electrochemical sensors applied for the determination of illicit drugs and contaminants in food and fuel samples (Continuation)

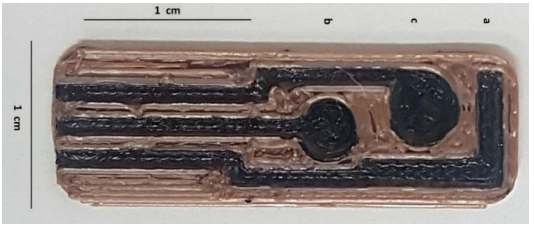
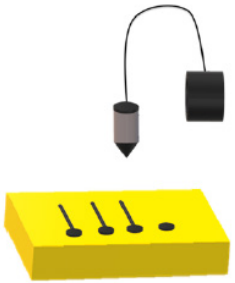
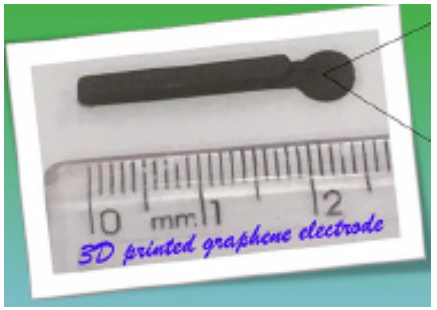
Electrochemical device	Treatment/ Activation	3D printing technique	Design	Analyte	Class	Analytical technique	Ref.
G/PLA (Line M)	Mechanical polishing and electrochemical activation	3D printing pen	—	Atropine	Illicit drugs	SWV	[96]
NR (Line N)	NR	FDM		Δ9-THC and 11-nor-9-carboxy-THC	Illicit drugs	CV	[99]
G/PLA (Line O)	Chemical and electrochemical activation	FDM		Mycotoxin	Food	CV	[100]
3DGrE/PB (Line P)	Electrochemical activation	FDM		H_2O_2	Food	BIA-AD	[106]

Table II. 3D printed electrochemical sensors applied for the determination of illicit drugs and contaminants in food and fuel samples (Continuation)

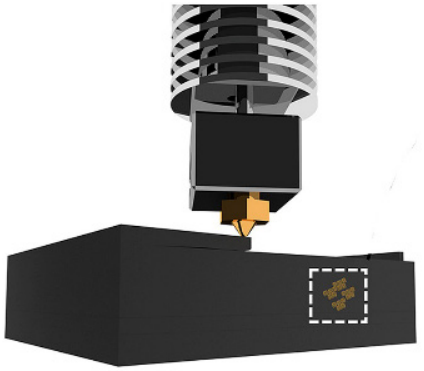
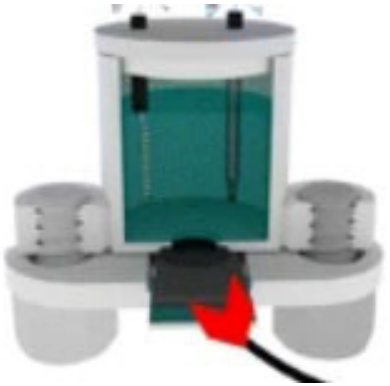
Electrochemical device	Treatment/ Activation	3D printing technique	Design	Analyte	Class	Analytical technique	Ref.
PB/G/PLA (Line Q)	Chemical activation	FDM		H_2O_2	Food	BIA-AD	[109]
CB/PLA (Line R)	Mechanical polishing and electrochemical activation	FDM		Cu^{2+}	Fuel	SWASV	[118]

Table II. 3D printed electrochemical sensors applied for the determination of illicit drugs and contaminants in food and fuel samples (Continuation)

Electrochemical device	Treatment/ Activation	3D printing technique	Design	Analyte	Class	Analytical technique	Ref.
CB/PLA (Line S)	Electrochemical activation	3D printing pen		Pb^{2+} and Cu^{2+}	Fuel	SWASV	[39]

Electrochemical device: G/PLA: graphene/polylactic acid; PGE: pencil graphite electrode; CB/PLA: carbon black/polylactic acid; NG/PLA: nanographite/polylactic acid; GS: graphite sheet; SPE: screen-printed graphite microelectrode; NR: not reported; 3DGrE/PB and PB/G/PLA: 3D printed graphene electrode with Prussian blue;
3D printing technique: SLA: stereolithography; FDM: fused deposition modelling
Analytical technique: SWV: square-wave voltammetry; FIA-AD: flow injection analysis with amperometric detection; HPLC-AD: high performance liquid chromatography with amperometric detection; CV: cyclic voltammetry; BIA-AD: batch injection analysis with amperometric detection; SWASV: square-wave anodic-stripping-voltammetry.

Clozapine is a therapeutic, antipsychotic drug approved for the treatment of schizophrenia and, due to its sedative effect, has been used as date rape drug or drug of abuse. This type of drug is commonly given to victims for non-medical reasons (abuse intentions). For this reason, the situation generates a demand for selective and sensitive methods to detect clozapine and its metabolites in biological samples collected from the victims [85]. In this context, Senel and Alachkar developed a 3D-printed sensing microfluidic device with amperometric detection using graphite pencil as working electrode (Table II – Line K) [86]. The 3D model of the electrochemical cell is entirely fabricated by 3D printing using a stereolithography (SLA) 3D printer. The authors highlighted the use of SLA for microchannel printing due to its ability to produce high-accurate and isotropic parts in a range of advanced materials with smooth surface finish. Moreover, the resolution of SLA printing is higher than FDM-based 3D printing technique for the formation of microstructures. As working and reference electrodes, 0.5 mm pencil graphite rod and 0.5 mm silver wire were inserted into commercially available threaded fittings along the 3D printed microfluidic channel. Nevertheless, the electrodes were not directly adapted in the 3D printed device, that was adapted in a threaded fitting and connected to the device. Before use, the pencil graphite electrode (formed by the pencil graphite rod) was electrochemically activated by 50 cyclic voltammograms in the range 0.0 V to +1.2 V (*vs*. PGE) with a scan rate of 100 mV s^{-1} in 0.1 mol L^{-1} phosphate buffer (pH = 7.4) [87]. The device was successfully applied to detect clozapine in serum samples with a limit of detection of 24 nmol L^{-1} and good recovery values (96-108%).

Novel psychoactive substances (NPS) are a class of compounds that have been designed to mimic recreational drugs and are commonly produced in clandestine laboratories [88,89]. An important class of NPS are phenethylamine derivatives, such as, 25F-NBOMe, 25C-NBOMe, and 25F-NBOMe. This class are potent agonist of the 5-HT2A receptor and can produce psychoactive effects [90,91]. Recently, Elbardisy *et al.* reported that a complete electrochemical system (wall jet flow cell and working electrodes) can be produced by 3D-printed technology in order to coupling high performance liquid chromatography and amperometric detection [92]. The 3D-printed flow cell was designed to allow the use of different working electrodes such as screen-printed sensors, graphite sheets, and FDM 3D-printed electrodes (fabricated with Proto-Pasta®, Black-Magic® and homemade conductive filaments [93]). The 3D printed flow cell was produced using an SLA 3D printer and photopolymer resin. The working electrodes (printed rectangular pieces) were produced using a FDM 3D printer and were positioned at the bottom of the flow cell and the geometric area was delimited with a rubber O-ring (single unprinted part) (Table II – Line L).

Before use, the 3D-printed working electrodes were activated using procedures described in the literature [29,34]. The 3D-printed carbon black electrode (produced with Proto-Pasta® filament) was electrochemically activated using 0.5 mol L^{-1} NaOH as supporting electrolyte (+1.4 V/200 s followed by -1.0 V/200 s) [34]. On the other hand, the 3D printed G/PLA (produced from Black Magic® filament) was electrochemical activated using the procedure described by Santos *et al*. (+1.8 V/900 s and cyclic voltammetric scans from 0.0 V to -1.8 V in 0.1 mol L^{-1} phosphate buffer; pH 7.4) [29]. As a proof-of-concept, the performance of the three 3D-printed electrodes was tested through the amperometric detection of four NBOMes (NBOMe derivates, 25F-NBOMe, 25C-NBOMe and 25F-NBOMe) after their chromatographic separation. The following ranges of limits of detection (10.2 – 15.3, 4.4 – 11.0, 3.2 – 5.0, and 14.4 – 16.1 μg mL^{-1}) and recoveries values in simulated drug samples (98 – 103, 96 – 100, 99 – 101, and 97 – 103%) were obtained with SPEs, activated 3D-printed CB/PLA, graphite sheets and 3D printed homemade filament as working electrodes, respectively.

According to the authors, the AM/3D printed flow cell had several advantages over the commercial systems: (i) simple geometrical configuration, (ii) short production time, (iii) low cost, (iv) higher sensitivity of the wall-jet design, (v) efficient mass transport of the analyte onto the electrode surface, (vi) simple assembly, (vii) versatility toward working electrode substrates and, (viii) high flow rate tolerance (2.5 mL min^{-1}).

Atropine is a natural tropane alkaloid from the *Datura stramonium* (jimsonweed) and *Atropa belladonna* (deadly nightshade) and usually employed for medicinal purposes. Moreover, atropine can be used in criminal activities by poisoning beverages. The most popular case using atropine as a deadly poison was the case of Dr. Paul Agutter who tried to murder his wife by spiking her beverage with atropine. Chromatographic methods are generally employed for determining atropine [94,95], however, this type of analytical method involves high cost and sample preparation in which are not attractive for forensic applications where a rapid and portable sensing approach is required. Electrochemical procedures can be used to overcome these limitations, because they enable versatile, low-cost and portable analytical methods. Therefore, João and coworkers [96] developed a method for detection of atropine in beverage samples (white wine, vodka, whisky and energy drink) using 3D-printed G/PLA electrodes (Table II – Line M). 3D pen was employed to construction of working electrode using customized acrylic substrates to guide the reproducible application of the G/PLA filament by the pen. Before use, the 3D-printed electrode was electrochemically activated in basic medium to expose the conductive sites, improving the analytical response of atropine.

Thus, using square-wave voltammetric determination, a linear concentration range between 5 and 60 μmol L^{-1}, with a limit of detection of 1 μmol L^{-1} and good recoveries values (104-120%) were achieved. Considering that the victim drinks around 250 mL of beverage, the atropine average fatal dose is 1.38 mmol L^{-1}, thus the proposed method is appropriate to determine atropine in this type of the sample.

Cannabis, also known as Marijuana, is the most commonly addictive drug used worldwide. It stimulates cells in the brain to release dopamine, creating euphoria and memory loss. The chemical responsible for most of marijuana's psychological effects is the tetrahydrocannabinol (THC) [97]. In the human body, THC is metabolized in 11-nor-9-carboxy-THC (also known as 11-COOH-THC), being found in biological fluids such as plasmas and oral fluid between 3 and 6 hours after consumption [98].

In this sense, Oiye *et al.* [99] proposed a method for the detection of THC in aqueous solution and its metabolite (11-COOH-THC) in saliva using 3D printing technology for construction of device. The electrode design was similar to screen-printed electrodes commercially-available on the market, as summarized in Table II – Line N. After printing, the reference electrode was covered with a silver ink to create a silver pseudo reference electrode. The authors showed a detection of 15 μmol L^{1} THC in aqueous solutions and 170 μmol L^{1} 11-COOH-THC in real saliva samples. Moreover, the analysis using different electrodes presented a variation of 8% in the current peak response that indicates good reproducibility of the proposed method. It is important to mention that Oiye and coworkers did not present important analytical parameters, such as limit of detection and quantification, linear range and recovery values for the analysis of spiked samples; however, the proposed method is a promising work to expand the results in forensic area.

In regard to the analytical performance of these sensors, all works presented herein show appropriate limit of detection values for forensic applications. Moreover, electrochemical procedures associated to 3D printed technology are versatile alternatives by reason of being low cost, good reproducibility, portable and acceptable limit of detection. However, the electrochemical response of the as printed electrodes is relatively poor if compared to other carbonaceous surfaces (glassy carbon, carbon paste, etc.). Thus, all works found in the literature highlighted the need of pre-treatments (activations) to reduce of insulating polymer, exposing of the conductive material.

FOOD AND FUELS

The 3D-printing technology, specially FDM, has also enabled the production of electrochemical sensors for food and fuel analysis and depicted in Table II. Food analysis is an important topic for discussion among international institutions, particularly, in view of the potential from terrorism, also known, bioterrorism [100]. This term is defined as deliberate release of biological agents (fungi, bacteria, viruses, and other microorganisms) to cause death or damage to health in humans, animals or plants. These agents are typically found in nature or synthetized and mutated by humans. This practice started during World War I, when the bacterium *Bacillus anthracis* was used to cause infection anthrax [10]. In this context, one class of compounds which has received great attention are the mycotoxins. Mycotoxins are naturally metabolites

produced by certain fungi in which can be found in foodstuffs, including cereals, nuts, spices, dried fruits, apple and coffee beans. This class of compounds can cause a variety of adverse human health effects such as immune deficiency and cancer [101].

In this sense, Nasir and coauthors proposed a 3D-printed electrode for the detection of the Zearalenone, a type of Mycotoxins, produced by the Fusarium fungi species, using commercially-available conductive filaments composed by G/PLA (Table II – Line O) [100]. For this purpose, the authors submitted the 3D-printed electrode an immersion in DMF for 10 minutes. After washing and drying, electrochemical activation was achieved at a potential of 2.5 V (*vs*. Ag|AgCl|$KCl_{(sat.)}$) in phosphate buffer solution (pH 7.2) [102]. The activated 3D-printed G/PLA electrodes displayed a good linear response in the concentration range of 10 to 300 μmol L^{-1} with a limit of detection of 0.340 μmol L^{-1}. The authors did not present results in real samples, but the results were promising for the electrochemical detection of mycotoxins in food samples.

The modification of 3D-printed electrode surfaces using chemical modifiers has been investigated for the development of electrochemical sensors for the detection of analytes in food samples with improved detectability, sensitivity and selectivity. Prussian blue (PB) is a relatively cheap and stable electrocatalyst used as an electrode modifier, due to its good spectroscopic and electrochemical characteristics [103,104]. Briefly, PB is a structure containing alternate Fe (II) and Fe (III) atoms connected by cyanide that can be easily oxidized or reduced according to the applied potentials. PB is also known as an artificial enzyme peroxidase due to its properties of electrocatalytic reduction of hydrogen peroxidase at potentials close to 0.0 V [103]. The electrochemical properties of this material make its use an attractive strategy for H_2O_2 sensing to control food adulteration, such as milk samples [105]. H_2O_2 has been commonly used a preservative in milk as well as disinfectant agent in milk processing equipment; however, this molecule is considered an adulterant and its content in milk requires routine monitoring.

Katic *et al*. reported the use of PB for the modification of 3D-printed G/PLA electrodes for selective detection of H_2O_2 in milk and mouthwash samples (Table II – Line P). The PB synthesis was achieved by applying a potential of +0.4 V (*vs*. Ag|AgCl|$KCl_{(sat.)}$) for 600 s in a solution containing 1 mmol L^{-1} $[Fe(CN)_6]^{3-}$ and 1 mmol L^{-1} $FeCl_3$, using 0.1 mol L^{-1} KCl acidified with 0.01 mol L^{-1} HCl as the supporting electrolyte [106]. The performance of the 3D-printed G/PLA electrode modified with PB was compared with results obtained with other working electrodes (glassy-carbon, platinum and gold). According to the authors, the PB film showed higher stability of the 3D-printed electrode surface in acid medium. Thus, the authors showed the applicability of the modified 3D printed electrode for detection of H_2O_2 in milk samples. For that, the 3D-printing modified electrode was coupled to a 3D-printed batch injection analysis (BIA) cell [107]. The proposed method presented a wide linear range (1.0 to 700 μmol L^{-1}) and a limit of detection of 0.37 μmol L^{-1} for the selective detection of H_2O_2 at 0.0 V (*vs*. Ag|AgCl|$KCl_{(sat.)}$). Moreover, milk and mouthwash samples were analyzed and good recoveries values (97 to 120%) were obtained, which indicates absence of sample matrix effects. Finally, an interference study was performed considering potential interfering species commonly found in biological fluids (dopamine, ascorbic acid, and uric acid). In this study, the selective detection of hydrogen peroxide using the modified 3D-printing electrode was confirmed.

Commercially conductive filaments for FDM 3D-printers have enabled the rapid development of new sensors, mainly the filament based on PLA and graphene. However, Browne and collaborators reported the presence of metallic impurities in this type of commercial filament (G/PLA from BlackMagic®). In addition, as already reported in the literature, the presence of metallic impurities can affect the electrochemical characteristics of the 3D-printed material, such as for water splitting [108]. PB films can be designed electrochemically in the presence of iron (III) cations and ferricyanide, as described above [104]. In this sense, Rocha and coworkers proposed the electrochemical synthesis of iron hexacyanoferrate, using the Fe(III) provided as impurities in the commercially-available filament used in the experiment (Table II – Line Q) [109]. The presence of iron impurities was confirmed by X-ray spectroscopic analysis. For the PB formation, two hundred voltammetric cycles were performed in presence of 1 mmol L^{-1} potassium ferricyanide, using 0.1 mol L^{-1} KCl acidified with 0.01 mol L^{-1} HCl over -0.3 to +1.2 V (*vs*. Ag|AgCl|$KCl_{(sat.)}$)

and scan rate of 50 mV s^{-1}. Before the surface modification, the 3D-printed electrode was submitted to DMF immersion for 30 min to remove insulating material [23].

After this procedure, a typical profile of PB-modified electrodes was observed by cyclic voltammetry. This modified 3D-printed electrode was then evaluated for amperometric detection of H_2O_2 and a linear response between 1 and 700 μmol L^{-1} and the limit of detection of 0.56 μmol L^{-1} were obtained. Moreover, the authors showed the detection of H_2O_2 in milk samples with good recoveries (between 94 and 101%).

The use of alternative fuels has increased dramatically over the last several years, as more people look for ways to save money, reduce environmentally harmful emissions, and decrease their dependence on fossil fuels [110]. Biofuels were introduced to reduce engine emissions and provide better environmental concerns and socioeconomic issues, in addition to being renewable energies over mineral fuel [111,112]. Fossil fuels are still being created today by underground heat and pressure, so they are being consumed more rapidly than they are being created. For that reason, fossil fuels are considered non-renewable; that is, they are not replaced as soon as we use them. Renewable energy is a promising alternative solution because it is clean and environmentally safe [113].

Modern society's quality of life undoubtedly depends on liquid fuels for the development of agricultural, commercial, and industrial activities [10]. Biofuel quality control involves the determination of metal and metalloid content. These species play an important role because they may modify the efficiency of biofuel production as well as the stability of these products. Furthermore, some metals are toxic and generate environmental pollution [114]. Due to the growing use and manufacture of biodiesel, both commercially and inhouse, the exposure to combustion of these fuels is increasing. Metals are adsorbed or attached on the structures of organic compounds or hydrocarbons emitted from combustion of vehicle engines and can be of different toxicological proprieties. The chemical composition of particle emission is related to the quality of the burned fuel and vehicular exhaust condition and may affect its toxicity. Many people who make biodiesel at home are working with several gallons of fuel at a time [110] and the attendants of gas stations are daily exposed to the inhalation of smoke from the engine combustion that carries particles of heavy metal and gradually degrades their health. Many death cases are reported regularly which are investigated forensically to determine the cause and manner of death so as to establish them as the cases of homicidal, suicidal or accidental metal poisoning and to know whether poisoning resulted from acute, chronic or acute-on-chronic exposure [115]. Studies suggest that exposure to nanoparticles causes serious damage to health, such as lung inflammation, asthma, chronic obstruction of arteries and lungs, cell death, obstruction and accumulation in the olfactory bulb, access to brain damage, tumor necrosis, oxidative stress, neural effects, heart problems, and even death [116].

Additionally, the content of metals in engine oils and fuels can be helpful in forensic cases involving automobile accidents [117]. The concentration profile of metals in used oil or fuels found at the accident scene can aid in the identification of the cars involved in the accident. Hence, the determination of metals in such samples (oils and fuels) can be an important evidence in the forensic scenario.

Recently, the upgrading and replacement of products have become rapidly increasing. On other hand, the 3D printing or additive manufacturing is emerging as a technology that could revolutionize how studies are conducted in numerous scientific research fields [102]. On the research front, additive manufacturing technology is creating new paradigms in different research fields, such as bioprinting, electronic printing, as well as environmental-related fields. Currently, 3D-printed conductive filaments containing carbonaceous materials have been presented as promising electrochemical sensors for control and monitoring bioethanol fuel quality [39,118].

3D-printed CB/PLA electrodes have been successfully applied for copper detection in fuel bioethanol samples [118]. The electrochemical cell and working electrodes were 3D-printed in a similar way that was previously reported in the first section. Copper is one of the metals controlled by regulatory agencies of fuels due to its catalytic action on the degradation of fuels. The main regulatory framework for ethanol biofuel quality monitoring are the American Society for Testing and Materials (ASTM D4814), the Brazilian National Agency for Natural Gas and Biofuels [119,120] and the European Standardization (EN 15376). These agencies establish the specificities and basic rules of fuel quality monitoring. In their protocols,

some contaminants, antioxidants, metal deactivators, and dispersants used to improve the stability of middle distillate fuels limits and physicochemical properties of biofuels are indicated. Considering the contamination with copper, techniques involving high instrumentation costs and bulky equipment, such as ICP OES, ICP-MS, and atomic absorption spectroscopy (AAS), are proposed as official methods. In this context, 3D-printing technologies provide a tool for prototyping simple or complex structures for electrochemical sensing, due to its ability to produce highly versatile, tailored-shaped devices in a low-cost and fast way with minimized waste. Table II – Line R shows the use of an electrode printed with CB-PLA filament was proposed for the determination of copper in bioethanol [118]. The analytical features of the proposed voltammetric method include a wide linear response concentration range of 10 to 300 μg L^{-1} ($R = 0.999$), high inter-day precision 8% ($n = 10$, for 20 μg L^{-1}) and a LOD of 0.097 μg L^{-1} using 180 s as deposition time. Bioethanol samples were simply diluted in the supporting electrolyte (0.1 mol L^{-1} HCl) before analysis (30:70 v/v ethanol:water proportion). The upper limit concentration of copper in bioethanol samples is 56 μg L^{-1} (according to the Brazilian and European agencies that present a stricter limit for this metal). Therefore, the method can be used for quality control of bioethanol samples.

Sensors used in previous works were prepared following laborious and time-consuming steps, such as synthesis of composites [121–123] or modification of sensor surface [124,125]. Furthermore, the use of mercury film modified electrodes, despite their excellent properties in electrochemical determination of metals, have fallen into disuse due to the toxicity of the metal to analysts and environment [126]. Therefore, the use of 3D-printing technology may provide advantages in the manufacture of electrochemical sensors with better detection properties than commercial gold disk [127] or screen-printed gold electrodes [128].

The combination of a 3D pen with 3D printers to fabricate low-cost electrochemical sensors was also explored to determine metals in bioethanol [39]. 3D-printed templates produced using a FDM printer and ABS filament were manually filled with conductive CB/PLA material using a 3D pen to fabricate sensors as previously reported (Table II – Line S) [33,35]. Bioethanol samples were similarly diluted in 0.1 mol L^{-1} HCl used as the supporting electrolyte for the simultaneous determination of copper and lead. The presence of lead as well copper in bioethanol can be associated with corrosion of metallic components. Although the content of lead is not controlled by regulatory agencies, the presence of lead in bioethanol results in higher emission of this toxic metal to the atmosphere. The analytical features of the development method using SWASV for the determination of both metals provided wide linear ranges, up to 200 μg L^{-1} for Pb(II) and up to 400 μg L^{-1} for Cu(II) and, LOD values of 1 and 2 μg L^{-1} for Pb(II) and Cu(II) using a deposition time of 100 s. The inter-electrode precision (for n = 3) was 2.8% which indicates that the electrode construction procedure is highly precise as well as the SWASV determination. The presence of other metallic interfering species, such as Fe(III), Cd(II), Zn(II) and Hg(II), was evaluated and no interference was verified in the detection of Pb(II) and Cu(II). Recovery values (ranged from 83% to 107%) for the analysis of fortified samples also attested for the absence of sample matrix. Some additional interesting observation of this work is the increase in the analytical response of both metals by the presence of mercury, which is likely to occur considering previous works using mercury-film electrodes and increase in the sensitivity of lead detection when copper is also present in solution (the literature also has shown benefits of copper films in the detection of lead).

The sensor obtained by combination of a 3D pen with 3D printers has better or equivalent performance in terms of detection limit when compared with previously published works. The limit of detection is closely dependent on the deposition time applied to the measurements. Nascimento *et al*. [129] and Tormin *et al*. [124] using shorter preconcentration times (30 s and 90 s, respectively) obtained lower LOD values for both metals; however, they used sensors which are not environmentally friendly and cannot be used as disposable electrodes. Several works employed commercial electrodes which have a relatively high cost compared to a 3D-printed electrode, and electrodes prepared following laborious and time-consuming steps, such as synthesis of composites [122,130] or modification of electrode surface [121,124,125].

CONCLUDING REMARKS AND PERSPECTIVES

This review shows that forensic chemistry can be largely benefited by the 3D printing technology. FDM 3D printers have enabled the fabrication of electrochemical sensors for a wide range of analytes of forensic interests. Carbon-based filaments have explored and quickly adapted for the printing of electrodes and devices applied for the detection of explosives, metallic GSR, illicit drugs, and contaminants in food and fuels. The knowledge on electrochemistry of carbon-based electrodes have been extended to understand the electrochemical processes occurred at the 3D-printed thermoplastic electrodes. New contributions have been reported, including the need for surface treatment for improved electrochemical activity. Other 3D-printing techniques, such SLM, have been explored to produce sensors, although they may be not so advantageous considering the high cost. However, many other 3D-printing techniques can still be explored for unlimited applications. Lab-on-a-chip devices taking the advantage of printing microfluidic channels with printed electrodes embedded along the channels by means of single-step fabrication protocols are highly promising for on-site analysis of complex forensic samples.

The preliminary works highlighted in this review using 3D-printed carbon-based electrodes have shown the excellent performance for sensing nitroaromatic explosives. Potentially, the simultaneous determination of two or more types of explosives is a challenge to be overcome. The same is true for the simultaneous determination of illicit drugs or at least the selective detection of single illicit drug in suspected sample. Most forensic samples require the selective identification of an illicit substance that can be attained by using chemical modifiers incorporated at the 3D-printed surface or within the polymeric matrix. The challenge still remains on the proper incorporation of the chemical modifier in a way that stable, reproducible, selective and sensitive electrode are obtained. PB-modified 3D-printed electrodes are a successful proof-of-concept case herein presented.

Due to the immeasurable possibilities that 3D printing offers, new applications can be envisaged going beyond the applications herein presented. Creative designs coupled with innovative surface modification protocols can be one feasible direction to be followed towards the development of high-performance electrochemical sensors and devices for forensic applications.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the agencies CAPES (001), CNPq (427731/2018-6, 307271/2017-0, and 208504/2018-6), INCTBio (CNPq grant no. 465389/2014–7), CNPq/TWAS (137634/2017-0), and FAPEMIG (RED-00042-16 and APQ-03141-18) for the financial support.

REFERENCES

1. Huang, S. H.; Liu, P.; Mokasdar, A.; Hou, L. *Int. J. Adv. Manuf. Technol.*, **2013**, *67*, pp 1191–1203 (https://doi.org/10.1007/s00170-012-4558-5).
2. Silver, A. *Nature*, **2019**, *565*, pp 123–124 (https://doi.org/10.1038/d41586-018-07853-5).
3. Kitson, P. J.; Glatzel, S.; Chen, W.; Lin, C. G.; Song, Y. F.; Cronin, L. *Nat. Protoc.*, **2016**, *11*, pp 920–936 (https://doi.org/10.1038/nprot.2016.041).
4. Zhang, M.; Mei, H.; Chang, P.; Cheng, L. *J. Mater. Chem. A,* **2020**, *8*, pp 10670–10694 (https://doi.org/10.1039/d0ta02099k).
5. Browne, M. P.; Redondo, E.; Pumera, M. *Chem. Rev*., **2020**, *120* (5), pp 2783–2810 (https://doi.org/10.1021/acs.chemrev.9b00783).
6. Cardoso, R. M.; Kalinke, C.; Rocha, R. G.; Santos, P. L.; Rocha, D. P.; Oliveira, P. R.; Janegitz, B. C.; Bonacin, J. A.; Richter, E. M.; Munoz, R. A. A. *Anal. Chim. Acta,* **2020**, *1118*, pp 73–91 (https://doi.org/10.1016/j.aca.2020.03.028).
7. Carrasco-Correa, E. J.; Simó-Alfonso, E. F.; Herrero-Martínez, J. M.; Miró, M. *TrAC - Trends Anal. Chem.,* **2021**, *136*, 116177 (https://doi.org/10.1016/j.trac.2020.116177).

8. Waheed, S.; Cabot, J. M.; Macdonald, N. P.; Lewis, T.; Guijt, R. M.; Paull, B.; Breadmore, M. C. *Lab Chip*, **2016**, *16* (11), pp 1993–2013 (https://doi.org/10.1039/c6lc00284f).
9. Carew, R. M.; Errickson, D. *J. Forensic Sci*., **2020**, *65* (5), pp 1752–1760 (https://doi.org/10.1111/1556-4029.14442).
10. Oliveira, L. P.; Rocha, D. P.; Araujo, W. R.; Muñoz, R. A. A.; Paixão, T. R. L. C.; Salles, M. O. *Anal. Methods*, **2018**, *10*, pp 5135–5163 (https://doi.org/10.1039/c8ay01389f).
11. To, K. C.; Ben-Jaber, S.; Parkin, I. P. *ACS Nano*, **2020**, *14* (9), pp 10804-10833 (https://doi.org/10.1021/acsnano.0c01579).
12. Hay, C. E.; Lee, J.; Silvester, D. S. *J. Electroanal. Chem*., **2020**, *872*, 114046 (https://doi.org/10.1016/j.jelechem.2020.114046).
13. Araujo, W. R.; Cardoso, T. M. G.; Rocha, R. G.; Santana, M. H. P.; Munoz, R. A. A.; Richter, E. M.; Paixão, T. R. L. C.; Coltro, W. K. T. *Anal. Chim. Acta*, **2018**, *1034*, pp 1–21. (https://doi.org/10.1016/j.aca.2018.06.014).
14. Yu, H. A.; DeTata, D. A.; Lewis, S. W.; Silvester, D. S. *TrAC - Trends Anal. Chem.,* **2017**, *97*, pp 374–384 (https://doi.org/10.1016/j.trac.2017.10.007).
15. Holubowitch, N. E.; Crabtree, C.; Budimir, Z. *Anal. Chem.,* **2020**, *92*, pp 11617-11626 (https://doi.org/10.1021/acs.analchem.0c01174).
16. Castro, S. V. F.; Cardoso, R. M.; Santana, M. H. P.; Richter, E. M.; Munoz, R. A. A. *Talanta*, **2019**, *203*, pp 106-111 (https://doi.org/10.1016/j.talanta.2019.05.048).
17. Wang, Z.; Liu, H.; Li, C.; Chen, X.; Weerasooriya, R.; Wei, J.; Lv, J.; Lv, P.; Wu, Y. *Talanta*, **2020**, *208*, 120410 (https://doi.org/10.1016/j.talanta.2019.120410).
18. Dettlaff, A.; Jakóbczyk, P.; Ficek, M.; Wilk, B.; Szala, M.; Wojtas, J.; Ossowski, T.; Bogdanowicz, R. *J. Hazard. Mater*., **2020**, *387*, 121672 (https://doi.org/10.1016/j.jhazmat.2019.121672).
19. Stefano, J. S.; Lima, A. P.; Nascentes, C. C.; Krzyzaniak, S. R.; Mello, P. A.; Gonçalves, J. M.; Richter, E. M.; Nossol, E.; Munoz, R. A. A. *J. Solid State Electrochem.,* **2020**, *24*, pp 121-129 (https://doi.org/10.1007/s10008-019-04465-5).
20. Tan, C.; Nasir, M. Z. M.; Ambrosi, A.; Pumera, M. *Anal. Chem*., **2017**, *89* (17), pp 8995–9001 (https://doi.org/10.1021/acs.analchem.7b01614).
21. Palenzuela, C. L. M.; Novotný, F.; Krupička, P.; Sofer, Z.; Pumera, M. *Anal. Chem*., **2018**, *90*, pp 5753–5757 (https://doi.org/10.1021/acs.analchem.8b00083).
22. Silva, V. A. O. P.; Fernandes-Junior, W. S.; Rocha, D. P.; Stefano, J. S.; Muñoz, R. A. A.; Bonacin, J. A.; Janegitz, B. C. *Biosensors and Bioeletronics*, **2020**, *170*, 112684 (https://doi.org/10.1016/j.bios.2020.112684).
23. Kalinke, C.; Neumsteir, N. V.; Aparecido, G. D. O.; Ferraz, T. V. D. B.; dos Santos, P. L.; Janegitz, B. C.; Bonacin, J. A. *Analyst*, **2020**, *145* (4), pp 1207–1218 (https://doi.org/10.1039/C9AN01926J).
24. Wirth, D. M.; Sheaff, M. J.; Waldman, J. V.; Symcox, M. P.; Whitehead, H. D.; Sharp, J. D.; Doerfler, J. R.; Lamar, A. A.; LeBlanc, G. *Anal. Chem.,* **2019**, *91* (9), pp 5553–5557 (https://doi.org/10.1021/acs.analchem.9b01331).
25. Redondo, E.; Muñoz, J.; Pumera, M. *Carbon,* **2021**, *175*, pp 413 – 419 (https://doi.org/10.1016/j.carbon.2021.01.107).
26. Gusmão, R.; Browne, M. P.; Sofer, Z.; Pumera, M. *Electrochem. Commun*., **2019,** *102*, pp 83–89 (https://doi.org/10.1016/j.elecom.2019.04.004).
27. Rocha, D. P.; Squissato, A. L.; Silva, S. M.; Richter, E. M.; Muñoz, R. A. A. *Electrochim. Acta*, **2020**, *335*, 135688 (https://doi.org/10.1016/j.electacta.2020.135688).
28. Rocha, R. G.; Ribeiro, J. S.; Santana, M. H.; Richter, E. M.; Munoz, R. A. A. *Anal. Methods*, **2021**, *13*, pp 1788–1794 (https://doi.org/10.1039/D1AY00181G).
29. Santos, P. L.; Katic, V.; Loureiro, H. C.; dos Santos, M. F.; dos Santos, D. P.; Formiga, A. L. B.; Bonacin, J. A. *Sensors Actuators B Chem*., **2019**, *281* (2), pp 837–848 (https://doi.org/10.1016/j.snb.2018.11.013).

30. Novotny, F.; Urbanova, V.; Plutnar, J.; Pumera, M. *Appl. Mater. Interfaces*, **2019**, *11*, pp 35371−35375 (https://doi.org/10.1021/acsami.9b06683).
31. Cardoso, R. M.; Castro, S. V. F.; Silva, M. N. T.; Lima, A. P.; Santana, M. H. P.; Nossol, E.; Silva, R. A. B.; Richter, E. M.; Paixão, T. R. L. C.; Muñoz, R. A. A. *Sensors Actuators B. Chem*., **2019**, *292*, pp 308–313 (https://doi.org/10.1016/j.snb.2019.04.126).
32. Silveira, G. D.; Di Turo, F.; Dias, D.; Silva, J. A. F. *J. Solid. State Electrochem*., **2020**, *24*, pp 2633–2652 (https://doi.org/10.1007/s10008-020-04720-0).
33. Cardoso, R. M.; Castro, S. V. F.; Stefano, J. S.; Muñoz, R. A. A. *J. Braz. Chem. Soc*., **2020**, *31* (9), pp 1764–1770 (https://dx.doi.org/10.21577/0103-5053.20200129).
34. Richter, E. M.; Rocha, D. P.; Cardoso, R. M.; Keefe, E. M.; Foster, C. W.; Munoz, R. A. A.; Banks, C. E. *Anal. Chem*., **2019**, *91*, pp 12844-12851 (https://doi.org/10.1021/acs.analchem.9b02573).
35. Cardoso, R. M.; Rocha, D. P.; Rocha, R. G.; Stefano, J. S.; Silva, R. A. B.; Richter, E. M.; Munoz, R. A. A. *Anal. Chim. Acta*, **2020**, *1132*, pp 10-19 (https://doi.org/10.1016/j.aca.2020.07.034).
36. Jodat, Y. A.; Kiaee, K.; Jarquin, D. V.; Hernández, R. L. D. G.; Wang, T.; Joshi, S.; Rezaei, Z.; Melo, B. A. G.; Ge, D.; Mannoor, M. S.; Shin, S. R. *Adv. Sci.*, **2019**, *7*, 1901878 (https://doi.org/10.1002/advs.201901878).
37. Gao, K.; Li, S.; Zhuang, L.; Qin, Z.; Zhang, B.; Huang, L.; Wang, P. *Biosensors and Bioelectronics*, **2018,** *102*, pp 150-156 (https://doi.org/10.1016/j.bios.2017.08.055).
38. Sempionatto, J. R.; Mishra, R. K.; Martín, A.; Tang, G.; Nakagawa, T.; Lu, X.; Campbell, A. S.; Lyu, K. M.; Wang, J. *ACS Sensors*, **2017**, *2* (10), pp 1531–1538 (https://doi.org/10.1021/acssensors.7b00603).
39. João, A. F.; Castro, S. V. F.; Cardoso, R. M.; Gamela, R. R.; Rocha, D. P.; Richter, E. M.; Muñoz, R. A. A. *J. Electroanal. Chem.*, **2020**, *876*, 114701 (https://doi.org/10.1016/j.jelechem.2020.114701).
40. Oliveira, F. M.; Melo, E. I.; Silva, R. A. B. *Sensors Actuators, B Chem.*, **2020**, *321*, 128528 (https://doi.org/10.1016/j.snb.2020.128528).
41. James, S.; Chishti, B.; Ansari, S. A.; Alothman, O. Y.; Fouad, H.; Ansari, Z. A.; Ansari, S. G. *Journal of Electronic Materials*, **2018**, *47*, pp 7505-7513 (https://doi.org/10.1007/s11664-018-6692-9).
42. Mahyari, M. *Int. J. Environ. Anal. Chem.*, **2016**, *96*, pp 1455-1468 (https://doi.org/10.1080/03067319.2016.1268606).
43. Zeng, W.; Manoj, D.; Sun, H.; Yi, R.; Huang, X.; Sun, Y. *J. Electroanal. Chem.***, 2019**, *833*, pp 527-535 (https://doi.org/10.1016/j.jelechem.2018.12.028).
44. Rani, S.; Sharma, B.; Kapoor, S.; Malhotra, R.; Varma, R. S.; Dilbaghi, N. *Appl. Sci.*, **2019**, *9* (22), 4952 (https://doi.org/10.3390/app9224952).
45. Ahmad, K.; Mohammad, A.; Mathur, P.; Mobin, S. M. *Electrochim. Acta*, **2016**, *215*, pp 435-446 (https://doi.org/10.1016/j.electacta.2016.08.123).
46. Dettlaff, A.; Jakóbczyk, P.; Ficek, M.; Wilk, B.; Szala, M.; Wojtas, J.; Ossowski, T.; Bogdanowicz, R. *Journal of Hazardous Materials*, **2020**, *387*, 121672 (https://doi.org/10.1016/j.jhazmat.2019.121672).
47. Lima, A. P.; Almeida, P. L. M. R.; Sousa, R. M. F.; Richter, E. M.; Nossol, E.; Munoz, R. A. A. *Journal of Electroanalytical Chemistry*, **2019**, *851*, 113385 (https://doi.org/10.1016/j.jelechem.2019.113385).
48. Niu, F.; Shao, Z.; Tao, L.; Ding, Y. *Journal of Colloid and Interface Scince*, **2021**, *594*, pp 848-856 (https://doi.org/10.1016/j.jcis.2021.03.091).
49. Leibl, N.; Duma, L.; Gonzato, C.; Haupt, K. *Bioelectrochemistry*, **2020**, *135*, 107541 (https://doi.org/10.1016/j.bioelechem.2020.107541).
50. Yew, Y. T.; Ambrosi, A.; Pumera, M. *Scientific Reports*, **2016**, *16*, 33276 (https://doi.org/10.1038/srep33276).
51. Goudsmits, E.; Sharples, G. P.; Birkett, J. W. *Trends Anal. Chem*., **2015**, *74*, pp 46–57 (https://doi.org/10.1016/j.trac.2015.05.010).
52. Charles, S.; Geusens, N.; Vergalito, E.; Nys, B. *Forensic Sci. Int*., **2020**, *2*, pp 416-418 (https://doi.org/10.1016/j.fsisyn.2020.01.011).
53. Feeney, W.; Pyl, C. V.; Bell, S.; Trejos, T. *Forensic Chem*., **2020**, *19*, pp 100250 (https://doi.org/10.1016/j.forc.2020.100250).

54. Woolever, C. A.; Starkey, D. E.; Dewald, H. D. *Forensic Sci. Int.*, **1999**, *102*, pp 45-50 (https://doi.org/10.1016/S0379-0738(99)00036-5).
55. Woolever, C. A.; Dewald, H. D. *Forensic Sci. Int.*, **2001**, *117*, pp 185-190 (https://doi.org/10.1016/S0379-0738(00)00402-3).
56. Erden, S.; Durmus, Z.; Kiliç, E. *Electroanalysis*, **2011**, *23* (8), pp 1967-1974 (https://doi.org/10.1002/elan.201000612).
57. Bandodkar, A. J.; O'Mahony, A. M.; Ramírez, J.; Samek, I. A.; Anderson, S. M.; Windmiller, J. R.; Wang, J. *Analyst*, **2013**, *138* (18), pp 5288–5295 (https://doi.org/10.1039/c3an01179h).
58. Mahony, A. M. O.; Windmiller, J. R.; Samek, I. A.; Bandodkar, A. J.; Wang, J. *Electrochem. Commun.*, **2012**, *23*, pp 52–55 (https://doi.org/10.1016/j.elecom.2012.07.004).
59. Mahony, A. M. O.; Samek, I. A.; Sattayasamitsathit, S.; Wang, J. *Anal. Bioanal. Chem.*, **2014**, *86*, pp 8031–8036 (https://doi.org/10.1021/ac5016112).
60. Hashim, N. H. M.; Zain, Z. M.; Jaafar, M. Z. *MATEC Web Conferences*, **2016**, *59*, 04005 (https://doi.org/10.1051/matecconf/20165904005).
61. Trejos, T.; Pyl, C. V; Menking-Hoggatt, K.; Alvarado, A. L.; Arroyo, L. E. *Forensic Chem.*, **2018**, *8*, pp 146–156 (https://doi.org/10.1016/j.forc.2018.02.006).
62. Castro, S. V. F.; Lima, A. P.; Rocha, R. G.; Cardoso, R. M.; Santana, H. P.; Richter, E. M.; Munoz, R. A. A.; Montes, R. H. O. *Anal. Chim. Acta*, **2020**, *1130*, pp 126–136 (https://doi.org/10.1016/j.aca.2020.07.033).
63. Trejos, T.; Pyl, C. V.; Menking-Hoggatt, K.; Alvarado, A. L.; Arroyo, L. E. *Forensic Chemistry*, **2018**, *8*, pp 146-156 (https://doi.org/10.1016/j.forc.2018.02.006).
64. Ott, C. E.; Dalzell, K. A.; Calderón-Arce, P. J.; Alvarado-Gámez, A. L.; Trejos, T. Arroyo, L. E. *J. Forensic Sci.,* **2020***, 65* (6), pp 1935-1944 (https://doi.org/10.1111/1556-4029.14548).
65. Vuki, M.; Shiu, K.; Galik, M.; Mahony, A. M. O.; Wang, J. *Analyst*, **2012**, *137*, pp 3265–3270 (https://doi.org/10.1039/c2an35379b).
66. United Nations Office on Drugs and Crime. *Crime Scene and Physical Evidence Awareness for Non-Forensic Personnel*. New York, **2009**, pp 1-36.
67. Shbair, M. K. S.; Lhermitte, M. *Ann. Pharm. Fr.*, **2010**, *69* (3), pp 136–147 (https://doi.org/10.1016/j.pharma.2010.03.005).
68. Jones, N. S.; Comparin, J. H. *Forensic Sci. Int. Synerg.*, **2020**, *2*, pp 608–669 (https://doi.org/10.1016/j.fsisyn.2020.01.019).
69. Foster, C. W.; Down, M. P.; Zhang, Y.; Ji, X.; Rowley-Neale, S. J.; Smith, G. C.; Kelly, P. J.; Banks, C. E. *Sci. Rep.*, **2017**, *7* (1), 42233 (https://doi.org/10.1038/srep42233).
70. Tully, J. J.; Meloni, G. N. *Anal. Chem.*, **2020**, *92* (22), pp 14853–14860 (https://doi.org/10.1021/acs.analchem.0c03299).
71. Silva, G. O.; de Araujo, W. R.; Paixão, T. R. L. C. *Talanta*, **2018**, *176*, pp 674–678 (https://doi.org/10.1016/j.talanta.2017.08.082).
72. Erickson, J. S.; Shriver-Lake, L. C.; Zabetakis, D.; Stenger, D. A.; Trammell, S. A. *Sensors,* **2017**, *17* (8), 1769 (https://doi.org/10.3390/s17081769).
73. Scott Jr, L. J. *Microgram*, **1973**, *6*, pp 179–181.
74. Marcelo, M. C. A.; Mariotti, K. C.; Ortiz, R. S.; Ferrão, M. F.; Anzanello, M. J. *Microchem. J.* , **2016**, *127*, pp 87–93 (https://doi.org/10.1016/j.microc.2016.02.012).
75. Osterloh, J. *Clin. Pharmacokinet.*, **1993**, *24* (5), pp 355–361 (https://doi.org/10.2165/00003088-199324050-00001).
76. Tsumura, Y.; Mitome, T.; Kimoto, S. *Forensic Sci. Int.*, **2005**, *155* (2–3), pp 158–164 (https://doi.org/10.1016/j.forsciint.2004.11.011).
77. Botelho, É. D.; Cunha, R. B.; Campos, A. F. C.; Maldaner, A. O. *J. Braz. Chem. Soc.*, **2014**, *25* (4), pp 611–618 (https://doi.org/10.5935/0103-5053.20140008).

78. Maldaner, A. O.; Botelho, É. D.; Zacca, J. J.; Melo, R. C. A.; Costa, J. L.; Zancanaro, I.; Oliveira, C. S. L.; Kasakoff, L. B.; Paixão, T. R. L. C. *J. Braz. Chem. Soc*., **2016**, *27* (4), pp 719–726 (https://doi.org/10.5935/0103-5053.20150321).
79. Freitas, J. M.; Ramos, D. L. O.; Sousa, R. M. F.; Paixão, T. R. L. C.; Santana, M. H. P.; Muñoz, R. A. A.; Richter, E. M. *Sensors Actuators B Chem*., **2017**, *243*, pp 557–565 (https://doi.org/10.1016/j.snb.2016.12.024).
80. Brunt, T. M.; Rigter, S.; Hoek, J.; Vogels, N.; van Dijk, P.; Niesink, R. J. M. *Addiction*, **2009,** *104* (5), pp 798–805 (https://doi.org/10.1111/j.1360-0443.2009.02532.x).
81. Rocha, D. P.; Ataide, V. N.; de Siervo, A.; Gonçalves, J. M.; Munoz, R. A. A.; Paixão, T. R. L. C.; Angnes, L. *Chem. Eng. Sci*, **2021**, *425*, 130594 (https://doi.org/10.1016/j.cej.2021.130594).
82. Rocha, D. P.; Dornellas, R. M.; Nossol, E.; Richter, E. M.; Silva, S. G.; Santana, M. H. P.; Munoz, R. A. A. *Electroanalysis*, **2017**, *29*, pp 2418-2422. (https://doi.org/10.1002/elan.201700437).
83. De Jong, M.; Florea, A.; Vries, A.; van Nuijs, A. L. N.; Covaci, A.; Durme, F. V.; Martins, J. C.; Samyn, N.; De Wael, K. *Anal. Chem.*, *90* (8), pp 5290-5297 (https://doi.org/10.1021/acs.analchem.8b00204).
84. Oiye, E. N.; Figueiredo, N. B.; Andrade, J. F.; Tristão, H. M.; Oliveira, M. F. *Forensic Sci. Int.*, **2009**, *192*, pp 94-97 (https://doi.org/10.1016/j.forsciint.2009.08.004).
85. Kang, M.; Kim, E.; Winkler, T. E.; Banis, G.; Liu, Y.; Kitchen, C. A.; Kelly, D. L.; Ghodssi, R.; Payne, G. F. *Biosens. Bioelectron*., **2017**, *95*, pp 55–59 (https://doi.org/10.1016/j.bios.2017.04.008).
86. Senel, M.; Alachkar, A. *Lab Chip*, **2021**, *21* (2), pp 405–411 (https://doi.org/10.1039/D0LC00970A).
87. Purushothama, H. T.; Nayaka, Y. A.; Vinay, M. M.; Manjunatha, P.; Yathisha, R. O.; Basavarajappa, K. V. *J. Sci. Adv. Mater. Devices*, **2018**, *3* (2), pp 161–166 (https://doi.org/10.1016/j.jsamd.2018.03.007).
88. Miliano, C.; Serpelloni, G.; Rimondo, C.; Mereu, M.; Marti, M.; De Luca, M. A. *Front. Neurosci*., **2016**, *10*, pp 1–21 (https://doi.org/10.3389/fnins.2016.00153).
89. Hondebrink, L.; Lonkhuyzen, J. J. N.; Van Der Gouwe, D.; Brunt, T. M. *Drug Alcohol Depend*., **2015**, *147*, pp 109–115 (https://doi.org/10.1016/j.drugalcdep.2014.11.033).
90. De Andrade, A. F. B.; Gonzalez-Rodriguez, J. *Analyst*, **2019**, *144* (9), pp 2965–2972 (https://doi.org/10.1039/c9an00062c).
91. Odoardi, S.; Romolo, F. S.; Strano-Rossi, S. *Forensic Sci. Int*., **2016**, *265*, pp 116–120 (https://doi.org/10.1016/j.forsciint.2016.01.037).
92. Elbardisy, H. M.; Richter, E. M.; Crapnell, R. D.; Down, M. P.; Gough, P. G.; Belal, T. S.; Talaat, W.; Daabees, H. G.; Banks, C. E. *Anal. Methods*, **2020**, *12* (16), pp 2152–2165 (https://doi.org/10.1039/d0ay00500b).
93. Foster, C. W.; Elbardisy, H. M.; Down, M. P.; Keefe, E. M.; Smith, G. C.; Banks, C. E. *Chem. Eng. J*., **2020**, *381*, 122343 (https://doi.org/10.1016/j.cej.2019.122343).
94. Zhang, P.; Li, Y.; Liu, G.; Sun, X.; Zhou, Y.; Deng, X.; Liao, Q.; Xie, Z. *J. Sep. Sci.*, **2014**, *37* (19), pp 2664-2674 (https://doi.org/10.1002/jssc.201400534).
95. Boros, B.; Farkas, A.; Jakabová, S.; Bacskay, I.; Kilár, F.; Felinger, A. *Chromatographia*, **2010**, *71*, pp 43-49 (https://doi.org/10.1365/s10337-010-1524-y).
96. João, A. F.; Rocha, R. G.; Matias, T. A.; Richter, E. M.; Petruci, J. F. S.; Munoz, R. A. A. *Microchem. J.*, **2021**, 167, 106324 (https://doi.org/10.1016/j.microc.2021.106324).
97. Kenney, J. E. *Drug Test. Anal.*, **2010**, *3* (3), pp 163-165 (https://doi.org/10.1002/dta.151).
98. Huestis, M. A. *Cheminform,* **2007**, *38* (47), pp 1770-1804 (https://doi.org/10.1002/chin.200747256).
99. Oiye, E. N.; Ribeiro, M. F. M.; Ferreira, B.; Botelho, R. C. B.; Oliveira, M. F. *Brazilian Journal of Forensic Sciences, Medical Law and Bioethics*, **2020**, *9* (4), pp 521-533 (https://doi.org/10.17063/bjfs9(4)y2020521-533).
100. Nasir, Z. M. M.; Novotný, F.; Alduhaish, O.; Pumera, M. *Electrochem. Commun*., **2020**, *115*, 106735 (https://doi.org/10.1016/j.elecom.2020.106735).
101. Maragos, C. *World Mycotoxin J*., **2010**, *3* (4), pp 369–383 (https://doi.org/10.3920/WMJ2010.1240).

102. Browne, M. P.; Novotný, F.; Sofer, Z.; Pumera, M. *ACS Appl. Mater. Interfaces*, **2018**, *10* (46), pp 40294–40301 (https://doi.org/10.1021/acsami.8b14701).
103. Karyakin, A. A.; Karyakina, E. E. *Sensors Actuators B Chem*., **1999**, *57* (1–3), pp 268–273 (https://doi.org/10.1016/S0925-4005(99)00154-9).
104. Nossol, E.; Zarbin, A. J. G. *Adv. Funct. Mater*., **2009**, *19* (24), pp 3980–3986 (https://doi.org/10.1002/adfm.200901478).
105. Heli, H.; Sattarahmady, N.; Zare, S. N. *RSC Adv.,* **2015**, *5* (27), pp 21005–21011 (https://doi.org/10.1039/c5ra01405k).
106. Katic, V.; Santos, P. L.; Santos, M. F.; Pires, B. M.; Loureiro, H. C.; Lima, A. P.; Queiroz, J. C. M.; Landers, R.; Munoz, R. A. A.; Bonacin, J. A. *ACS Appl. Mater. Interfaces*, **2019**, *11*, pp 35068–35078 (https://doi.org/10.1021/acsami.9b09305).
107. Cardoso, R. M.; Mendonça, D. M. H.; Silva, W. P.; Silva, M. N. T.; Nossol, E.; da Silva, R. A. B.; Richter, E. M.; Muñoz, R. A. A. *Anal. Chim. Acta*, **2018**, *1033*, pp 49-57 (https://doi.org/10.1016/j.aca.2018.06.021).
108. Browne, M. P.; Pumera, M. *Chem. Commun.*, **2019**, *55*, pp 8374–8377 (https://doi.org/10.1039/c9cc03774h).
109. Rocha, R. G.; Stefano, J. S.; Cardoso, R. M.; Zambiazi, P. J.; Bonacin, J. A.; Richter, E. M.; Munoz, R. A. A. *Talanta*, **2020**, *219*, 121289 (https://doi.org/10.1016/j.talanta.2020.121289).
110. Goodman, M. R.; Kaley, E. A.; Finney, E. E. *Forensic Sci. Int.*, **2016**, *263*, pp 10-26 (https://doi.org/10.1016/j.forsciint.2016.03.040).
111. Ogunkunle, O.; Ahmed, N. A. *Energy Reports*, **2019**, *5*, pp 1560-1579 (https://doi.org/10.1016/j.egyr.2019.10.028).
112. Nogueira, T.; Cordeiro, D. S.; Muñoz, R. A. A.; Fornaro, A.; Miguel, A. H.; Andrade, M. F. Bioethanol and Biodiesel as Vehicular Fuels in Brazil: Assessment of Atmospheric Impacts from the Long Period of Biofuels Use. In: Krzysztof Biernat (Ed.). *Biofuels - Status and Perspective*, 1th edition. InTech, **2015**, vol. 18, pp 377-412 (https://doi.org/10.5772/60944).
113. Demirbas, A. *Biodiesel: A Realistic Fuel Alternative for Diesel Engines*. Springer, **2008**, pp 1-108.
114. Sánchez, R.; Sánchez, C.; Lienemann, C.; Todolí, J. *J. Anal. At. Spectrom.*, **2015**, *30*, pp 64-101 (https://doi.org/10.1039/C4JA00202D).
115. Verma, A. *International Journal for Research in Applied Science & Engineering Technology*, **2018**, *6* (1), pp 1089-1092 (https://doi.org/10.22214/ijraset.2018.1165).
116. Guarieiro, L. L. N.; Guarieiro, A. L. N. *Biofuels – Status and Perspective: Chapter 11*, **2015** (http://dx.doi.org/10.5772/60110).
117. Kim, Y.; Kim, N. Y.; Park, S. Y.; Lee, D.; Lee, J. H. *Forensic Sci. Int.*, **2013**, *230*, pp 58-67 (https://doi.org/10.1016/j.forsciint.2013.01.013).
118. João, A. F.; Squissato, A. L.; Richter, E. M.; Muñoz, R. A. A. *Anal. Bioanal. Chem*., **2020**, *412*, pp 2755-2762 (https://doi.org/10.1007/s00216-020-02513-y).
119. Agência Nacional do Petróleo, Gás Natural e Biocombustíveis. Resolução n° 807, de 23 de janeiro de 2020. Brasil, ANP, **2020**. Available at: https://www.in.gov.br/web/dou/-/resolucao-n-807-de-23-de-janeiro-de-2020-239635261 [Accessed July 2021].
120. Agência Nacional do Petróleo, Gás Natural e Biocombustíveis. Resolução n° 30, de 23 de junho de 2016. Brasil, ANP, **2016**. Available at: https://www.in.gov.br/web/guest/materia/-/asset_publisher/Kujrw0TZC2Mb/content/id/23064622/do1-2016-06-24-resolucao-n-30-de-23-de-junho-de-2016-23064478 [Accessed July 2021].
121. Takeuchi, R. M.; Santos, A. L.; Padilha, P. M.; Stradiotto, N. R. *Talanta*, **2007**, *71* (2), pp 771-777 (https://doi.org/10.1016/j.talanta.2006.05.035).
122. Cesarino, I.; Marino, G.; Cavalheiro, E. T. G. *Fuel*, **2010**, *89* (8), pp 1883-1888 (https://doi.org/10.1016/j.fuel.2009.11.037).

123. Neto, S. Y.; Viégas, H. D. C.; Almeida, J. M. S.; Cavalheiro, E. T. G.; Araújo, A. S.; Marques, E. P.; Marques, A. L. B.; *Electroanalysis*, **2015**, *28* (5), pp 1035-1043 (https://doi.org/10.1002/elan.201500619).
124. Tormin, T. F.; Narciso, L. C. D.; Richter, E. M.; Munoz, R. A. A. *Fuel*, **2014**, *117*, pp 952-956 (https://doi.org/10.1016/j.fuel.2013.10.038).
125. Oliveira, M. F.; Saczk, A. A.; Okumura, L. L.; Fernandes, A. P.; Moraes, M.; Stradiotto, N. R. *Anal. Bioanal. Chem.*, **2004**, *380*, pp 135-140 (https://doi.org/10.1007/s00216-004-2733-8).
126. Ariño, C.; Serrano, N.; Díaz-Cruz, J. M. Esteban, M. *Anal. Chim. Acta*, **2017**, *990*, pp 11-53 (https://doi.org/10.1016/j.aca.2017.07.069).
127. Munoz, R. A. A.; Angnes, L. *Microchem. J.*, **2004**, *77* (2), pp 157-162 (https://doi.org/10.1016/j.microc.2004.02.010).
128. Almeida, E. S.; Richter, E. M.; Munoz, R. A. A. *Anal. Chim. Acta*, **2014**, *837*, pp 38-43 (https://doi.org/10.1016/j.aca.2014.05.031).
129. Nascimento, D. S.; Insausti, M.; Band, B. S. F.; Lemos, S. G. *Fuel*, **2014**, *137*, pp 172-178 (https://doi.org/10.1016/j.fuel.2014.07.100).
130. Saciloto, T. R.; Cervini, P.; Cavalheiro, É. T. G. *Electroanalysis*, **2014**, *26* (12), pp 2664-2676 (https://doi.org/10.1002/elan.201400282).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 106-117
doi: 10.30744/brjac.2179-3425.AR-17-2021

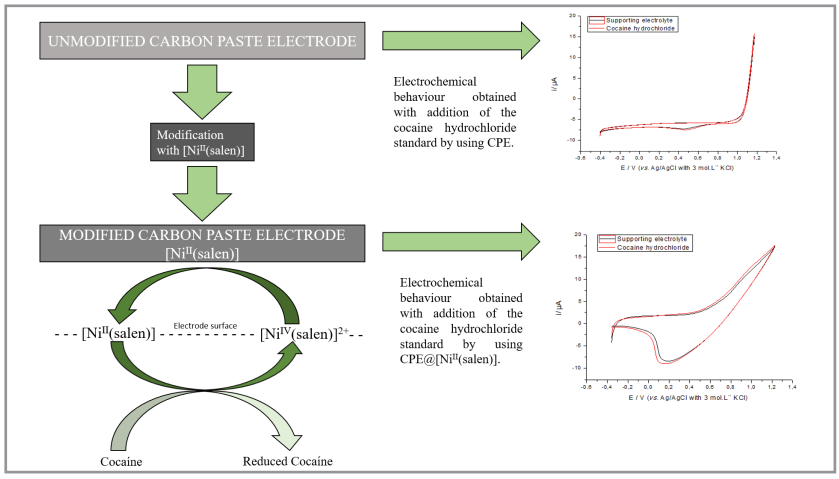
BrJAC

ARTICLE

Development of Carbon Paste Electrode Chemically Modified with Schiff Base Complexes for Forensic Analysis of Cocaine

Ana Beatriz Defendi da Silva, Alex Soares Castro, Marcelo Firmino de Oliveira*

Grupo de Eletroquímica, Eletroanalítica e Química Forense (GEEQFor), Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo (DQ/FFCLRP/USP), Avenida Bandeirantes, 3900, Ribeirão Preto, SP, Brazil



In this article we have studied the electrochemical behavior of cocaine hydrochloride on the surface of a carbon paste electrode chemically modified with a Schiff base complex, namely [Fe^{III}(salen)], [ZrO^{II}(salen)], or [Ni^{II}(salen)], during voltammetric analyses. Among the tested complexes, [Ni^{II}(salen)] provided amperometric and thermal stability and it was only degraded at temperatures above 400 ºC. To prepare the cocaine hydrochloride was used hydrochloric acid (HCl 1 mol L^{-1}) after we tested the electrode, the HCl did not cause electrode passivation. In this study we can see that the voltammetric analyses revealed a satisfactory result, that the peak current obtained between 0.1 and 0.2 V (*vs.* Ag/AgCl) varied linearly with cocaine hydrochloride concentration and the average amperometric sensitivity, the LOD, and the LOQ were 5.5 µmol L^{-1}, 0.945 µmol L^{-1}, and 3.16 µmol L^{-1}, respectively.

Keywords: forensic chemistry, cocaine, chemically modified electrodes, voltammetry, Schiff base complexes

INTRODUCTION

About 275 million people use drugs worldwide. Among drug users, 21 million people, aged between 15 and 64, use cocaine (Figure 1, IUPAC nomenclature: methyl (1R,2R,3S,5S)-3-(benzoyloxy)-8-methyl-8-azabicyclo-[3.2.1]-octane-4-carboxylate, $C_{17}H_{21}NO_4$). Cocaine is an alkaloid that stimulates the central nervous system, causing addiction, high blood pressure, and psychiatric disorders [1-3]. It is used as a recreational drug, but it is also taken by people who live on the streets and have to deal with adverse everyday circumstances [4-7].

Cite: da Silva, A. B. D.; Castro, A. S.; de Oliveira, M. F. Development of Carbon Paste Electrode Chemically Modified with Schiff Base Complexes for Forensic Analysis of Cocaine. *Braz. J. Anal. Chem.,* 2022, *9* (34), pp 106-117. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-17-2021

Submitted 02 February 2021, Resubmitted 11 June 2021, 2nd time Resubmitted 13 July 2021, Accepted 16 August 2021, Available online 03 September 2021.

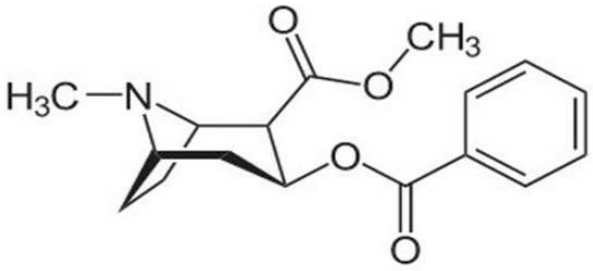


Figure 1. Molecular structure of cocaine.

Cocaine can be found in *Erythroxylon* bushes, which are native to South America. Coca leaves have been found in the burial tombs of early Incas; only the royalty used cocaine at the time. Later, in the sixteenth century, Europeans gave coca leaves to slaves to take hunger and fatigue away and hence make them work harder. In 1855, the German Friedrich Gaedecke extracted the active ingredient from coca leaves and called it *Erythroxylon*. In 1859, the chemist Albert Niemann isolated an alkaloid from coca leaves and called it cocaine. This allowed cocaine to be transported to other countries because it circumvented the issue of leaf degradation and the consequent loss of its properties. Cocaine thus became an illicit drug and since then has been cause for concern [8-9].

The dire consequences of drugs of abuse to our society have encouraged research into electrochemical analyses to identify and to quantify illicit drugs in seized samples. Indeed, public agencies that are responsible for drug seizures worldwide need reliable, practical, and fast tests to help fight crime and to aid the judicial system in passing fair sentences. In this scenario, tests relying on fast and simple techniques are essential for preliminary analysis of the seized drug and for moving on to further analysis. Most preliminary tests are based on color reaction and turbidity. They are inexpensive and easy to produce, and they can be transported everywhere.

Numerous methods for cocaine analysis have been described in the literature, including voltammetry, which offers advantages such as high sensitivity in a linear concentration range, low limits of detection and quantification, and good precision. Voltammetry allows various matrixes to be analyzed within a short time (within seconds), requires only a small volume of the sample (usually in the range of microliters), and is simple to perform.

Electrodes used in voltametric analyses can be chemically modified. The modifier is of paramount importance because it will interact with the target analyte. Chemically modified electrodes have high sensitivity [10] for several target compounds, are inexpensive and easy to produce (given that their surface can be renewed), and can have the electrodynamic potential range adjusted depending on the target analyte [11-12].

Recently, Schiff base complexes have been employed to develop chemically modified electrodes for the production of voltammetric sensors for forensic purposes. Great results have been achieved for voltammetric sensors that can detect and quantify cocaine in seized samples, as described by Oliveira et al. (2013) [13,17] and Ribeiro et al. (2015) [14], who used Schiff base complexes to modify electrodes.

In this study, we have developed new carbon paste electrodes chemically modified with a Schiff base complex, namely [Fe^{III}(salen)], [ZrO^{II}(salen)], or [Ni^{II}(salen)]. We have studied the electrochemical activity of cocaine hydrochloride on the surface of these electrodes and assessed them as voltammetric sensors for cocaine hydrochloride detection and quantification, with a view to their application in the analysis of seized cocaine samples.

MATERIAL AND METHODS

Thermogravimetric analyses

The stability of the [Fe^{III}(salen)], [ZrO^{II}(salen)], and [Ni^{II}(salen)] complexes was evaluated by TGA studies on the TGA Q 5000 V3.13 build 261 apparatus. The melting point of these complexes was established by DSC studies on the DSC Q 1000V9.9 build 303 device.

SEM

The carbon paste electrodes chemically modified with [Fe^{III}(salen)], [ZrO^{II}(salen)], or [Ni^{II}(salen)] were analyzed by scanning electron microscopy (SEM) (Carl Zeiss AG - EVO® 50 Series) and dispersive energy spectrometry (DES) before and after voltammetric analysis of cocaine hydrochloride. The SEM and EDS analyses were performed on 500 Digital Processing equipment; IXRF Systems (Houston, TX, USA).

Electrochemical measurements

The electrochemical behavior of cocaine hydrochloride in carbon paste electrodes chemically modified with a Schiff base complex, electron exchange between cocaine hydrochloride and [Fe^{III}(salen)], [ZrO^{II}(salen)], or [Ni^{II}(salen)], and voltammetric sensors to quantify cocaine in seized samples were analyzed by voltammetry on a Metrohm® model AUTOLAB 128N potentiostat coupled to a computer. The voltammograms had the baseline corrected with the NOVA 1.8.17 software.

The seized cocaine samples were provided by the local scientific police laboratory, in a work partnership between our research group and Scientific Police of Sao Paulo State.

An electrochemical cell made up of three electrodes was employed. The reference electrode was Ag/AgCl Metrohm®; the auxiliary electrode was a platinum spiral, and the working electrode was a carbon paste electrode chemically modified with [Fe^{III}(salen)], [ZrO^{II}(salen)], or [Ni^{II}(salen)]. The supporting electrolyte was 0.1 mol L^{-1} KCl.

The carbon paste electrodes chemically modified with a Schiff base complex were prepared by adding graphite (conductor) and Schiff base complex (modifier), at 70:30 ratio, and paraffin, as binding agent, to a crucible. The crucible was heated to 60 ºC, and the mixture was macerated until a homogeneous paste was achived. After the paste inside the electrode solidified, voltammetric analyses were carried out. The voltammetric measurements were conducted from -0.4 to 1.8 V, at scan rates varying from 5 to 100 mV s^{-1}.

RESULTS AND DISCUSSION

Amperometric stability of the chemically modified carbon paste electrodes

We evaluated the factors that could limit cocaine hydrochloride identification and quantification. First, we verified the stability of the amperometric current of the carbon paste electrode (CPE) chemically modified with [Fe^{III}(salen)], [ZrO^{II}(salen)], or [Ni^{II}(salen)].

Performing these electrode stability tests before starting the analysis with the analyte of interest is extremely important, since within a laboratory where there is a high demand for forensic analysis. In this work, we look for a modified electrode that loses its amperometric signal after (at least) five voltammetric cycles, as it is suggested that any chemical analysis, whether qualitative or quantitative, be performed in at least triplicate (ours were performed in quintuplicates). Therefore, the first primary analysis was the amperometric signal stability test, in which a screening was performed: a complex that had amperometric signal loss below 10% after the 5^{th} scan was considered stable. Higher loss of amperometric signal could jeopardize the use of the modified working electrode to identify cocaine hydrochloride.

It is noteworthy that stability tests are performed in the absence of the analyte of interest (cocaine hydrochloride), thus, we performed the voltametric analyses of the chemically modified carbon paste electrodes in 0.1 mol L^{-1} KCl, as supporting electrolyte, for potential values ranging from -0.4 to 1.8 V (*vs.* Ag/AgCl) and scan rate of 100 mV s^{-1}. Figure 2 shows the electrochemical stability analysis of the carbon paste electrode chemically modified with [Fe^{III}(salen)]. An anodic peak emerged at 1.3 V (*vs.* Ag/AgCl), but it disappeared after the first scan.

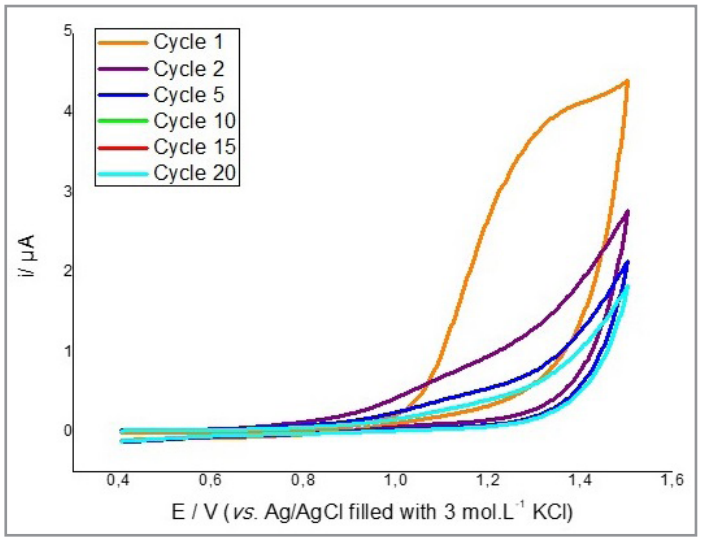


Figure 2. Cyclic voltamograms shown the amperometric stability of the carbon paste electrode chemically modified with [Fe^{III}(salen)], at a scan rate of 100 mV s^{-1}, in 0.1 mol L^{-1} KCl.

Figure 3 depicts the electrochemical stability analysis of the carbon paste electrode chemically modified with [ZrO^{II}(salen)]. An anodic peak (oxidation) emerged at 1.2 V (*vs*. Ag/AgCl), but it lost amperometric amplitude after the first scan.

As described above, the CPE[Fe^{III}(salen)] and CPE[ZrO^{II}(salen)] lose signal more than 30% of their amperometric signal before the 5th cycle. This phenomenon of instability was not interesting in the development of this study, since the demand for day-to-day analyzes is large in number of repetitions, and this could make it difficult for a professional to perform this analysis to perform the work, or generate false negatives, since as we will see later in the work, the detection of cocaine hydrochloride happens from the redox activity of the complex used as a working electrode modifier. Therefore, it is essential that the amperometric peak remains stable after consecutive sweep cycles.

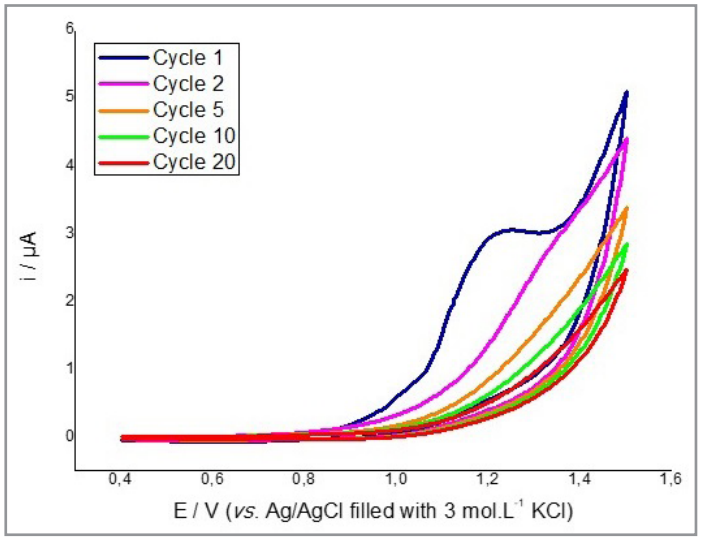


Figure 3. Cyclic voltamogram of the amperometric stability of the carbon paste electrode chemically modified with [ZrO^{II}(salen)], at a scan rate of 100 mV s^{-1}, in 0.1 mol L^{-1} KCl.

In contrast, Figure 4 presents the amperometric stability analysis of the carbon paste electrode chemically modified with [Ni^{II}(salen)]. A cathodic peak with amperometric stability emerged at 0.2 V (*vs.* Ag/AgCl). After 25 scans, the amplitude of the reduction peak decreased by only 6.36%. Therefore, the complex remained stable after many scans, and the loss of signal amplitude did not affect cocaine hydrochloride analysis. In other words, as we do not have a significant loss of amperometric signal even after 25 scans, this stability results in the discarding of false negatives, thus, an analyst can carry out his analyzes with greater security to issue his report.

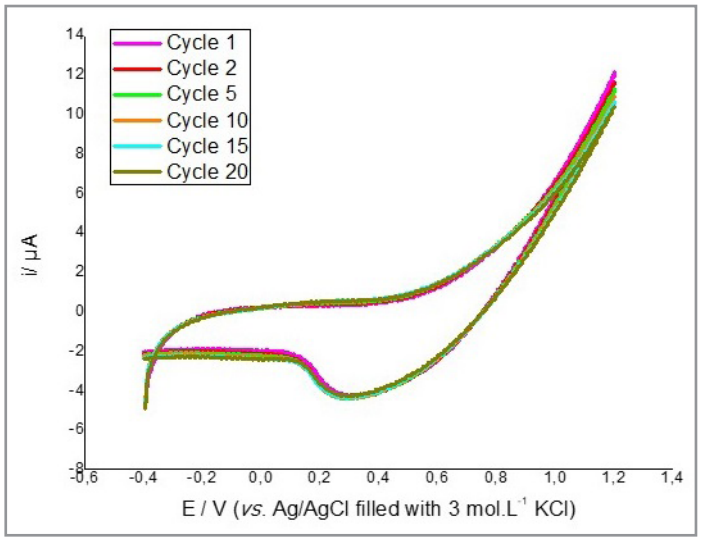


Figure 4. Cyclic voltamogram of the amperometric stability of the carbon paste electrode chemically modified with [Ni^{II}(salen)], at a scan rate of 100 mV s^{-1}, in KCl 0.1 mol L^{-1} KCl.

The second primary analysis before we begin to describe the analysis of cocaine hydrochloride is the CPE[Ni^{II}(salen)] passivation test, since the solvent used to prepare the standard cocaine hydrochloride solution – hydrochloric acid (HCl 1 mol L^{-1}) – could degrade the CPE[Ni^{II}(salen)], causing it to corrode or melt, causing passivation (damage to the [Ni^{II}(salen)]) and not making it viable for use in analysis in a forensic laboratory.

The carbon paste electrode chemically modified with [Ni^{II}(salen)], designated CPE@[Ni^{II}(salen)] hereafter, displayed good electrochemical stability, so we submitted it to a passivation test. Our aim was to evaluate whether the solution we used to prepare the cocaine hydrochloride solution degraded CPE@[Ni^{II}(salen)]. To this end, we added aliquots of 100, 200, 300, or 400 µL of HCl solution (pH 3) to the electrochemical cell containing the supporting electrolyte, 0.1 mol L^{-1} KCl, and conducted experiments at a scan rate of 100 mV s^{-1} across the potential range. On the basis of Figure 5, CPE@[Ni^{II}(salen)] did not undergo passivation/degradation in acidic medium. Therefore, we used CPE@[Ni^{II}(salen)] to detect and to quantify cocaine hydrochloride.

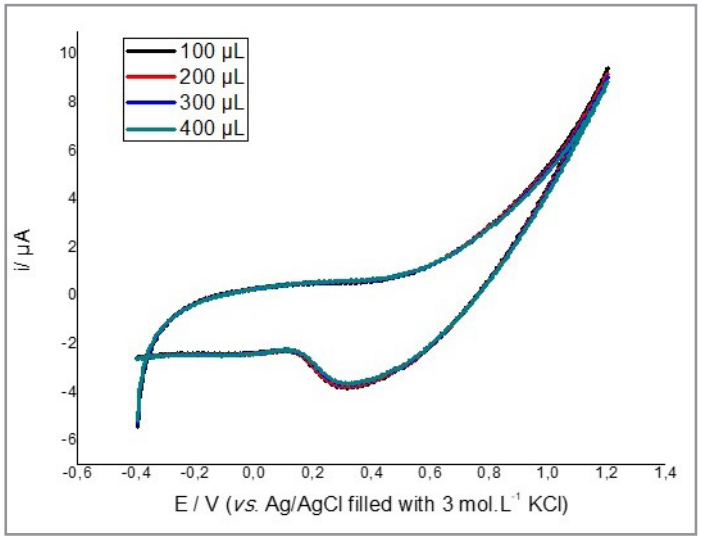


Figure 5. Passivation test of the carbon paste electrode chemically modified with [Ni^{II}(salen)] by HCl 1 mol L^{-1}, at a scan rate of 100 mV s^{-1}, in 0.1 mol L^{-1} KCl.

Finally, we evaluated which scan rate provided the highest amperometric peak, and hence the best amperometric sensitivity. The scanning speed at which the amperometric signal had the greatest amplitude was sought, as this reflects the greater efficiency of qualitative and quantitative analyses. According to

Figure 6, the optimal scan rate was 100 mV s^{-1} (without the addition of cocaine hydrochloride), which gave the highest anodic peak amplitude. Thus, we accomplished qualitative and quantitative analyses of cocaine hydrochloride at a scan rate of 100 mV s^{-1} in 0.1 mol L^{-1} KCl.

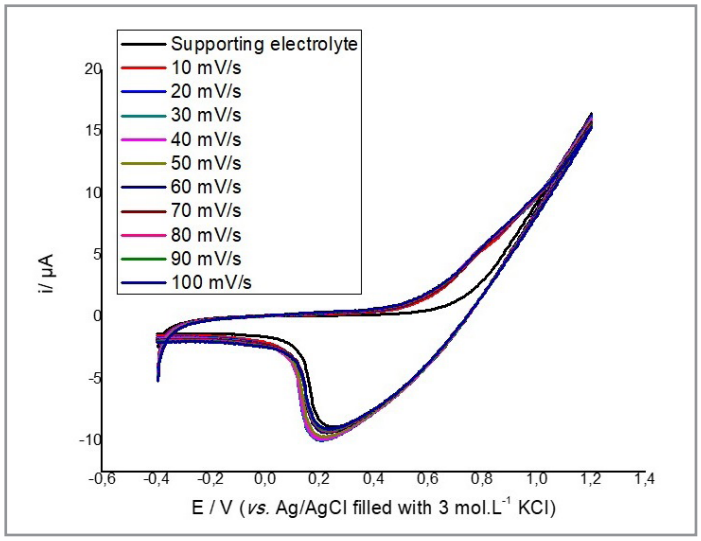


Figure 6. Scan speed study of CPE[Ni^{II}(salen) in 0.1 mol L^{-1} KCl.

Electrochemical profile of the carbon paste electrode with and without modification

Given that CPE@[Ni^{II}(salen)] was the only chemically modified carbon paste electrode that presented satisfactory amperometric stability along successive potential scans, we carried out cyclic voltammetry analysis of the carbon paste electrode without modification and modified with the [Ni^{II}(salen)] complex.

On the basis of Figure 7, the carbon paste electrode without modification was not electrochemically active in the analyzed potential range between -0.4 and 1.2 V (*vs*. Ag/AgCl). After chemical modification, a cathodic peak emerged at 0.2 V (*vs*. Ag/AgCl).

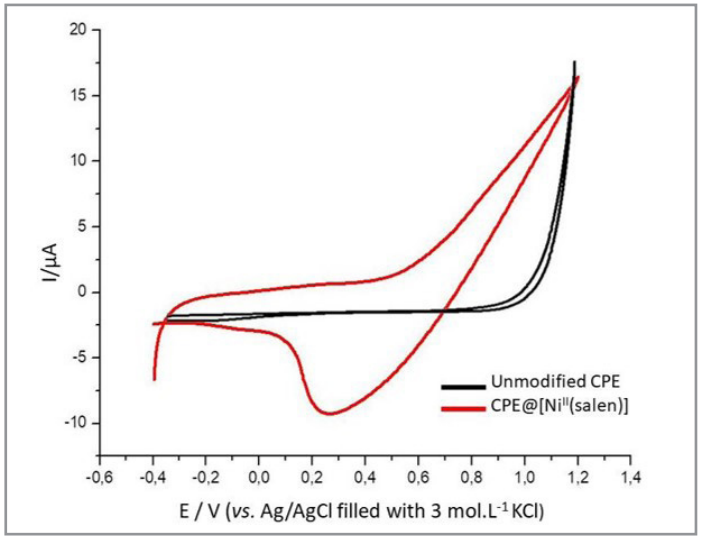


Figure 7. Voltammetric profile of the unmodified carbon paste electrode and of the carbon paste electrode chemically modified with the [Ni^{II}(salen)] complex, at a scan rate of 100 mV s^{-1}, in 0.1 mol L^{-1} KCl.

CPE@[Ni^{II}(salen)] characterization

When we prepared the chemically modified carbon paste electrode, we heated the mixture to 60 ºC to promote agglutination of the paste. Therefore, we had to evaluate whether any [Ni^{II}(salen)] was lost along the process.

Figure 8 shows the TG curve of the [Ni^{II}(salen)] complex, which indicated total weight loss of 79.2%. The complex underwent degradation at 400 °C, when nickel oxide emerged after a major mass loss. DSC revealed an exothermic peak at 400 °C and a heat flow of 160 Wg^{-1}, associated with decomposition of the complex.

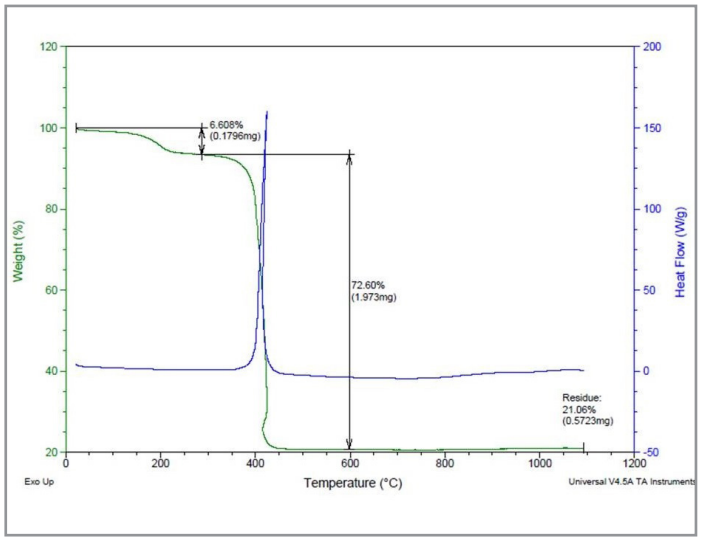


Figure 8. TGA and DSC of the [Ni^{II}(salen)] complex.

We also evaluated the uniformity and homogeneity of CPE@[Ni^{II}(salen)]. SEM analysis (Figure 9) showed that the complex was uniformly and homogeneously distributed on the CPE@[Ni^{II}(salen)] surface.

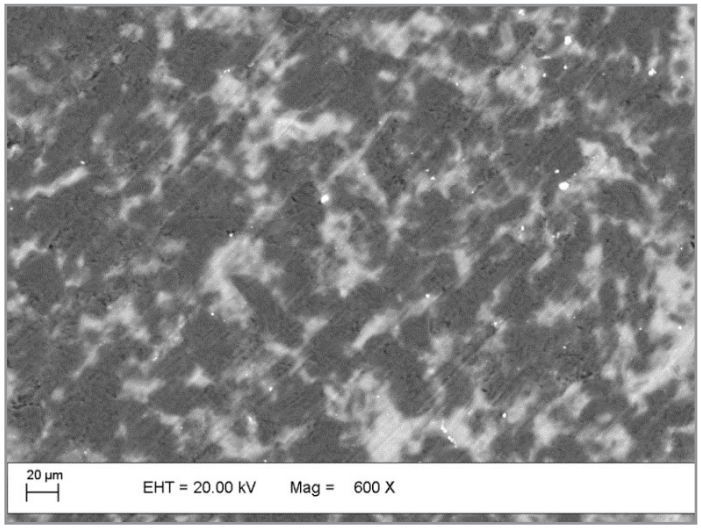


Figure 9. Uniformity and homogeneity of the carbon paste electrode chemically modified with [Ni^{II}(salen)] complex as analyzed by SEM.

Analysis of cocaine hydrochloride by cyclic voltammetry

After we conducted the stability tests and optimized the scan rate, we obtained satisfactory voltammograms for the cocaine hydrochloride standard at concentrations ranging from 1 to 15 µmol L^{-1} in 0.1 mol L^{-1} KCl. Figure 10A shows the voltammograms obtained at different cocaine hydrochloride concentrations, in which an increase in the cathodic peak was observed in the range of 0.1 V – 0.2 V (*vs.* Ag/AgCl) as the standard of cocaine hydrochloride was added. Whereas Figure 10B shows the analytical curve profile for this analysis. The intra and inter-day repeatability studies (n = 3) indicated deviations of 1.68 and 3.72%. The recovery test gave 94.43% recovery.

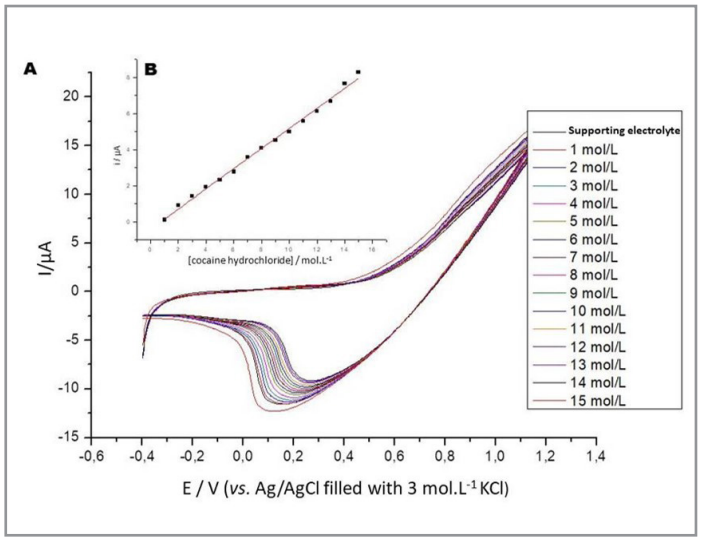


Figure 10. (A) cyclic voltammograms obtained with addition of the cocaine hydrochloride standard by using CPE@[Ni^{II}(salen)] in 0.1 mol L^{-1} KCl and **(B)** analytical curve obtained for the addition of cocaine hydrochloride concentrations ranging from 1 to 15 µmol L^{-1}, where a reduction activity in the range of 0.1 V – 0.2 V (*vs*. Ag/AgCl) is observed.

The chemical modification of CPE using Ni^{II}(salen) presented an increase in voltammetric response for cocaine species. In fact, Figure 11 exihibits the voltammetric response for cocaine in a CPE without chemical modification, being possible to observe an unsatisfactory and less specific response for cocaine.

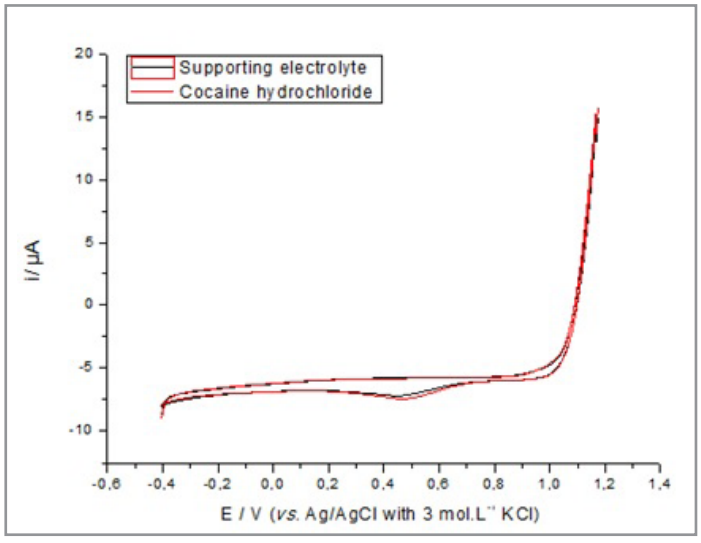


Figure 11. Cyclic voltammograms obtained with addition of the cocaine hydrochloride standard in 1.0 µmol L^{-1} by using CPE in 0.1 mol L^{-1} KCl.

Table I shows the linear correlation coefficient (r), standard deviations, amperometric sensitivities (s), limits of detection (LOD, 3DP/s), and limits of quantification (LOQ, 10DP/s) for nine replicates on three different days.

Table I. Statistical data from the analyses of cocaine hydrochloride standard by using CPE@[Ni^{II}(salen)], performed on different days

	Sensitivity [μmol L^{-1}] / [μA]	Intercept [μA]	Number of points	Standard deviation [μA]	LOD [μmol L^{-1}]	LOQ [μmol L^{-1}]	r
Measurement 1, day 1	5.60	-4.91	15	1.16	0.62	2.07	0.992
Measurement 2, day 1	5.24	-3.14	15	3.77	2.15	7.19	0.991
Measurement 3, day 1	5.57	-3.97	15	0.94	0.50	1.68	0.996
Measurement 1, day 2	5.46	-4.12	15	1.58	0.87	2.89	0.993
Measurement 2, day 2	5.32	-4.87	15	2.55	1.44	4.79	0.992
Measurement 3, day 2	5.24	-3.78	15	4.45	2.55	8.49	0.995
Measurement 1, day 3	5.57	-3.87	15	0.95	0.51	1.70	0.995
Measurement 2, day 3	5.13	-3.99	15	1.01	0.59	1.96	0.993
Measurement 3, day 3	5.25	-4.15	15	0.89	0.51	1.69	0.998

As we can see in Table II, the new electrode developed on this article shows itself superior when compared on the literature, with anothers methods and anothers working electrodes. Table II shows various methods and theirs respectives working electrodes and their value of LOD. The best value of LOD is from the electrode developed on this study, showing its efficiency.

Table II. Comparison of different electrochemical results for quantification and determination of cocaine

Method	Working Electrode	LOD [μmol L^{-1}]	Ref.
Cyclic voltammetry	GCE@[$U^{II}O$(salen)]	0.50	[1]
Cyclic voltammetry	PtE@[$U^{II}O$(salen)]	0.29	[1]
Cyclic voltammetry	CP@[UO2(5-MeOSalen)(H2O)]	0.15	[13]
Potentiometry	Selective membrane electrode	0.4	[15]
Linear sweep voltammetry	PANI-β-CD/fMWCNT/GCE	1.02	[16]
Cyclic voltammetry	CP@[$U^{II}O$(salen)]	0.326	[17]
Cyclic voltammetry	CP@[Mn(salen)]	0.9320	[18]
Cyclic voltammetry	CP@[Ni^{II}(salen)]	2.55	This work

CP: Carbon Paste Electrode; PtE: Platinum Electrode; GCE: Glassy Carbon Electrode; β-CD: Beta-Cyclodextrin.

Kinetics and mechanism of cocaine hydrochloride electroreduction on the CPE@[Ni^{II}(salen)] surface

Komorsky-Lovric et al. (1999) [19] and Pavlova et al. (2004) [20] reported that electron exchange between cocaine and the electrode surface occurs by diffusion. We evaluated this process by analyzing 8 µmol L^{-1} cocaine hydrochloride at scan rates of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mV s^{-1}. In Figure 12, we present the curve ip *vs.* $v^{1/2}$. The curve suggested that the exchange of two electrons during cocaine hydrochloride oxidation at CPE@[Ni^{II}(salen)] was controlled by diffusion, as suggested in the literature (Equation 1).

$$i_{pc} = 0.37899\ v^{1/2} - 1.3380 \qquad r = 0{,}9907 \qquad n = 10 \qquad \text{Equation 1}$$

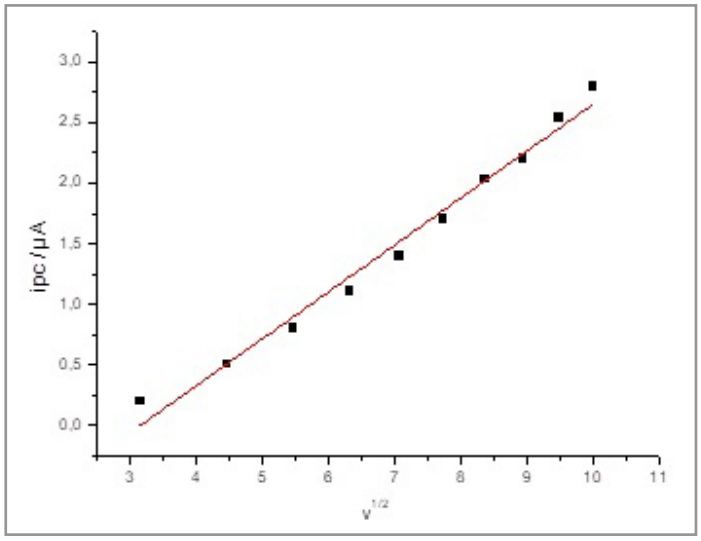


Figure 12. Study of cocaine mass transport for 8 µmol L^{-1} cocaine hydrochloride by using CPE@[Ni^{II}(salen)] in 0.1 mol L^{-1} KCl.

To understand the mechanism of electrochemical cocaine hydrochloride reduction on the CPE@[Ni^{II}(salen)] surface, it is necessary to revisit the description of the fragmentation of cocaine during cocaine hydrochloride reduction on the surface of graphite electrodes reported by Komorsky-Lovric et al. (1999) and Pavlova et al. (2004). These authors found that the diffusion-controlled cathodic reaction occurred in the ester group; that is, the oxygen-containing groups were the electroactive centers of the cocaine molecule when the analyte was in close contact with the graphite surface [19-20].

Through the mechanism proposed by Castro et al. (2019) [18], we can assume that the exchange of electrons between the cocaine molecule and the complex [Ni^{II}(salen)] occurs in two concomitant steps: in the first step, when the oxidation potential is applied to the working electrode, the complex [Ni^{II}(salen)] is electrochemically oxidized, producing [Ni^{IV}(salen)]$^{2+}$ on the electrode surface (this step was not observed in the worked potential range). In the second step (as noted), in scanning the cathode potential, the complex [Ni^{II}(salen)] is electrochemically regenerated; that is, the complex in the reduced form reacts with the reduced cocaine, consequently oxidizing the [Ni^{II}(salen)] complex to [Ni^{IV}(salen)]$^{2+,}$ which is electrochemically reduced, thus, as seen in Figure 7B, only the increase in the magnitude of the peak cathodic current obtained between 0.1 and 0.2 V (*vs.* Ag/AgCl) was proportional to the concentration of cocaine hydrochloride in solution.

CONCLUSIONS

TGA and DSC analyses proved that the [Ni^{II}(salen)] complex that we used to modify a carbon paste electrode was thermally stable up to 400 °C, which was five times higher than the temperature that we used for the carbon paste agglutination. During voltammetric analyses, the cathodic peak current obtained between 0.1 and 0.2 V (*vs*. Ag/AgCl) increased linearly with the cocaine hydrochloride concentration. The prototype of the chemically modified carbon paste electrode developed in this work presented good and specific electrochemical activity for cocaine hydrochloride, and we can see that the complex reacts with cocaine, this behavior can be explained in two steps when we do the voltammetric analyses.

The first step occurs when the oxidation potential is applied, the complex [Ni^{II}(salen)] is electrochemically oxidized, producing [Ni^{IV}(salen)]$^{2+}$, this product can be found on the surface of the working electrode. The second step occurs in the cathode potential, when the complex [Ni^{II}(salen)] is electrochemically regenerated, thus the complex in the reduced form reacts with reduced cocaine, oxidizing the [Ni^{II}(salen)] in to [Ni^{IV}(salen)]$^{2+}$, this process increases the magnitude of the peak cathodic current obtained between 0.1 and 0.2 V (*vs*. Ag/AgCl), that was proportional to the concentration of the cocaine hydrochloride in the solution.

The linear correlation coefficient was 0.9760, the average amperometric sensitivity was 5.5 μmol L^{-1}, and the LOD and LOQ were 0.945 μmol L^{-1} and 3.16 μmol L^{-1}, respectively.

Conflicts of interest

The authors have no financial conflicts of interest or lack thereof.

Acknowledgments

The authors thank CAPES (PROCAD 16/2020), FAPESP (2016/23825-3) and CNPq (301864/2019-5) for financial support. The authors also thank Dr. Cynthia Maria de Campos Prado Manso for help with the English language.

REFERENCES

1. Oliveira, L. S.; Balbino, A. M.; Menezes, M. M. T.; Dockal, E. R.; Oliveira, M. F. *Microchem. J.*, **2013**, *110*, pp 374–378 (https://doi.org/10.1016/j.microc.2013.04.017).
2. Goldstein, R. A.; DesLauriers, C.; Burda, A.; Johnson-Arbor, K. *Semin. Diagn. Pathol.*, **2009**, *26* (1), pp 10-17 (https://doi.org/10.1053/j.semdp.2008.12.001).
3. Trifilieff, P.; Martinez, D. Cocaine: Mechanism and Effects in the Human Brain. In: Madras. B.; Kuhar, M. (Eds.) *The Effects of Drug Abuse on the Human Nervous System*, Elsevier, **2014**, Chapter 5, pp 103-133 (https://doi.org/10.1016/B978-0-12-418679-8.00005-8).
4. https://www.unodc.org/doc/wdr2018/WDR_2018_Press_ReleaseENG.PDF [Accessed Feb 2020].
5. https://www.nytimes.com/2019/08/18/world/europe/paris-crack-cocaine-la-colline.html [Accessed Feb 2020].
6. https://www.statista.com/statistics/264738/number-of-worldwide-users-of-cocaine-by-region/ [Accessed Feb 2020].
7. https://www.unodc.org/unodc/en/frontpage/2019/June/world-drug-report-2019_-35-million-people-worldwide-suffer-from-drug-use-disorders-while-only-1-in-7-people-receive-treatment.html [Accessed Feb 2020].
8. Gootenberg, P. (Ed.) *Cocaine: Global Histories*, Routledge, London, **1999**.
9. Karch, S. B. *A Brief History of Cocaine*, CRC Press, **1998**.
10. Harris, D. C. *Análise Química Quantitativa*. Livros Técnicos e Científicos (LTC), Rio de Janeiro, **2008**.
11. Skoog, D. A.; West, D. M.; Holler, F. J.; Crouch, S. R. *Fundamentos de Química Analítica*, 8th Ed. Thomson, São Paulo, **2005**.
12. De Souza, D.; Machado, S. A. S.; Avaca, L. A. *Quim. Nova,* **2003**, *26* (1) pp 81-89 (https://doi.org/10.1590/S0100-40422003000100015).

13. Oliveira, L. S.; Balbino, M. A.; Menezes, M. M. T.; Dockal, E. R.; Oliveira, M. F. *Microchem. J*., **2013**, *110*, pp 374–378 (https://doi.org/10.1016/j.microc.2013.04.017).
14. Ribeiro, M. F. M.; Da Cruz Junior, J. W.; Dockal, E. R.; McCord, B. R.; Oliveira, M. F. *Electroanalysis*, **2016**, *28* (2), pp 320-326 (https://doi.org/10.1002/elan.201500372).
15. Watanabe, K.; Okada, K.; Oda, H., Furuno, K.; Gomita, Y.; Katsu, T. *Anal. Chim. Acta,* **1995**, *316* (3), pp 371–375 (https://doi.org/10.1016/0003-2670(95)00374-9).
16. Garrido, J. M. P. J.; Borges, F.; Brett, C. M. A.; Garrido, E. M. P. J. *Ionics*, **2016**, *22* (12), pp 2511–2518 (https://doi.org/10.1007/s11581-016-1765-3).
17. de Oliveira, L.; Poles, A. S.; Balbino, M.; de Menezes, M. T.; de Andrade, J.; Dockal, E.; de Oliveira, M. *Sensors*, **2013**, *13* (6), pp 7668–7679 (https://doi.org/10.3390/s130607668).
18. Castro, A. S.; de Menezes, M. M. T.; Alves, G. M.; de Oliveira, M. F. *Microchem. J*., **2020**, *153*, 104399 (https://doi.org/10.1016/j.microc.2019.104399).
19. Komorsky-lovric, S.; Galic, I.; Penovski, R. *Electroanalysis*, **1999**, *11* (2), pp 120–123 (https://doi.org/10.1002/(SICI)1521-4109(199902)11:2<120::AID-ELAN120>3.0.CO;2-R).
20. Pavlova, V.; Mirčeski, V.; Komorsky-Lovrić, Š.; Petrovska-Jovanović. S.; Mitrevski, B. *Anal. Chim. Acta*, **2004**, *512* (1) pp 49–56 (https://doi.org/10.1016/j.aca.2004.02.035).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 118-137
doi: 10.30744/brjac.2179-3425.AR-18-2021

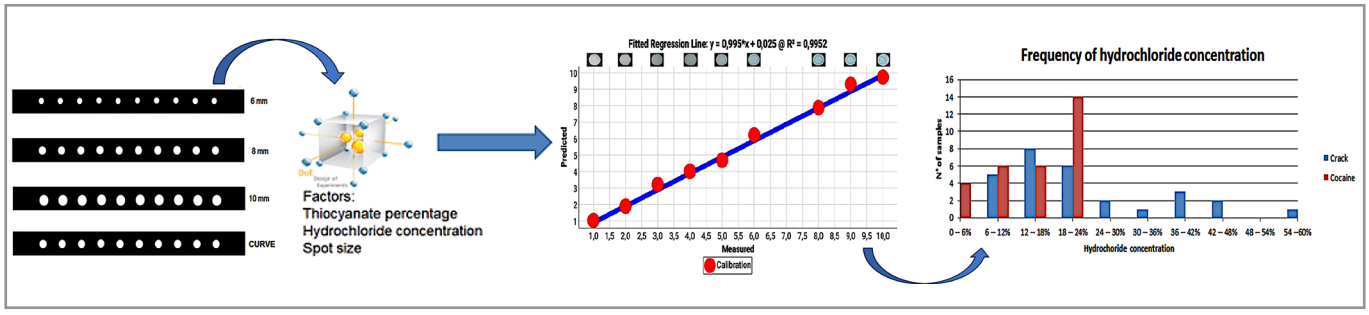
BrJAC

ARTICLE

Use of Paper Microdevices in the Identification and Quantification of Cocaine in Seized Street Samples

Jadson Zeni dos Reis, Wanderson Romão*

Laboratório de Petroleômica e Forense, NCQP, Universidade Federal do Espírito Santo, 29075-910, Vitória, ES, Brazil



The growing consumption of illicit drugs in Brazil is becoming increasingly problematic for society. It is therefore critical to develop technologies to combat drug trafficking that allow for rapid, non-invasive evaluation of drug samples. Microfluidics is a technology that manipulates and studies small amounts of fluids, using structures with dimensions from ten to hundreds of micrometers (microdevices). The main advantages of microfluidic approaches are its low cost, speed, and ability to provide results in loco. Here, paper microfluidics were developed to perform the modified Scott test to calculate the cocaine hydrochloride content in seized samples of cocaine (n = 30) and crack (n = 30). A smartphone with the Photometrix® app was used to construct a model for quantifying the samples. A factorial model was developed to optimize microfluidic analytical parameters such as spot size (6, 8 and 10 mm), reagent content (50, 75, and 100% cobalt thiocyanate II), cocaine hydrochloride concentration (4, 6 and 8 mg mL^{-1}) and response time (or analyte detection; t = 0, 0.5, 1, 12 and 24 h). After experimental planning, a diameter of MPADs = 8 mm - $[Co(SCN)_2]$ = 100% and a 1 h response time were identified as the best conditions. We observed that the cocaine hydrochloride concentration did not influence the model. A sample concentration of 15 mg mL^{-1} was used to quantify cocaine hydrochloride in street samples apprehended by the Forensic Police of Espírito Santo state (with n = 60). The quantification curve constructed to determine the cocaine hydrochloride concentration showed a determination coefficient, R^2, of 0.98246 and RMSEC (root mean squares error calibration - mean square error of the calibration) of 0.39480, with a LOD and LOQ of 0.09 and 0.30 mg mL^{-1}, respectively. For the crack samples, the cocaine hydrochloride concentrations ranged from 2.5 to 60.8 wt% with an average purity content of 21.3 ± 13.3 wt%. For the seized cocaine samples, variation in hydrochloride content from 1.2 to 22.6 wt% was observed with a mean percentage of 14.19 ± 6.92 wt%. Finally, chemometric tools such as principal component analysis were used to assess the similarity among the samples.

Cite: dos Reis, J. Z.; Romão, W. Use of Paper Microdevices in the Identification and Quantification of Cocaine in Seized Street Samples. *Braz. J. Anal. Chem.,* 2022, *9* (34), pp 118-137. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-18-2021

Submitted 02 March 2021, Resubmitted 01 June 2021, Accepted 12 June 2021, Available online 09 August 2021.

Keywords: cocaine, crack, microfluidic, Chemometrics, Photometrix®.

INTRODUCTION

The commonly abused substances that cause psychological and physical dependence can be grouped into three major classes according to their main actions on the central nervous system (CNS): CNS depressants, CNS stimulants, and CNS disruptors. CNS depressants reduce brain activity and also have analgesic properties. People under the influence of these drugs become drowsy, lazy, careless, and unfocused. Among the main substance classes are highlighted: opioids, ethanol and barbiturates. CNS stimulants, which increase brain activity, accelerate the activity of particular neuronal systems, producing an exaggerated state of alertness, and insomnia. Cocaine, amphetamines, and methamphetamines (such as MDMA), and anorectic are examples of CNS stimulants. CNS disruptors distort brain functioning, producing altered states such as delusions, hallucinations, and changes in sensory perception. For this reason, CNS disruptors are also referred to as hallucinogens. Examples of CNS disruptors include LSD, psilocybin, mescaline, and cannabinoids [1-5].

Among these drugs, the cocaine, that is composed of a tropane alkaloid, inhibits the action of acetylcholine. Cocaine has a high local anesthetic action and is a potent stimulant of the central nervous system [6]. Cocaine belongs to the family of alkaloids (compounds with aromatic nitrogen) obtained from the leaves of the coca plant, Erythroxylum coca. The alkaloid content of the coca leaves varies according to different growing regions and varieties of the plant. The shrub grows in South America, particularly in Peru and Bolivia, and is found in the eastern Andes and above the Amazonic watershed. There are about 200 plant species, but only 17 of them are used for cocaine extraction and production. Cocaine can be consumed as salt, cocaine hydrochloride, or as a free base (crack) [7].

Forensic sciences

Forensic sciences are based on the concept that crimes cannot occur without producing some evidence, such as skin cell fragments, microscopic fibers, or a small amount of poison present in a drop of blood. Increasingly, forensic sciences have evolved to include the use of sensitive and precise instruments [8]. In this context, forensic chemistry is a branch of forensic science aimed at identifying material evidence for criminal justice proceedings, through the analysis of substances such as licit and illicit drugs, poisons, accelerants and fire residues, explosives, waste firearms, fuels, paints, and fibers [9]. These new technologies allow for efficient confirmation of crimes by laboratory analysis [10]. However, the ability to confirm samples in loco could solve several problems more quickly.

Chewing and ingesting coca leaves has been a common practice in South America for generations due to the stimulating effects of coca. However, cocaine purified from coca leaves is a schedule II drug since it has a high potential for abuse. In the street, cocaine has the appearance of a fine white powder and is mixed with a wide variety of adulterants, such as benzocaine, lidocaine, caffeine and procaine, in order to inflate the mass of the product, increasing profits from sales. For forensic identification of cocaine, it is common to use colorimetric kits that react to the presence of cocaine hydrochloride; however, these tests can be interfered with, producing false positive results. Other analytical techniques, including infrared spectroscopy by Fourier transform (ATRFTIR) and visible in ultraviolet (UV-VIS), can be used to confirm samples [11-13].

Microfluidics

Microfluidics, the study of the behavior of small fluid samples in small channels, has been applied in biochemical and pharmaceutical tests. However, a more detailed definition describes microfluidics as the science and technology of systems that manipulate and study small amounts of fluids, using structures (microdevices) with dimensions from tens to hundreds of micrometers [14].

To reduce the use of reagents and lower operating costs, microfluidics is increasingly being used in new equipment used in the chemical industry, and, consequently, in the forensic sciences. This trend is due

to the increasing miniaturization of electronic devices. Miniaturization began in the 1960s, when analog devices began to be replaced by digital devices which have smaller dimensions but equivalent efficiency. The miniaturization of electronics is apparent when comparing a television from the 70s with a current television or even when comparing a notebook to the first desktops [15].

In the 1990s, Manz et al. proposed the use of microsystems for total analysis, or simply μTAS. With the development of μTAS, it became possible to integrate in a single device several analytical steps, such as sample introduction, sample pre-treatment, chemical reactions, analytical separation, and detection [16,17]. Since several analytical steps, normally developed in a laboratory, were consolidated on a single chip, μTAS are also called “lab-on-a-chip” (LOC) [14]. μTAS, or LOC, transform chemical information into electrical or optical signals, enabling easy automation, becoming relevant to the clinical and environmental field in which the term “point-of-care” has been used [14,18]. These factors, coupled with portability, have driven the massive development of analytical microscale systems in recent years [19].

In the early 2000s, the 3D printer modified and accelerated the direction of microdevices. This technology uses a resin that stiffens in the presence of ultraviolet light, producing a sculpture perfectly aligned with the determinations indicated in specific computer programs. It is possible to easily create, for example, a channel system with micrometric measurements and printing.

New methods that use microfluidics for analysis have increased in number as 3D printing technology technique has become more popular. Among these innovations, paper-based microdevices stand out in terms of their low manufacturing cost and high portability.

Microfluidic paper-based analytical devices (μPADs)

Paper-based microfluidic devices (or μPADs, from the term “microfluidic paper-based analytical devices”) were created in 2007 by Martinez et al., from Harvard University. The purpose of these devices is to perform colorimetric sensing in a low-cost biological analysis. The paper is mainly composed of a cellulose polymer, which makes it porous, allowing it to favor the liquid fluidity between the fibers due to capillarity action [20].

Advantages to using paper for devices include the following: great abundance, low cost compared to other platforms for sensing, easy to obtain and handle, compatibility with large-scale production of microfluidic devices, possibility of long-term storage, easy physical modification and surface chemistry for bioassays, easy disposal through incineration making it more environmentally friendly, ability to use reduced volumes of samples (microliters to nanoliters depending on the resolution of the barriers created on the paper), and white color (suitable for colorimetric tests). However, some factors can also hinder the use of paper devices such a matrix effect, humidity and the homogeneity of the structure [21]. It is worth noting that substances such as benzocaine, lidocaine, caffeine, and procaine, if mixed with the samples, can generate a false positive result [13].

While there are clear advantages to using μPADs for colorimetric analysis, there are some drawbacks. Applying μPAD technology cannot always be carried out directly, since pretreatment of the sample is necessary to avoid interference in the color of the solution, inconsistencies in lighting, lack of uniformity, or the presence of particulate contaminants that can confuse the interpretation of the colorimetric result. However, it is possible to couple these pretreatment processes in a single disposable paper device.

Wax printing is a method of producing μPADs by depositing wax using solid ink printers. After printing, the paper must be heated to the melting point of the wax so that it penetrates through the cellulose fibers. Heating can be carried out with a heating plate, which is a common piece of equipment in laboratories. The use of μPADs as colorimetric sensors is already widespread in the scientific literature, such as research on monitoring acid-base titrations, food analysis, forensic analysis, and tumor biomarker identification [22-27].

Smartphone app

Growing technological advances have led to a dramatic increase in digital photographic technology, both in terms of software and hardware, which is expected to continue in the coming years due to the easiness and low cost of acquiring images through digital and smartphone cameras. The relationship

between digital image and colorimetric tests favors obtaining both qualitative data and quantitative analytical measurements.

Digital images have been widely used to analyze food, beverages, fuels and other substances due to the low cost and speed of analysis and data collection. Studies to monitor and evaluate food quality, for example, are carried out visually where the use of digital image minimizes the recurrent subjectivity in these types of analyses. Digital images are matrices in which a point in the image is identified by lines and columns. The basic elements of this matrix are called pixels or image elements. Each pixel reports the intensity of the red, green, and blue colors, which are the three primary colors that produce different types of colors [28].

One of the software used is Photometrix®, which is used for image processing in the quantification of chemical substances. This application was developed by Professor Gilson A. Helfer and collaborators at the University of Santa Cruz do Sul in Rio Grande do Sul [29].

Use of chemometric tools

One of the most important tools in the current development of analytical methods is the design of experiments, which helps to reduce the high variability of results, analysis times and the costs involved [30]. Routine applications of chemometric methods flow from analytical chemistry literature, such as in extraction methods, since there are several influential factors in this process.

Experimental planning seeks both to describe the experiment and to explicitly identify inferences about the causes of the process and/or the relationships of the conditions. This allows us to infer what we produced or contributed to these events. While several approaches can be used during experimental planning, the best approach is selected based on the type of evaluation or response. Thus, planning can be carried out using the factorial model, the fractional factorial, the Doehlert, the central compound (Central Composite Design - CCD), the Box-Behnken (Box-Behnken Design - BBD), among other approaches [31].

Principal component analysis (PCA) is a widely used chemometric technique in analytical chemistry that utilizes pattern recognition without supervision. Thus, PCA was used in this work to identify cluster formation, enabling the detection of anomalous samples [32]. In addition, PCA forms the basis for numerous processes of classification, pattern recognition and multivariate calibration [33].

PCA is used for classification, pattern recognition, and multivariate calibration processes [33]. PCA derives the analysis of multi-point principal components (multiway principal component analysis - MPCA). Thus, MPCA is an extension of PCA for data sets with a high degree of complexity, in which the data cube X is unfolded in a matrix X (i, j, k). PCA is a pattern recognition technique without supervision that allows the identification of natural cluster formation and the detection of anomalous samples [32]. Another technique applied in this work was partial least squares regression (PLS), a technique that reduces predictors to a smaller set of non-correlated components and performs least squares regression for these components in place of the original data. PLS regression is particularly useful when predictors are highly collinear or when there are more predictors than observations Because PLS does not assume that predictors are fixed, as opposed to multiple regressions, predictors can be measured in error, making PLS more robust to measurement uncertainty [34].

Thus, this work seeks to design and optimize μPAD systems for the detection and quantification of cocaine. For this study, six factorial experimental designs with central points were developed using three different factors: spot diameter (6, 8 and 10 mm), cobalt II thiocyanate content (50, 75 and 100% in relation to a standard commercial supply provided by the Civil Police of the state of ES, CP - ES), and concentration of cocaine hydrochloride (4, 6 and 8 mg mL^{-1}), where a different sample exposure time was used to assess the stability of the system produced (t = 0, 0.5, 1, 12, and 24 h). With the aid of analytical tools such as ANOVAs, Pareto Graphs, and Response Surfaces, the factors will be optimized to determine the ideal point for the analysis. The light intensity recorded by the Photometrix® application will be compared to an analytical curve with nine points of known concentrations. In addition, chemometric tools such as PCA and PLS will be used to group and predict the cocaine hydrochloride content present in seized samples.

MATERIALS AND METHODS

Materials and reagents

The MPADs were built with the aid of a ColorQube 8880 wax printer on a sheet of A4 paper. The standard solutions were prepared from 100 mg of cocaine (standard produced by the Federal Police of Rio Grande do Sul). The photos were generated with a Samsung J8 Smartphone with a Photometrix® application. In total, 60 street samples were analyzed, including 30 of cocaine and 30 of crack.

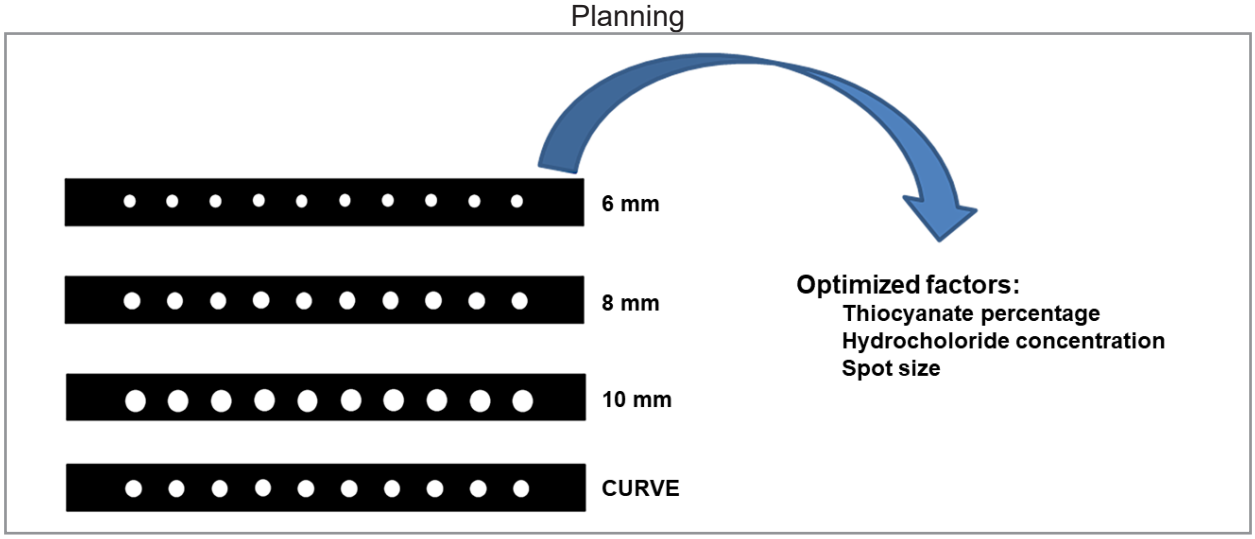


Figure1. Optimization of µPADs.

µPADs were designed with the help of the Windows power point program. µPADs of three different diameters – 6, 8 and 10 mm – were designed, since this was one of the factors studied with experimental planning. The ColorQube 8880 printer was used to print µPADs on standard A4 chamex sheets. After printing, the sheet with the wax was fully impregnated with wax by placing the µPADs in a greenhouse at 120 °C for 5 minutes.

The standard solutions for the analytical curve for cocaine hydrochloride were prepared. Exactly 100 mg of sample was weighed and dissolved in 10 mL of ultrapure water. Using the prepared 10 mg mL^{-1} stock solution, parts of the solution were diluted to prepare eight additional standards of different concentrations – 9, 8, 6, 5, 4, 3, 2 and 1 mg mL^{-1} – which were used to construct the analytical curve. It is worth mentioning that constructing the analytical curve is important because it is another factor studied in planning – the influence of the cocaine hydrochloride concentration on the efficiency of the sensitivity of µPADs. The third factor studied was the percentage of cobalt thiocyanate II, $[Co(SCN)_2]$ added that could react with the drug. The original test uses the solution provided by the cooperation agreement, process 23068.011398/2012-72, with the CP-ES. The study also assessed if the thiocyanate concentration increases the selectivity of the technique for cocaine. In this work, we tested 50, 75 and 100% values relative to the standard solution provided by PC-ES [35].

With the prepared µPADs and the cocaine hydrochloride and $[Co(SCN)_2]$ solutions, the next step was the construction of the experimental design. A factorial design was chosen, since there was no understanding of how each factor would influence the model. A total of six experiments were carried out. In the first, the factors chosen for planning were cocaine hydrochloride concentration, $[Co(SCN)_2]$ concentration and the diameter of the µPAD, keeping the detection time fixed. In the first experiment, the detection time was defined as t = 0 s, and the colorimetric test images were acquired immediately after the experiment. For the other five time periods, the waiting time factor was treated in a unidimensional way with values of 30 min, 1 hour, 12 hours and 24 hours – waiting for the acquisition of images and analysis in the Photometrix® application.

A factorial design was set up with three factors, totaling eight experiments, with three experiments added to the central point, totaling 11 experiments (see Table I).

Table I. Experimental factorial design with 3 factors

Test	Factor 1	Factor 2	Factor 3	Answer*
1	100	10	8	
2	50	10	8	
3	100	6	8	
4	50	6	8	
5	100	10	4	
6	50	10	4	
7	100	6	4	
8	50	6	4	
9	75	8	6	
10	75	8	6	
11	75	8	6	

Factor 1 (% of thiocyanate), Factor 2 (Diameter (mm)) and Factor 3 (Cocaine Hydrochloride ($mg \cdot mL^{-1}$)). Answer * is the accuracy of the model in prediction of the cocaine concentration obtained by predicting whether the application curve matches the actual sample concentration.

Each experiment used 3 µL of cocaine hydrochloride solution and 3 µL of Cobalt II Thiocyanate solution provided by CP-ES. For example, in experiment 1, 3 µL of 100% of the $[Co(SCN)_2]$ solution was pipetted, followed by waiting 5 minutes for the total and uniform impregnation of the µPAD. Next, 3 µL of hydrochloride solution was applied at a concentration of 8 mg mL^{-1}. The other experiments were carried out using the same procedures. Following this, six experiments were built including one containing four factors, with the last factor being the waiting time for the response, which is used to determine the cocaine concentration using the Photometrix® app. In the other five schedules, the waiting time factor was treated in a unidimensional way with its waiting values – t = 0 s, 30 min, 1 hour, 12 hours and 24 hours – for analysis with the Photometrix®.

A PLS model was constructed using three different levels of concentrations and nine levels on the analytical curve. Since the concentrations of the solutions were known, the efficiency/accuracy – comparison between the measured and real values – was used as a response for each experiment performed, thus obtaining five plans.

Analyzed samples

With the planning completed and the analytical curve constructed, we analyzed the samples seized by the CP-ES, which included cocaine (n =30) and crack (n = 30) samples.

Each sample was processed using the same procedure, in which a mass of 30 mg was dissolved in 2 mL of ultrapure water. After the construction of the µPADs with 8 mm in diameter, 3 µL was pipetted of 100% $[Co(SCN)_2]$ solution, where a total and uniform impregnation of the MPAD was carried out for five

minutes, and then 3 µL of solution was applied for each unknown sample. After 1 hour, the Photometrix® software was used to build the PLS model and predict the cocaine hydrochloride content in each sample.

Finally, a PCA analysis was performed in order to determine the similarity between the seized samples of cocaine and crack.

Photometrix

To analyze the data, the Photometrix® software developed by the chemometrics research group of the University of Santa Cruz do Sul (RS, Brazil) was used. This software is available for free in the Android® and IOS® application store (http://www.photometrix.com.br). The software contains multivariate analysis tools and allows the user to perform principal component analysis (PCA), partial least squares regression (PLS), and hierarchical cluster analysis (HCA).

The application interface includes univariate and multivariate analyses. In the configuration interface, the user can determine the region of interest, camera settings, and register an email to export the data.

The PCA analysis method performed by the application was rapid and easy to carry out, with a few steps to the final data and without the need for image processing to interpret the results. For the construction of the graphs, channels R, G, B, H, S, V, L, and I was established with data centered on the mean.

To perform this test, the Samsung smartphone model J8 (16-megapixel camera, aperture F 1.7, digital stabilization, 4032x3024 pixel resolution and Android 8.1 operating system) was used. The samples were deposited in the MPADs followed by the reagents. After the pre-established time, a photo was taken with the µPADs nailed to a white background. Then the data was processed.

RESULTS AND DISCUSSION

Experimental plans

The experimental planning satisfied the imposed needs, allowing us to evaluate the best conditions to analyze the optimum concentration of cocaine hydrochloride. A total of six experiments were carried out as a result of five different times and a sixth planning in which the time was a fourth influential factor in the planning.

The analytical curve was constructed using the efficiency of the response obtained by the PLS model (Figure 5). Since the concentrations used at the three levels were known, the precision of the method – that is, how close the actual concentration is to the model – was used as a response for the experiments. This analysis was based on the modified Scott test, a forensic screening test widely used to identify cocaine hydrochloride, which was created by Scott in 1973 and modified by Fasanello and Higgins for analyzing crack samples. Scott's test, equation 1, is performed using a solution of [Co $(SCN)_2$] in an acid medium, which produces a blue-colored cobalt II complex in the presence of cocaine hydrochloride [35].

$$Co^{2+} + 4SCN^- + 2B: \leftrightarrow [Co(SCN)_4B_2]^{2-} \qquad \text{Equation 1}$$

Pink **Blue**

Of the points analyzed, 3 µL was used from a 1 mg mL^{-1} solution, demonstrating that it is possible to produce a qualitative response to 3 µg of cocaine hydrochloride sample at low cost. Figure 5 shows the analytical curve of the analysis.

With the result of the calibration, tables can be constructed with responses for each experiment (supplementary material). Pareto graphs (Figure 1S), ANOVAs (Table 1S), and response surfaces (Figures 2S and 3S) were constructed with the values obtained.

The ANOVAs assessed the fit of the models as well as the predictability of the models (supplementary material Table 1S). Two models with three factors with immediate waiting times (t = 0 min) and (t = 30 min) showed a lack of adjustment (supplementary material, Table 1S – A and B). Thus, these models were discarded for analysis. Therefore, only other models were used in the study.

The study was carried out based on the experimental design with t = 1 hour, since the other times make the analysis unpracticable for a routine exam. However, the plans for t = 12 h and 24 h revealed that the image coloring became stable after a certain amount of time and was not influenced after t = 1 h. However, μPADs can be stored for a longer time, making their use very versatile for possible counter-proof. This was demonstrated through the factorial planning of four factors, which confirmed that time is not an influential factor in the efficiency of the model's response (Pareto graph, Figure 2).

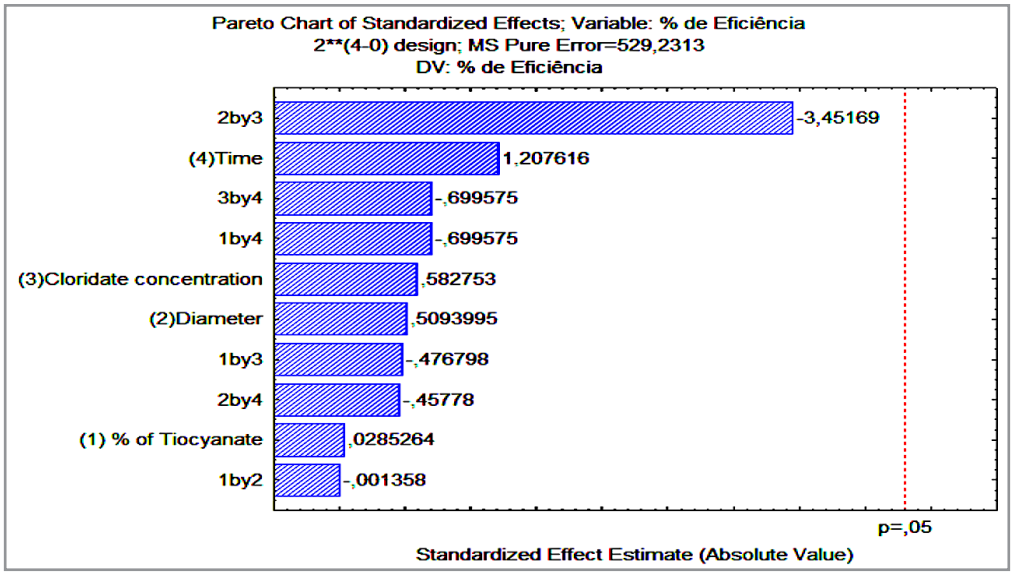


Figure 2. Pareto chart of planning with four factors.

The Pareto chart (supplementary material Figure 1S C – with t = 1 h) shows that the only influential factor is the concentration of hydrochloride in the sample in a negative form. It is due to its low concentration values, thus influencing, the photo resolution obtained that is directly proportional to the amount of revealed substance.

Thus, determining the response surfaces (Figures 2S and 3S of the supplementary material) were helpful in choosing the optimal points of the model (Figure 3). As described, the choice of the ideal points for the analysis was decided by observing the best efficiencies for each factor, which are found around the central points towards the ends of the model.

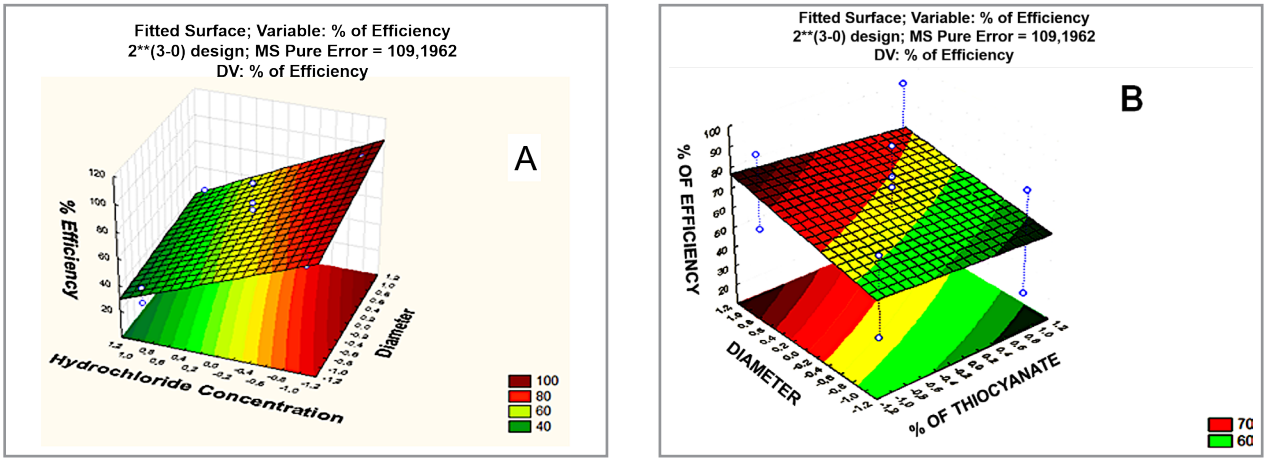


Figure 3. Response surfaces of hydrochloride concentration (A) *versus* spot diameter; and (B) spot diameter *versus* % cobalt thiocyanate II.

With the analytical parameters of μPADs defined, including diameter information (8 mm), the percentage of the cobalt thiocyanate II solution (100%), and a response time of 1 h, the quantification of cocaine hydrochloride in seized street samples (n = 60) was carried out. It is worth mentioning that for the use of the technique in the field, all this construction of MPADs is only necessary once, and can be updated over time in order to make the analysis more accurate. For daily use, it is only necessary to transport the printed sheet, being activated and already impregnated with the Cobalt II Thiocyanate reagent [$Co(SCN)_2$]. Thus, the qualitative result is instantly generated with the gain of the preliminary quantitative analysis, in case of using the PLS model previously loaded in the application already installed on any *smartphone*.

Seized samples

The methodology used for the analyses was similar to the methodology previously discussed. All samples analyzed (n = 60) showed a positive response for the cocaine hydrochloride identification (Figure 4), and in some cases, the less intense bluish color is apparent (samples C16, C33, C35, and C44, for example) [13].

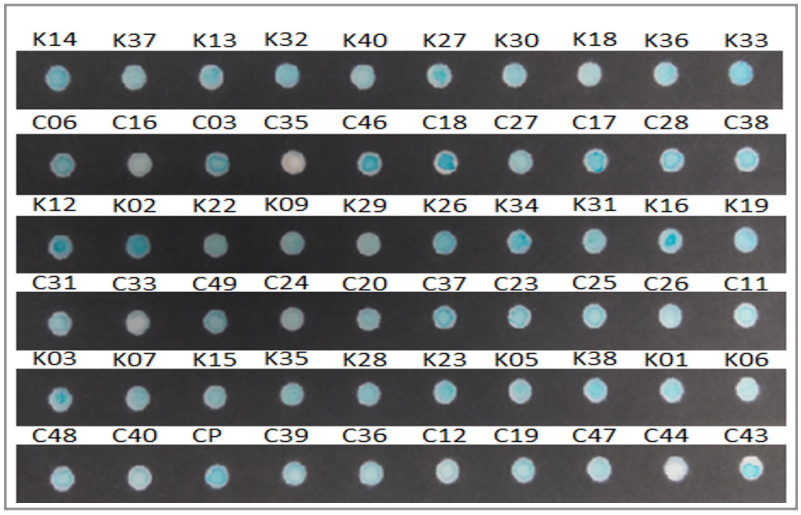


Figure 4. Result of the modified Scott test using the MPADs devices for seized samples of cocaine and crack.

The presence of a blue color confirms that the qualitative test has a good efficiency. According to the SWGDRUG recommendations, colorimetric tests are classified as C class, being recommended as preliminary test. Therefore, it is necessary to perform other chemical analysis that are classified as A or B (NMR, GC-MS, LC-MS, etc.). However, the positive result for the colorimetric analysis allows to conclude negative results, being valid as an initial proof and opening precept for flagrante delicto. Traditional colorimetric analysis requires cobalt thiocyanate II reagent and is normally applied to solid samples, being difficulty its detection in dark liquids such as wines and grape juices. Hence, the technique presented herein uses volumes so small of the analyte that even when dissolved, the contrast with the white of the leaf allows to obtain visually positive results. Thus, the main gain for the insertion of this analysis in the forensic scope with a simple preparation, having the possibility of preliminary identification even the samples being dissolved in dark liquids [36]. Each spot of μPAD consumes only 3 μL of aqueous solution, with virtually no sample loss.

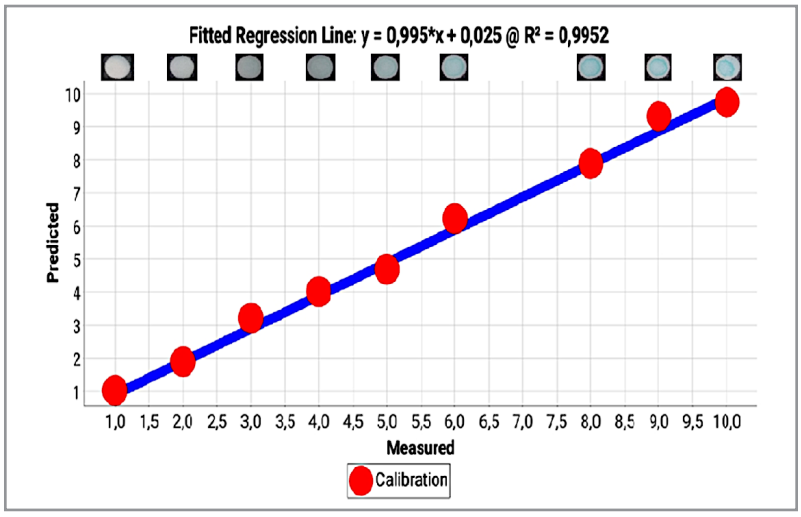


Figure 5. Cocaine hydrochloride analytical curve constructed by PLS.

The analytical curve and construction of a regression model for PLS was calculated (Figure 5). The curve produced a determination coefficient, R^2, of 0.98246, in addition to a RMSEC (root mean squares error calibration: mean square error of the calibration) of 0.39480. The analytical curve generated a regression equation: y = 0.995 * x + 0.025 with a LOD and LOQ of 0.09 mg mL^{-1} and 0.30 mg mL^{-1}, respectively [37].

For the crack samples, the hydrochloride concentrations ranged from 2.5 to 60.8 wt% with a mean average of 21.3 ± 13.3 wt% (Table II).

Table II. Hydrochloride concentrations (mg mL^{-1} and wt%) in crack samples

Sample	(mg mL^{-1})	wt%	Sample	(mg mL^{-1})	wt%
K14	9.12	60.8	**K26**	1.35	9.00
K37	6.14	40.94	**K34**	2.96	19.74
K13	4.03	26.87	**K31**	2.86	19.07
K32	6.81	45,40	**K16**	2.34	15.60
K40	3.59	23.94	**K19**	1.29	8.60
K27	6.89	45.94	**K03**	2.08	13.87
K30	4.41	29,40	**K07**	2.70	18.00
K37	6.14	40.94	**K34**	2.96	19.74
K18	2.63	17.54	**K15**	1.94	12.94
K36	5.06	33.74	**K35**	1.51	10.07
K33	5.73	38.20	**K28**	2.83	18.87
K12	1.76	11.74	**K23**	2.32	15.47
K02	0.37	2.47	**K05**	1.66	11.07
K22	2.03	13.54	**K38**	0.54	3.60
K09	2.79	18.60	**K01**	1.97	13.14
Average percentage = 21.30			Standard Deviation = 13.35		

For the seized cocaine samples, the hydrochloride content variated from 1.2 to 22.6 wt%, with an average cocaine hydrochloride percentage of approximately 14.19 ± 6.92 wt% (Table III), similar to values previously reported in the literature [13]. It is important to note that the hydrochloride values present in the seized samples may indicate cocaine as well as some impurities such as adulterants like lidocaine and phenacetin. Previous evidence has shown that the Scott test provides false-positive results for these two substances [38]. However, it is important to note that, like the traditional method of analysis, the technique applied in this work is not able to differentiate interfering substances. On other hand, colorimetry test in microfluids scale allows to produce qualitative and quantitative results simultaneously, with minor sample preparation, lower cost than the traditional one and being less environmentally invasive, since uses much less sample and is based on a sheet of paper.

Table III. Hydrochloride concentrations (mg mL^{-1} and wt%) in cocaine samples

Sample	(mg mL^{-1})	wt%	Sample	(mg mL^{-1})	wt%
C06	0.97	6.47	**C37**	1.19	7.94
C16	3.39	22.60	**C23**	2.95	19.67
C03	0.19	1.27	**C25**	3.00	20.00
C35	3.22	21.47	**C26**	3.11	20.74
C46	1.01	6.74	**C11**	2.16	14.40
C18	1.02	6.80	**C48**	2.19	14.60
C27	2.75	18.34	**C40**	2.92	19.47
C17	1.94	12.94	**CP**	1.23	8.20
C28	0.22	1.47	**C39**	1.56	10.40
C38	-0.13	-0.87	**C36**	3.04	20.27
C31	0.74	4.94	**C12**	2.79	18.60
C33	3.19	21.27	**C19**	2.33	15.54
C49	2.54	16.94	**C47**	3.25	21.67
C24	2.86	19.07	**C44**	3.07	20.47
C20	2.29	15.27	**C43**	2.87	19.14

Average percentage = 14.19 Standard Deviation = 6.92

Figure 6 shows a graph of the frequency of the concentration of hydrochloride for cocaine and crack samples, showing a higher concentration present in crack samples: 14 samples had concentrations ranging from 18 to 24 wt%, while cocaine samples, had concentrations ranging from 12 to 18 wt%, similar to street sample values previously reported [38].

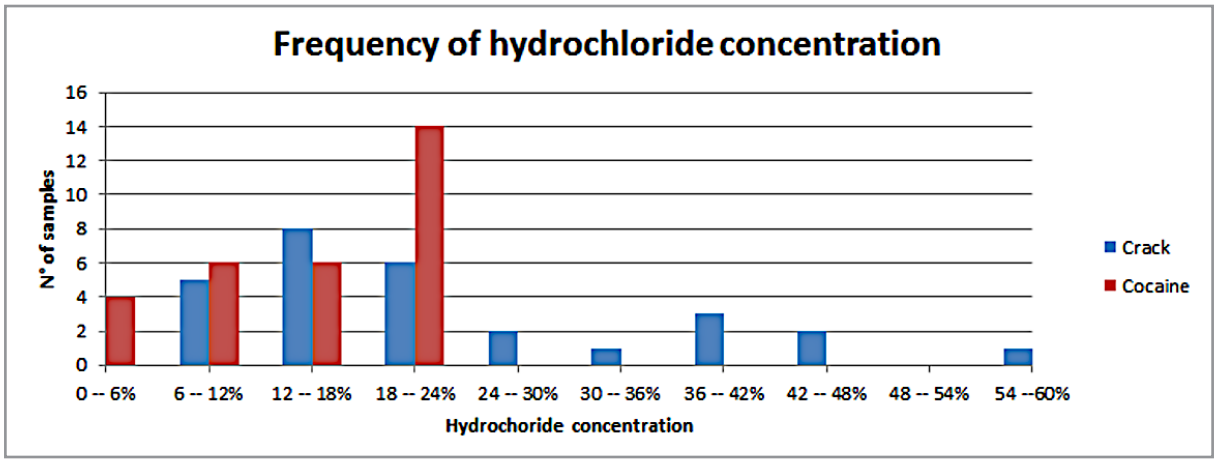


Figure 6. Frequency graph of hydrochloride concentrations for cocaine and crack samples.

Finally, PCA analysis was performed using 60 street samples, which were classified into two groups: cocaine (identified by "C") and crack ("K"; see Figure 7). Selection of the PC1 vs PC3 plot was based on the best visualization of separation of the groups. The K13, K27, K30, K32, K36, K37 and K40 samples (with [hydrochloride] from 27 to 45 wt%), which have a higher content of cocaine, were grouped together, analyzing the PC1>0 region. The first component separated the groups in an acceptable way and was responsible for explaining of more than 50% of variance; this component probably represents the hydrochloride content in the sample. The PC1 vs PC2 and PC2 vs PC3 plots did not clearly group the samples, therefore these plots are not shown.

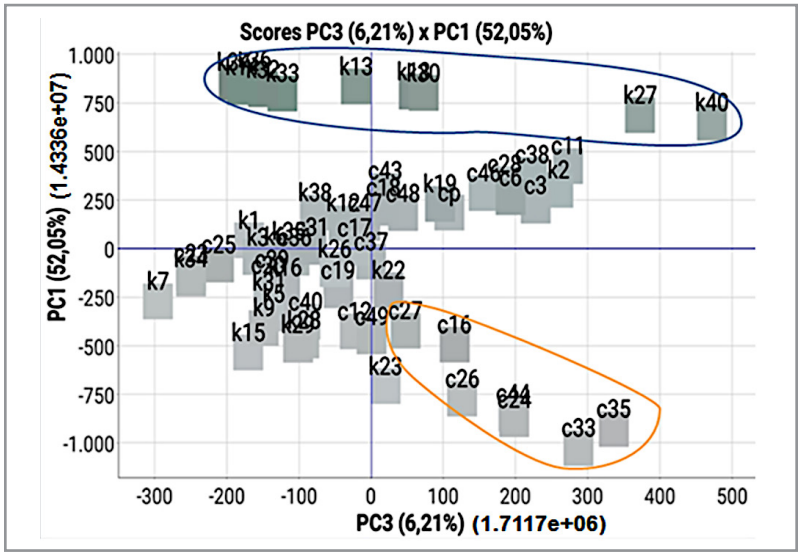


Figure 7. PC1 vs PC3 plot for cocaine and crack (represented by C_n and K_n symbols, respectively. With the loadings that explain 80.6% of the variance.

In general, many seized cocaine samples were distributed around the center of the PC1 vs PC3 plot, likely indicating a lower concentration of cocaine. On the other hand, cocaine samples located in the PC1<0 region (samples C16, C23, C24, C26, C33, C35 and C44, with [hydrochloride] ≈ 20% w/w) had similar analyte concentrations. Therefore, PC1 was capable of separating samples into three large groups based on the concentration of cocaine hydrochloride present.

CONCLUSIONS

In this work, choosing effective experimental designs saved hours of variation in factors, countless experiments as well as saving on reagents. The samples were preserved well, since there was a loss of approximately 3 µg per sample analyzed. The accuracy of the concentration can only be confirmed by cataloged analysis.

As our results show, it is possible to quickly classify samples as crack or cocaine. In addition, an analytical curve with R^2 greater than 0.98 with hydrochloride content variated from 1.2 to 22.6 wt%, with an average percentage of approximately 14.19 ± 6.92 wt% for street samples of cocaine. For crack samples, the hydrochloride concentrations ranged from 2.5 to 60.8 wt% with a mean average of 21.3 ± 13.3 wt%.

These findings demonstrate that MPAD is a useful approach for analyzing cocaine and crack samples. This analytical approach is still evolving, given the possibility of combining this approach with high-tech resources such as a *smartphone*. It is also important to note that the financial savings of the analysis is satisfactory, since the application is free and the possibility of up to 70 analyzes on a sheet of plain paper has already been proven, requiring only wax printing, at a standard cost of 25 cents per sheet, and activation in the greenhouse. In addition, the curves can be saved and incremented by new samples making the analysis more refined in the application, facilitating its application *in loco*. Thus, we present a quick, non-invasive analysis that allows the analysis of the substrate even dissolved in liquids.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank FAPES (EDITAL CNPq/FAPES Nº 23/2018 – Programa de Apoio a Núcleos Emergentes – PRONEM grant number 596/2018) and CNPq (422555/2018-5 and 305359/2017-7) for financial support. The authors would also like to thank the IFES (edital EDITAL PRPPG 07/2020) by funding the English grammar correction.

REFERENCES

1. United Nations, Brazil. Available at: https://nacoesunidas.org/29-milhoes-de-adultos-dependem-de-drogas-aponta-relatorio-do-unodc/ [Accessed 10 June 2019].
2. Rocha, W. W. F.; Leite, J. A.; Correia, R. M.; Tosato, F.; Madeira, N. C. L.; Filgueiras, P. R.; Lacerda Jr, V.; Freitas, J. C. C.; Romão, W.; Neto, A. C. *Anal. Methods*, **2018**, *10* (15), pp 1685-1694 (https://doi.org/10.1039/C7AY03000B).
3. United Nations Office on Drugs and Crime (UNODC). *World Drug Report, **2013***. Available at: http://www.unodc.org/unodc/secured/wdr/wdr2013/World_Drug_Report_2013.pdf [Accessed 30 March 2019].
4. United Nations Office on Drugs and Crime (UNODC), *World Drug Report*, **2017**. Available at: http://www.unodc.org/wdr2017/field/Booklet_1_EXSUM.pdf [Accessed 02 abril 2019].
5. Passagli, M. F. *Toxicologia Forense: teoria e prática*, 4th Edition. Editora Millennium, Campinas, **2013**.
6. Plowman, T.; Rivier, L. *Annals of Botany*, **1983,** *51* (5), pp 641-659 (https://doi.org/10.1093/oxfordjournals.aob.a086511).
7. Chitwood, D. D. Patterns and consequences of cocaine use. In: Kozel, N. J.; Adams, E. H. (Eds.) *Cocaine Use in America: Epidemiologic and Clinical Perspectives*. National Institute on Drug Abuse, NIDA Research Monograph 61, **1985,** pp 111-129.
8. Lesney, M. S. *Today's Chemist at Work*, **October 2004**, pp 15-16. Available at: https://pubsapp.acs.org/subscribe/archive/tcaw/13/i10/pdf/1004instruments.pdf [Accessed June 2021].
9. Romão, W.; Schwab, N. V.; Bueno, M. I. M. S.; Sparrapan, R.; Eberlin, M. N.; Martiny, A.; Sabino B. D.; Maldaner, A. O. *Quim. Nova*, **2011**, *34* (10), pp 1717-1728 (https://doi.org/10.1590/S0100-40422011001000005).

10. Sabino, B. D.; Romão, W.; Sodré, M. L.; Correa, D. N.; Pinto, D. B. R.; Alonso, F. O. M.; Eberlin, M. N. *Am. J. Anal. Chem.*, **2011**, *2* (6), pp 658-664 (https://doi.org/10.4236/ajac.2011.26075).
11. Calatayud, J.; González, A. *Anesthesiology*, **2003**, *98* (6) pp 1503-1508 (https://doi.org/10.1097/00000542-200306000-00031).
12. Goldstein, R. A.; DesLauriers, C.; Burda, A.; Johnson-Arbor, K. *Seminars in Diagnostic Pathology*, **2009**, *26* (1), pp 10-17 (https://doi.org/10.1053/j.semdp.2008.12.001).
13. Conceição, V. N.; Souza, L. M.; Merlo, B. B.; Filgueiras, P. R.; Poppi, R. J.; Romão, W. *Quim. Nova*, **2014**, *37* (9), pp 1538-1544 (https://doi.org/10.5935/0100-4042.20140240).
14. Martinez, A. W.; Phillips, S. T.; Butte, M. J.; Whitesides, G. M. *Angew. Chem., Int. Ed.*, **2007**, *46*, pp 1318-1320 (https://doi.org/10.1002/ange.200603817).
15. Terry, S. C.; Jerman, J. H.; Angell, J. B. *IEEE Trans. Electron Devices*, **1979**, *26* (12), pp 1880-1886 (https://doi.org/10.1109/T-ED.1979.19791).
16. Manz, A.; Graber, N.; Widmer, H. M. *Sens. Actuators, B*, **1990**, *1* (1-6), pp 244-248 (https://doi.org/10.1016/0925-4005(90)80209-I).
17. Lo, R. C. *Chem. Eng. Process Tech.*, **2013**. Available at: http://www.jscimedcentral.com/ChemicalEngineering/Articles/chemicalengineering-1-1002.pdf [Accessed June 2021].
18. Coltro, W. K. T.; Piccin, E.; Carrilho, E.; Jesus, D. P.; Silva, J. A. F.; Silva, H. D. T.; Lago, C. L. *Quim. Nova*, **2007**, *30* (8), pp 1986-2000 (https://doi.org/10.1590/S0100-40422007000800034).
19. Nguyen, N.-T.; Wereley, S. T.; Shaegh, S. A. M. *Fundamentals and Applications of Microfluidics*. Artech house, Norwood, MA, **2018**.
20. Martinez, A. W.; Phillips, S. T.; Butte, M. J.; Whitesides, G. M. *Angew. Chem., Int. Ed.*, **2007**, *46* (8), pp 1318-1320 (https://doi.org/10.1002/anie.200603817).
21. Araujo, W. R. *Development of electrochemical and colorimetric sensors for applications in forensic interest samples*. Doctoral thesis, **2016**, Institute of Chemistry, University of São Paulo, São Paulo, SP, Brazil (https://doi.org/10.11606/T.46.2016.tde-18082016-084906).
22. Garcia, P. T. *Development of colorimetric and electrochemical sensors for clinical and forensic applications*. Doctoral thesis, **2017**, Universidade Federal de Goiás, Goiânia, GO, Brazil (http://repositorio.bc.ufg.br/tede/handle/tede/8180).
23. Krauss, S. T.; Holt, V. C.; Landers, J. P. *Sens. Actuators, B*, **2017**, *246*, pp 740-747 (https://doi.org/10.1016/j.snb.2017.02.018).
24. Hidayat, M. A.; Maharani, D. A.; Purwanto, D. A.; Kuswandi, B.; Yuwono, M. *Biotechnol. Bioprocess Eng.*, **2020**, *25* (2), pp 255-263 (https://doi.org/10.1007/s12257-019-0299-8).
25. Balu, B.; Berry, A. D.; Hess, D. W.; Breedveld, V. *Lab on a Chip*, **2009**, *9* (21), pp 3066-3075 (https://doi.org/10.1039/B909868B).
26. Caivano, S.; Ferreira, B. J.; Domene, S. M. A. *Ciência & Saúde Coletiva*, **2014**, *19*, pp 1437-1446 (https://doi.org/10.1590/1413-81232014195.13932013).
27. Trinh, T. N. D.; Lee, N. Y. *Lab on a Chip*, **2019**, *19* (8), pp 1397-1405 (https://doi.org/10.1039/C8LC01389F).
28. Helfer, G. A.; Magnus, V. S.; Böck, F. C.; Teichmann, A.; Ferrão, M. F.; Costa, A. B. *J. Braz. Chem. Soc.*, **2017**, *28* (2), pp 328-335 (https://doi.org/10.5935/0103-5053.20160182).
29. Button, S. T. *Metodologia para Planejamento Experimental e Análise de Resultado*s. Mechanical Engineering Post Graduate Program Workbook, Mechanical Engineering Faculty, University of Campinas, **2005**. Available at: http://www.fem.unicamp.br/~sergio1/pos-graduacao/IM317/apostila.pdf [Accessed June 2021].
30. Neto, B. B.; Scarminio, I. S.; Bruns, R. E. *Como Fazer Experimentos: Pesquisa e Desenvolvimento na Ciência e na Indústria*. Bookman, Porto Alegre, **2010**.
31. Naef, R.; Jaquier, A.; Velluz, A.; Bachofen, B. *Chemistry & Biodiversity*, **2004**, *1* (12), pp 1870-1879 (https://doi.org/10.1002/cbdv.200490143).

32. Moniruzzaman, M.; Rodríguez, I.; Ramil, M.; Cela, R.; Sulaiman, S. A.; Ganc, S. H. *Talanta*, **2014**, *129*, pp 505-515 (https://doi.org/10.1016/j.talanta.2014.06.019).
33. Geladi, P.; Kowalski, B. R. *Anal. Chim. Acta*, **1986**, *185*, pp 1-17 (https://doi.org/10.1016/0003-2670(86)80028-9).
34. Caligiorne, M. S.; Marinho, A. P. *Revista Criminalística e Medicina Legal*, **2016**, *1* (1), pp 34-45.
35. Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO). De Acreditação, Coordenação Geral. *Orientação sobre validação de métodos analíticos*. Documento de carácter orientativo, **2010**. Available at: http://www.inmetro.gov.br/credenciamento [accessed June 2021].
36. Silva, G. R. *Perfil de drogas de abuso apreendidas e admitidas no Instituto de Polícia Científica entre os meses de janeiro a novembro de 2017*. Trabalho de Conclusão de Curso, **2018**, Centro de Ciências da Saúde, Universidade Federal da Paraíba, João Pessoa, PB, Brasil (https://repositorio.ufpb.br/jspui/handle/123456789/17642).
37. Watson, S.; Chandler, N.; *Howstuffworks: Manufacturing crack cocaine*. Available at: https://science.howstuffworks.com/crack2.htm. [Accessed 28 January 2020].
38. Kiefer, S. *Estudo mostra que cocaína comercializada em Minas é a mais "batizada" do Brasil*. Estado de Minas Gerais, Belo Horizonte, 04/04/**2014**. Available at: https://www.em.com.br/app/noticia/gerais/2014/04/04/interna_gerais,515369/estudo-mostra-que-cocaina-comercializada-em-minas-e-a-mais-batizada-do-brasil.shtml [Accessed 25 July 2020].

SUPPLEMENTARY MATERIAL

Table 1S. ANOVA of the six statistical models: Immediate (a), 30 min (b), 1 hour (c), 12 hours (d), 24 hours (e) and time as variable (f), respectively.

Factor	SS	df	MS	F	p
(1) % Thiocyanate	244.76	1	244.76	5.11	0.15
(2) Diameter	4.13	1	4.13	0.08	0.8
(3) Hydrochloride Concentration	2664.5	1	2664.5	55.63	0.02
1 by 2	144.5	1	144.5	3.01	0.22
1 by 3	453.76	1	453.76	9.47	0.09
2 by 3	79.69	1	79.69	1.66	0.33
Lack of Fit	2784.96	2	1392.48	29.07	0.03
Pure Error	95.79	2	47.89		
Total SS	6472.1	10			**A**

Table 1S. ANOVA of the six statistical models: Immediate (a), 30 min (b), 1 hour (c), 12 hours (d), 24 hours (e) and time as variable (f), respectively. (Continuation)

Factor	SS	df	MS	F	p
(1) % Thiocyanate	10.99	1	10.99	0.37	0.60
(2) Diameter	1474.92	1	1474.92	49.15	0.02
(3) Hydrochloride Concentration	295.55	1	295.55	9.85	0.09
1 by 2	57.11	1	57.11	1.90	0.30
1 by 3	16.89	1	16.89	0.56	0.53
2 by 3	7773.49	1	7773.49	259.05	0.004
Lack of Fit	2392.73	2	1196.36	39.87	0.02
Pure Error	60.01	2	30.01		
Total SS	12081.69	10			**B**

Factor	SS	df	MS	F	p
(1) % Thiocyanate	88.61	1	88.61	0.81	0.46
(2) Diameter	310.94	1	310.94	2.85	0.23
(3) Hydrochloride Concentration	5043.85	1	5043.85	46.19	0.02
1 by 2	5.08	1	5.08	0.05	0.85
1 by 3	254.53	1	254.53	2.33	0.27
2 by 3	26.74	1	26.74	0.24	0.67
Lack of Fit	1342.52	2	671.26	6.15	0.14
Pure Error	218.40	2	109.20		
Total SS	7290.65	10			**C**

Factor	SS	df	MS	F	p
(1) % Thiocyanate	67.57	1	67.57	0.19	0.70
(2) Diameter	47.53	1	47.53	0.13	0.75
(3) Hydrochloride Concentration	2701.12	1	2701.12	7.68	0.11
1 by 2	540.38	1	540.38	1.54	0.34
1 by 3	4.13	1	4.13	0.01	0.92
2 by 3	1188.28	1	1188.28	3.38	0.21
Lack of Fit	1993.22	2	996.61	2.83	0.26
Pure Error	702.87	2	351.44		
Total SS	7245.11	10			**D**

Table 1S. ANOVA of the six statistical models: Immediate (a), 30 min (b), 1 hour (c), 12 hours (d), 24 hours (e) and time as variable (f), respectively. (Continuation)

Factor	SS	df	MS	F	p
(1) % Thiocyanate	450.00	1	450.00	0.56	0.53
(2) Diameter	569.53	1	569.53	0.71	0.49
(3) Hydrochloride Concentration	625.69	1	625.69	0.79	0.47
1 by 2	1075.32	1	1075.32	1.35	0.36
1 by 3	2295.03	1	2295.03	2.88	0.23
2 by 3	55.12	1	55.12	0.07	0.82
Lack of Fit	260.97	2	130.48	0.16	0.86
Pure Error	1591.61	2	295.80		
Total SS	6923.28	10			**E**

Factor	SS	df	MS	F	p
(1) % Thiocyanate	0.43	1	0.43	0.0008	0.98
(2) Diameter	137.33	1	137.33	0.26	0.66
(3) Hydrochloride Concentration	179.73	1	179.73	0.34	0.62
(4) Thiocyanate Time	771.80	1	771.80	1.46	0.35
1 by 2	0.001	1	0.001	0.00	1.00
1 by 3	120.31	1	120.31	0.28	0.68
1 by 4	259.01	1	259.01	0.49	0.56
2 by 3	6305.35	1	6305.35	11.91	0.07
2 by 4	110.91	1	110.91	0.21	0.69
3 by 4	259.01	1	259.01	0.49	0.56
Lack of Fit	443.87	6	73.98	0.14	0.97
Pure Error	1058.46	2	529.23		
Total SS	9646.21	18			**F**

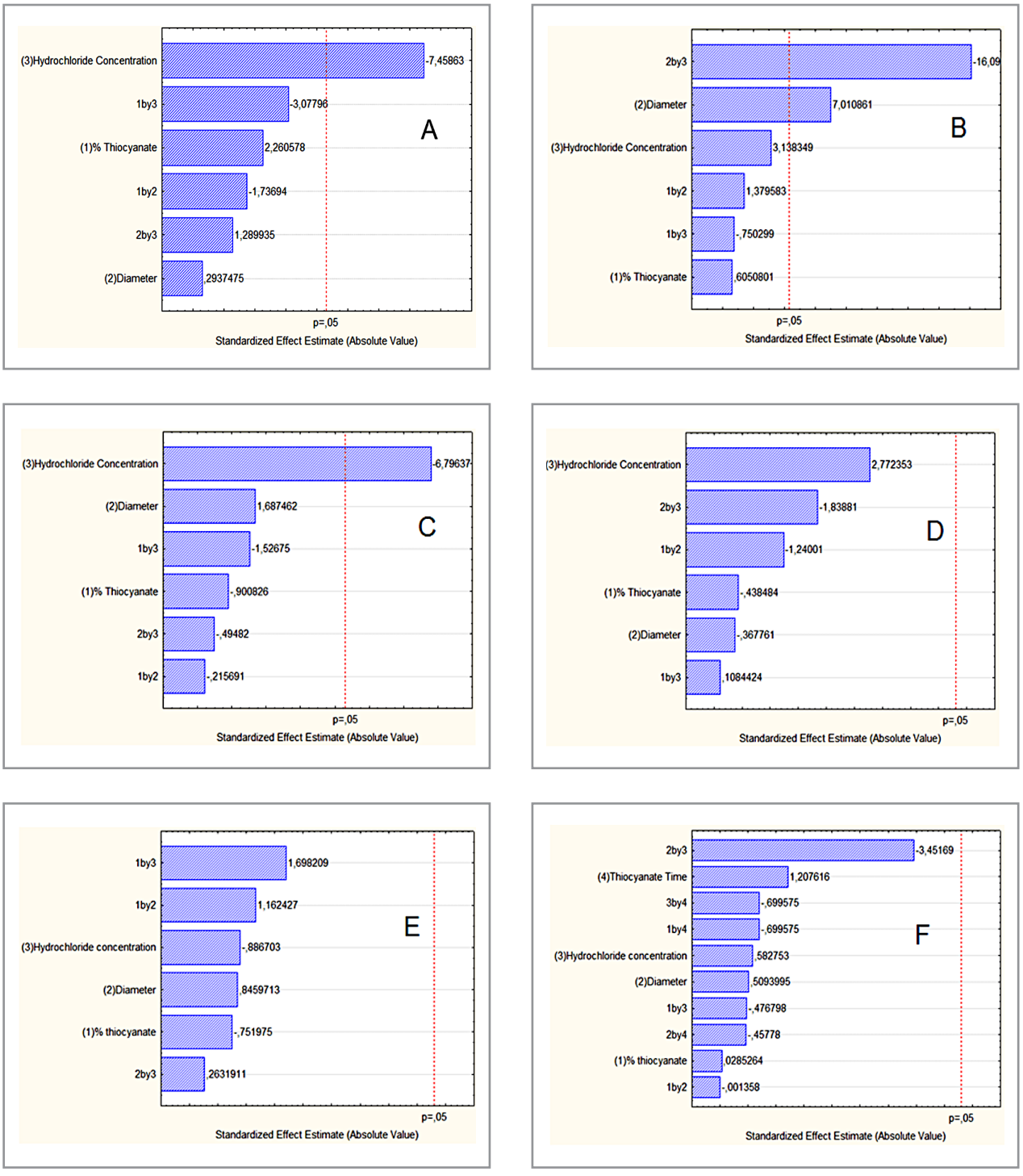


Figure 1S. Pareto charts of the six statistical models: Immediate (a), 30 min (b), 1 hour (c), 12 hours (d), 24 hours (e) and time as variable (f), respectively.

Response Surfaces Confronting Hydrochloride Concentration X Diameter

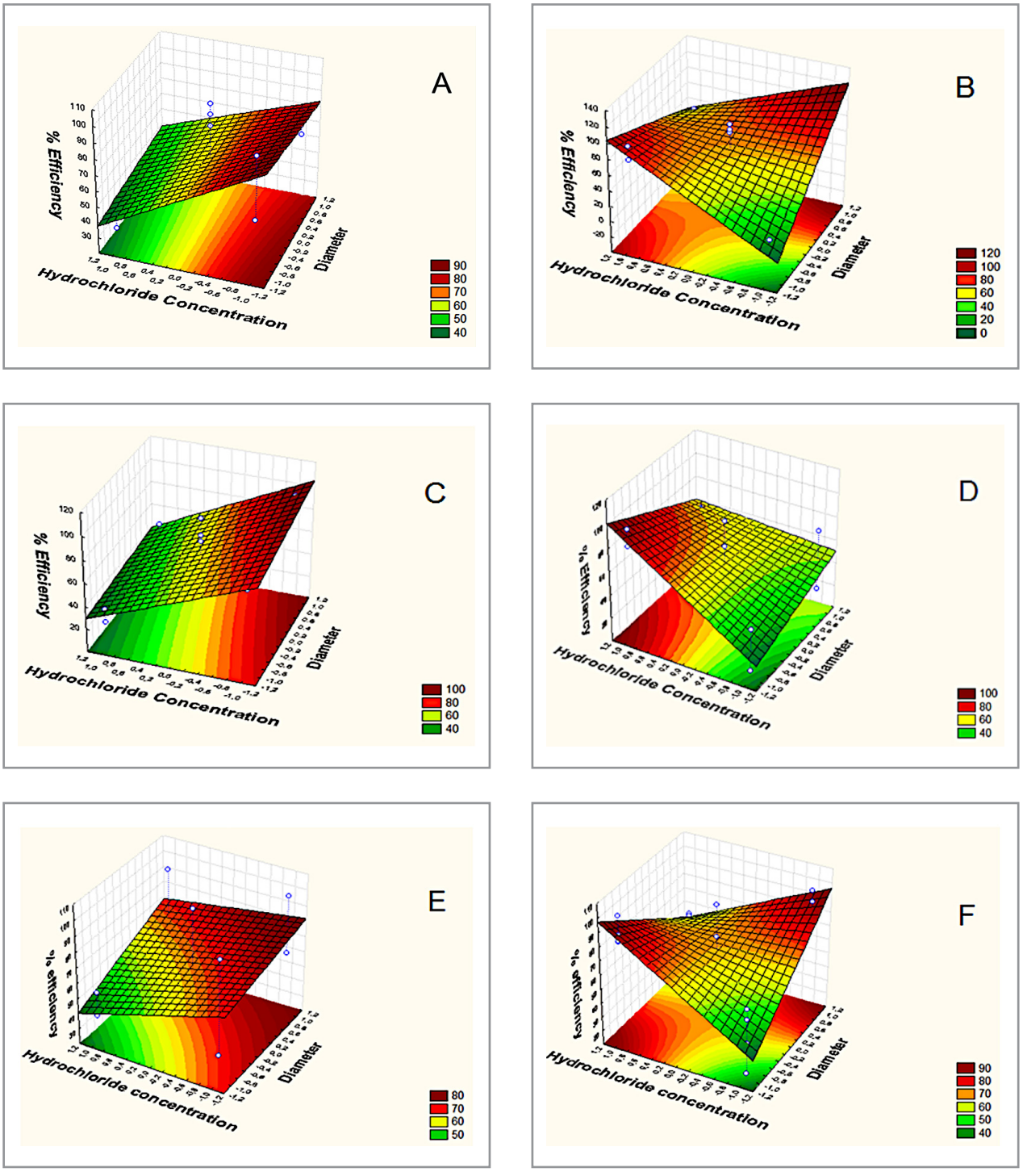


Figure 2S. Response surface graphs of the six statistical models: Immediate (a), 30 min (b), 1 hour (c), 12 hours (d), 24 hours (e) and time as variable (f), respectively.

Response Surfaces Confronting Hydrochloride Concentration X % Thiocyanate

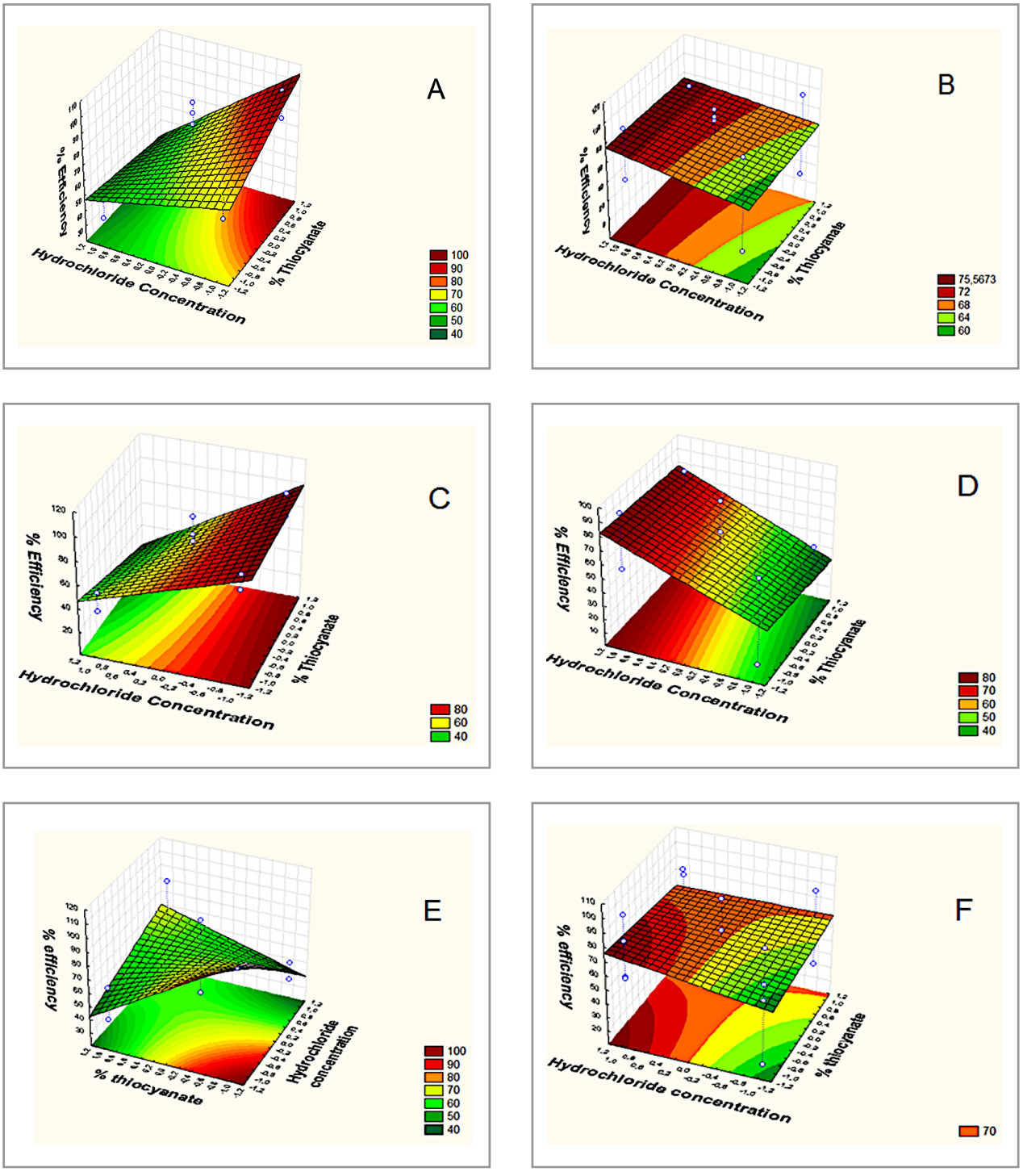


Figure 3S. Response surface graphs of the six statistical models: Immediate (a), 30 min (b), 1 hour (c), 12 hours (d), 24 hours (e) and time as variable (f), respectively.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 138-161
doi: 10.30744/brjac.2179-3425.AR-34-2021

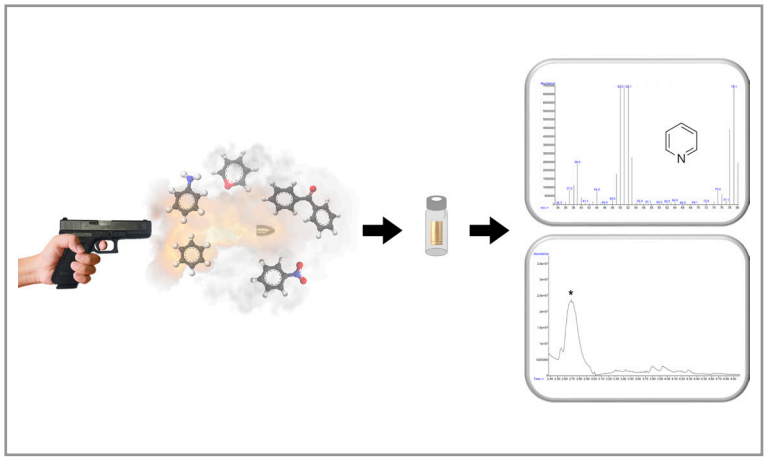
BrJAC

ARTICLE

Chromatographic Analysis of Byproducts from a Non-Toxic Ammunition and a Marked Ammunition: An Assessment of Toxicity

Aline Marcelino Arouca[1]*, Maurício Leite Vieira[2], Márcio Talhavini[2], Ingrid Távora Weber[3]

[1]*Instituto Federal de Brasília – IFB, Subcentro Leste, Complexo Boca da Mata, Lote 02, Samambaia Sul, 72302-300, Brasília, DF, Brazil*
[2]*Instituto Nacional de Criminalística, Polícia Federal, SAIS Quadra 07 Lote 23, 70610-200, Brasília, DF, Brazil*
[3]*Instituto de Química, Universidade de Brasília – UnB, P.O. Box 04478, 70904-970, Brasília, DF, Brazil*



One way to access the toxicity of a fired ammunition is by analyzing the byproducts generated by shooting. This work has analyzed compounds produced by firing non-toxic ammunition (NTA) by Gas Chromatography-Mass Spectrometry (GC-MS). In addition to standard NTA, NTA containing luminescent markers were also analyzed. Luminescent markers have been shown to be an excellent tool in the identification of Gunshot Residues (GSR) produced from NTA. As these markers are designed to tag NTA, they must not produce toxic byproducts. In this work, we focused on identification of volatile products that can be inhaled by shooter when firing and can represents risk to their health by acute and chronic exposition. For the NTA ammunition several toxic compounds, such as benzonitrile and naphthalene were found. They were related to de degradation of explosives, sensitizers, stabilizers, and other materials added to the gunpowder, indicating possible toxicity by shooters' long exposure. Moreover, as some of the compounds found are classified as GSR indicators, the used methodology could be adapted for GSR identification. Besides the compounds identified in NTA samples, in marked samples, pyridine and benzene were identified. Pyridine was provided by dipicolinic acid and benzene was provided by trimesic or terephthalic acid, all used as binder in the structures of the markers. However, it can be concluded that the possible toxicity of the NTA is mainly not altered by the presence of the markers because of the small amount of marker added to ammunition and because only a small part of the marker is degraded, requiring an unreal number of shots to produce some acute effect.

Cite: Arouca, A. M.; Vieira, M. L.; Talhavini, M.; Weber, I. T. Chromatographic Analysis of Byproducts from a Non-Toxic Ammunition and a Marked Ammunition: An Assessment of Toxicity. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 138–161. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-34-2021

Submitted 05 March 2021, Resubmitted 14 April 2021, Accepted 02 May 2021, Available online 19 May 2021.

Keywords: ammunition tagging, toxicity, non-toxic ammunition, luminescent marker, gas chromatography.

INTRODUCTION

The long-term exposure to gunshot residues (GSR) can be harmful to the health of frequent shooters, such as police and forensic experts, being the most known effect related to Pb-contamination [1]. After a series of studies [1–6] reported that frequent shooters had a high rate of lead in their blood, the ammunition industry developed heavy metals free ammunition (also called non-toxic ammunition, NTA). Despite the elimination of heavy metals represents an effective gain for the health of these professionals, the toxicity derived from other ammunition components, such as from degradation of the organic part of ammunition, has never been studied.

The GSR produced by NTA is hardly identified due to the lack of characteristic metals (Pb, Sb and Ba) or a unique chemical signature [7,8]. In fact, chemical markers are already being used for NTA in Germany [9]. In order to overcome this problem, luminescent markers has proven to be quite helpful in identifying the GSR and in analyzing the crime scene [10,11]. Interesting results were obtained using some Lanthanide Metal-Organic Frameworks (LMOFs) as luminescent markers in GSR. This materials exhibit chemical and optical signatures with high quantum yield and very defined emission spectra, which allow the unequivocal identification as LGSR (luminescent gunshot residues) by several nondestructive analytical methods [12].

In the last years a series of compounds [13–18] have been developed and tested in situations that simulated the routine analysis of forensic experts. The addition of luminescent markers to ammunition helped to identify the shooter, the weapon used, the location of the shooter and the distance at which the shot was fired [19–21], using only and UV lamp. Besides, LGSR was able to be visualized in different types of fabrics [22]. Furthermore, markers with different chemical and optical signatures can be used to mark specific batches of ammunition, allowing an ammunition coding system [23,24]. However, as the markers are intended primarily for NTA identification, luminescent markers are, naturally, expected to be non-toxic.

To access the toxicity of markers that could be used in NTA, Lucena *et al*. [25] evaluated the toxicity of the LMOF $_{\infty}$[Eu(BTC)] by acute oral test (OECD #432). This compound, that acted as an efficient luminescent marker in ammunition, was classified in the least toxic Globally Harmonized System (GHS) category (5), with a LD50 of 5000 mg kg^{-1}, which strongly suggests a wide security range for its application [25]. Also, Talhari *et al.* [26] evaluated the acute oral and inhalation toxicity of the MOF $_{\infty}$[Eu(DPA)(HDPA)] (OECD #432 and #436), another efficient luminescent marker for ammunition. Even when used in high doses (1 mg L^{-1} of air in the inhalation test and 2000 mg kg^{-1} of body weight in the oral test), the marker presented no toxic effect. Based on the acute oral test, it was also classified in GHS category 5 (which is the least toxic one), with a LD_{50} of 5000 mg kg^{-1} [26].

Although these markers have not been shown to be toxic, presenting a wide safety margin for oral and inhalation acute poisoning, all studies were conducted with markers itself, adapting some protocols usually related to drugs or biomaterials. Furthermore, since these markers are considered thermally and chemically stable, so far, no study has been carried out considering a possible degradation of part of the marker during the shot, as well as the possible byproducts of this degradation. In this way, additional studies are required to understand the toxicity aspects better, not only of the pure markers and of the LGSR generated by them, but also of the NTA-GSR itself, since the removal of heavy metals from NTA does not assures that toxic residues cannot be generated after the shot.

In this work, the byproducts found in cartridges of marked and non-marked NTA were studied. The byproducts are expected to derive from degradation of the gunpowder and stabilizers, and in the case of marked ammunition, from the degradation of the marker itself. With this, we expected to shed some light on possible toxic effects for frequent shooters due to continuous exposure to GSR as well as to investigate if the addition of Metal-Organic Framework (MOF)-based luminescent markers can alter the toxicity of the non-toxic ammunition (NTA).

MATERIALS AND METHODS

Luminescent markers

The LMOFs used as luminescent markers were hydrothermally prepared, using a microwave reactor and autogenous pressure, with a maximum power of 400 W (Monowave 300 Anton Paar), as described by Arouca *et al*. [20]. The following markers were synthesized: EuDPA ($_{\infty}$[Eu(DPA)(HDPA)]), EuBDC ($_{\infty}[Eu_2(BDC)_3(H_2O)_2]_n$) and EuBTC ($_{\infty}$[Eu(BTC)]), as shown in Figure 1. Each marker was individually added to a sample of NTA gunpowder (Clean Rage, CBC®) in the proportion of 10% wt, and ammunitions were reassembled for shots.

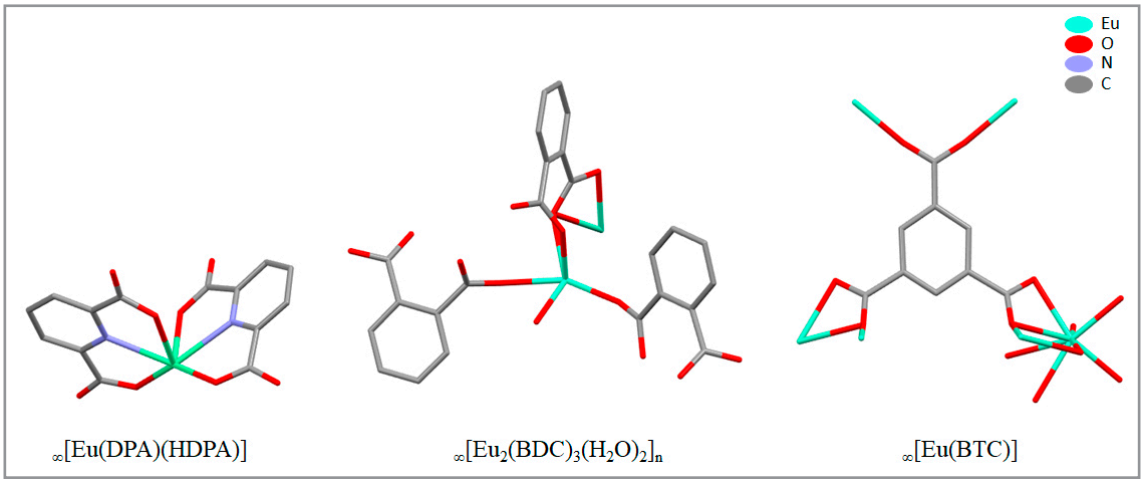


Figure 1. Asymmetric unit of the LMOFs: EuDPA, EuBDC and EuBTDC. The images were created using crystallographic information (CIF) from references [27–29], respectively.

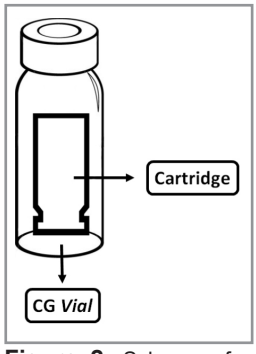


Figure 2. Scheme of a 9 mm cartridge in a GC vial to extract the volatile material after firing.

Sample collection

After shots, cartridges were immediately collected and kept in a 20 mL vial. The extraction of the volatile compounds was performed by headspace solid-phase microextraction (HS-SPME) using a 65 µm PDMS/DVB fiber (SUPELCO), as shown in Figure 2. Samples were collected from a single fired cartridge of NTA and a cartridge of marked-NTA. The samples were analyzed by gas chromatography - mass spectrometry (GC-MS) fitted with a single quadrupole mass analyzer. Two samples (replicates #1 and #2) of each marked ammunition and the pure NTA ammunition were collected.

SPME extraction

The SPME extraction was carried out in this sequence: incubation at 80 °C, at 600 rpm, for 3 min. The equilibration time was 16 min and the desorption in the injector occurred for 20 min.

GC-MS condition

The analyses were conducted using a mass spectrometer (Agilent model 5973), coupled to a gas chromatograph (Agilent model 6890N), with a phase capillary column poli(5% diphenyl / 95% dimethylsiloxane), dimensions 30 m x 0.25 mm x 0.25 µm (Rtxi-5ms RESTEK), equipped with CTC PAL multipurpose sampler, with the SPME module.

The injector temperature was maintained at 280 °C, in Splitless mode with Split valve closed for 4 min. The column was maintained with a constant flow of Helium for 1.3 mL min^{-1}. The chromatographic oven programming: initial temperature of 40 °C, maintained for 1 min and then heated at a rate of 10 °C min^{-1}

to 120 °C, remaining at this temperature for 1 min. Then the oven was heated at a rate of 5 °C min^{-1} up to 180 °C, maintaining at this temperature for 1 min, and finally, the oven was heated at a rate of 60 °C min^{-1} to 315 °C, maintaining this temperature for 25 min. The total analysis time was 50 min and 25 seconds.

The GC-MS interface was maintained at 280 °C, with the mass spectrometer operated in scan mode in the scan range from 30 to 500 *m/z*.

After the analysis, the chromatograms obtained for the marked ammunition were compared with those from NTA. Also, all mass spectra obtained at each chromatogram retention time were analyzed using the Chemstation Data Analysis program and the NIST Search program (version 2.0, NIST/EPA/NIH EI Mass Spectral Library).

Given the complexity of the matrix used (fired cartridge) and the fact that this is an exploratory study, we used only compounds that showed a match above 50% between the fragmentation pattern of the compound and the NIST data bank or those that were related to the degradation of the marker. For benzene and pyridine, standard solutions were used in other to determinate the retention time of each compound.

RESULTS AND DISCUSSION

NTA byproducts

To identify the byproducts produced after the shot of an NTA, the mass spectra found in the chromatograms were compared with the NIST data base. A large variety of compounds were identified. These variations in the same ammunition type were already expected and can be associated with the complexity of the matrix and the volatility of some compounds present in the cartridges. Ammunition is not a homogeneous “sample”, just as pistols are not precision instruments. So, changes in the “burning pattern” can be expected, resulting in the formation of different byproducts. In addition, small variations in the sampling time, ambient temperature, and humidity of the moment that the experiment was carried out can also lead to some variability in volatilization before the cartridge was placed in the vial. Despite this, some trends could be observed.

The following compounds were found in the pure NTA fired cartridge: glycidol (2.720–2.727 min), oxime-methoxy-phenyl (5.191 min), benzonitrile (6.668 min), naphthalene (9.992 min), 2,6-di-tert-butylbenzoquinone (15.703 min), 2,4-ditert-butyl-6-nitrophenol (20.058–20.445 min), N,N’-diethyl-N,N’-diphenylurea (24.059–24.452 min), as shown in Figure 3. N,N’-diethyl-N,N’-diphenylurea is used as stabilizer in the gunpowder [30], and naphthalene has already been described in GSR [31]. The other compounds found can be related to de degradation of explosives, sensitizers, stabilizers, flash inhibitors, plasticizers and others materials added to the gunpowder [32].

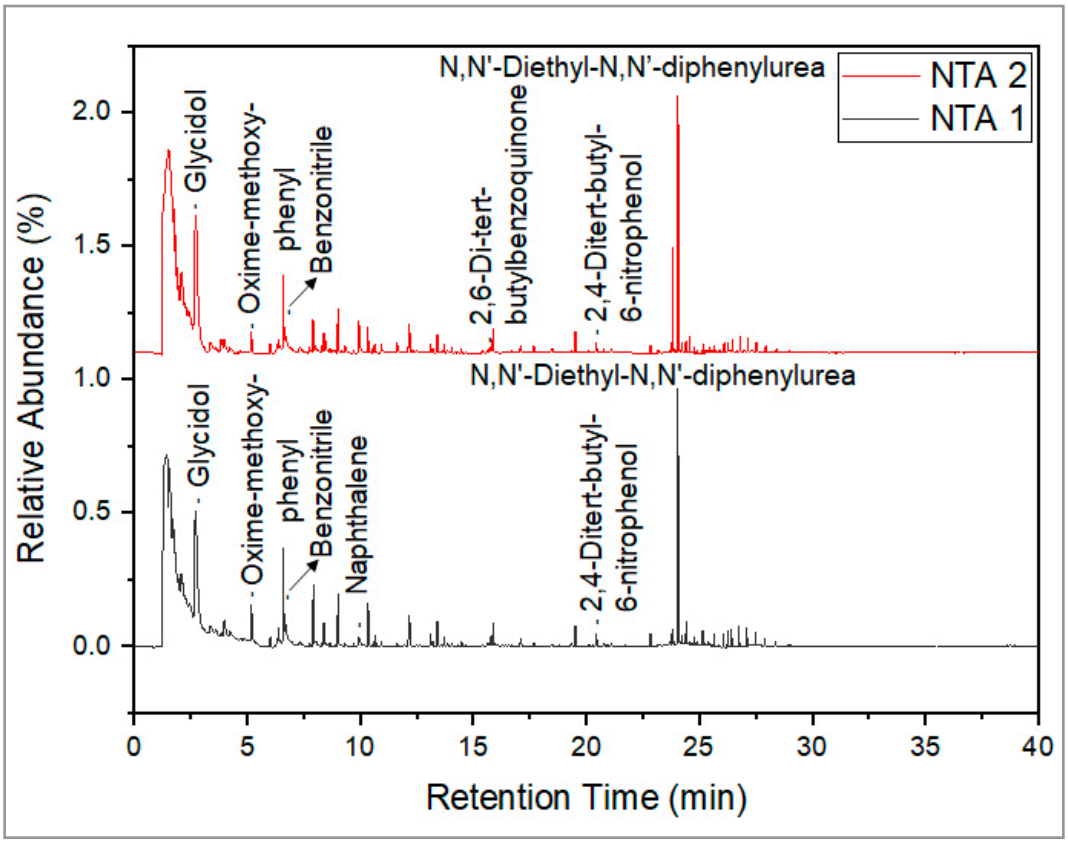


Figure 3. Chromatograms obtained from as-fired cartridge of pure NTA.

This result highlights an important aspect: despite being considered non-toxic, pure NTA also displays some hazardous byproducts after the shot. In fact, NTA is called as nontoxic ammunition because it is free from heavy metals. Nevertheless, it cannot be considered nontoxic because it produces some organic compounds that have known toxic effects. One of these compound found is naphthalene, a bicyclic aromatic hydrocarbon that is classified as Category 2B for as Carcinogenicity (possibly carcinogenic to humans) [33]. Carcinogenicity have been seen in rats studies, in which nasal tumors and non-neoplastic inflammatory changes has been identified [34]. In humans, naphthalene can cause hemolytic anemia with associated jaundice and cataract formation [34]. Also, form an acute exposure, hemolytic anemia and cataracts were observed [35].

Another compound found was benzonitrile. In humans, it was reported to cause severe respiratory distress, tonic convulsions, and periods of unconsciousness [36]. In animals, toxicity data are lethality and non-lethal effects in rats, mice, and rabbits [37].

Several other compounds are described as organic GSR by Goudsmits *et al.* [32], such as pyrene and anthracene, polycyclic aromatic hydrocarbons (PAH) classified as 3 and 2B, respectively, as well as benzyl nitrile, which have known toxic effects. Despite the presence of notoriously hazardous compounds derived from degradation of organic part of ammunition, a study about the toxicity of the organic GSR from both NTA or conventional ammunition (as was done with the Pb/Ba particles of the inorganic GSR) has not yet been carried out. The presence of the compounds listed above in the GSR may indicate that chronic exposure to vapors resulting from the firing of a NTA ammunition can produce toxic effects, requiring further studies.

LGSR byproducts

The study was focused on three previously studied MOF-based markers, in order to identify the presence of benzene in trimesic acid (H_3BTC) and in terephthalic acid (H_2BDC); or pyridine structures, in dipicolinic acid (H_3DPA). These three compounds (H_3BTC, H_2BDC and H_3DPA) are used as ligands (now on called BTC, BDC and DPA) to build up the LMOF structure (Figure 4). It is important to point out that these three markers have been studied before and have demonstrated potential as luminescent markers in ammunition (conventional and NTA) [19,20,24]. Moreover, EuBTC and EuDPA have been evaluated by oral or inhalation acute tests and shown as not harmful [25,26].

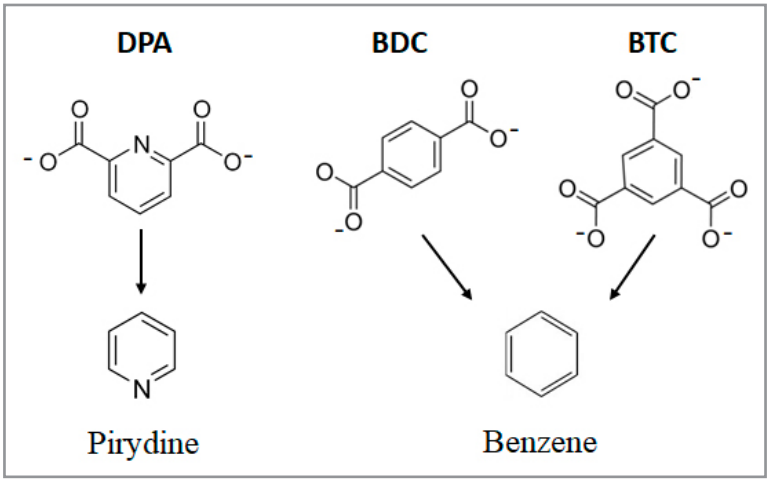


Figure 4. Ligands present in structures of tested markers and target byproducts.

For GSR-NTA marked with EuDPA, it was possible to observe, at 3.002–3.015 min, a peak referring to pyridine, as pointed in Figure 5. Also, less intense peaks corresponding to the following compounds were found: 2,4,7,9-tetramethyl-5-decyne-4,7-diol (14.357–14.362 min), 6-tert-butyl-4-ethyl-1,1-dimethylindan (19.155–19.156 min) and 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (24.104 min). Their structures are shown in Figure 6. Other compounds found in the NTA, such as N,N'-Diethyl-N,N'-diphenylurea and glycidol, were also found in the EuDPA´s chromatograms.

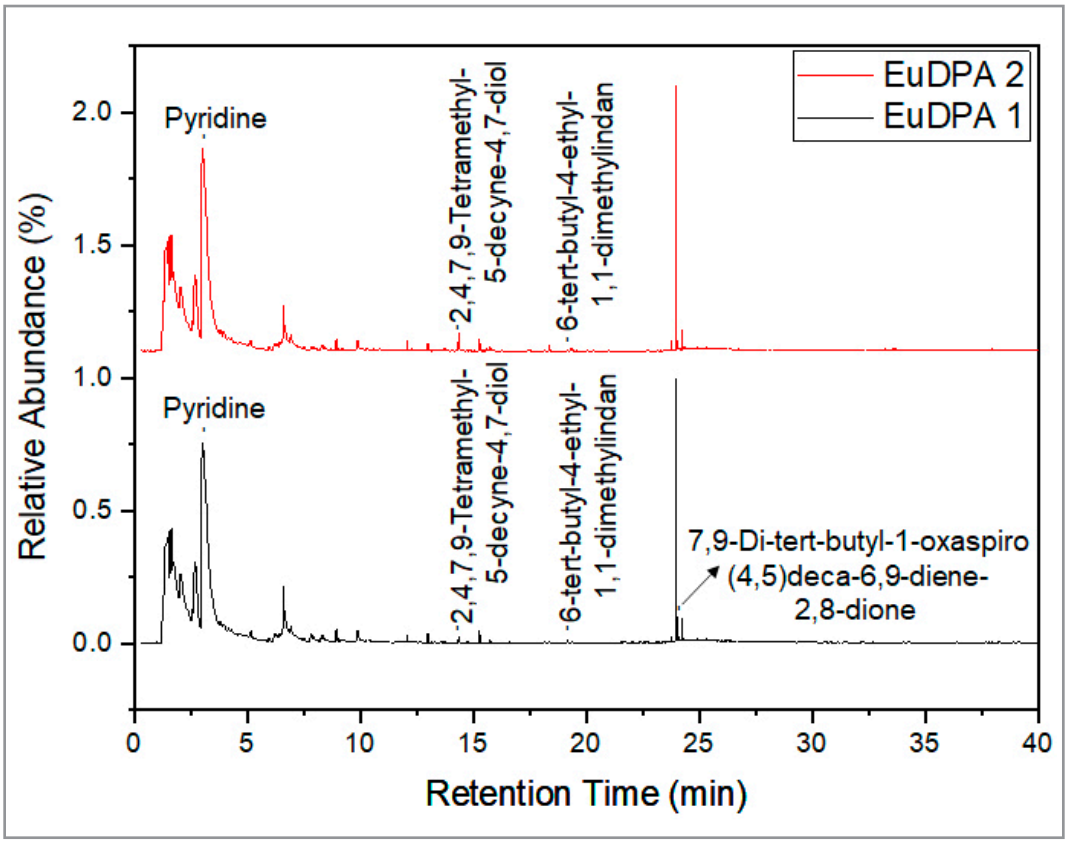


Figure 5. Chromatograms obtained from as-fired cartridge of NTA marked with EuDPA.

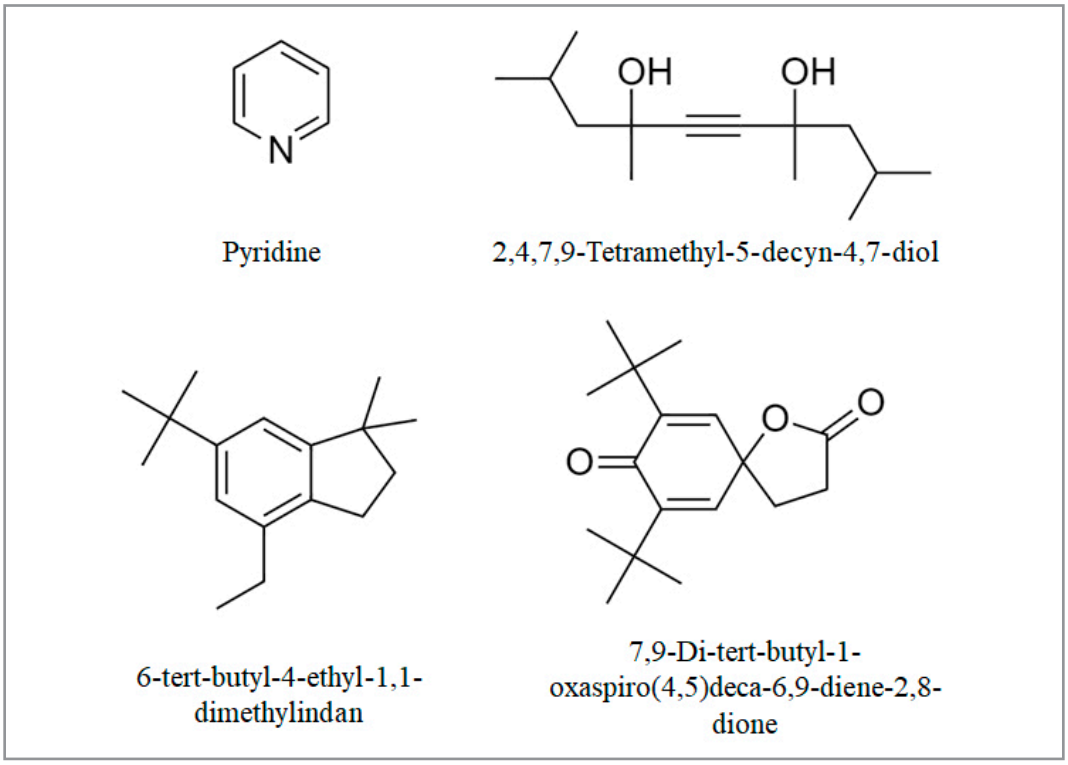


Figure 6. Structures of compounds found in NTA-GSR marked with EuDPA.

For GSR-NTA marked with EuBDC and EuBTC, a peak corresponding to benzene was observed at 2.231–2.248 min (Figure 7) and 2.260–2.268 min (Figure 8), respectively. Moreover, for GSR marked with EuBDC, the following compounds were found: alfa-methylbenzeneethanamine (3.298 min), benzaldehyde (6.235 min), nitrobenzene (8.244–8.249 min), biphenyl (13.820–13.872 min), acenaphthylene (15.346–15.347 min), dibenzofuran (16.833–16.834 min) and benzophenone (19.255–19.258 min). While for GSR-NTA marked with EuBTC, the listed compounds were found: 3-(3-Carboxy-4-hydroxyphenyl)-d-alanine (5.121 min), benzaldehyde (6.237 min), phenol (6.460 min), biphenyl (13.809–13.813 min) and acenaphthylene (15.347 min). All structures are shown in Figure 9. Again, the compounds found for the pure NTA ammunition were also found for the EuBDC and EuBTC marked GSR.

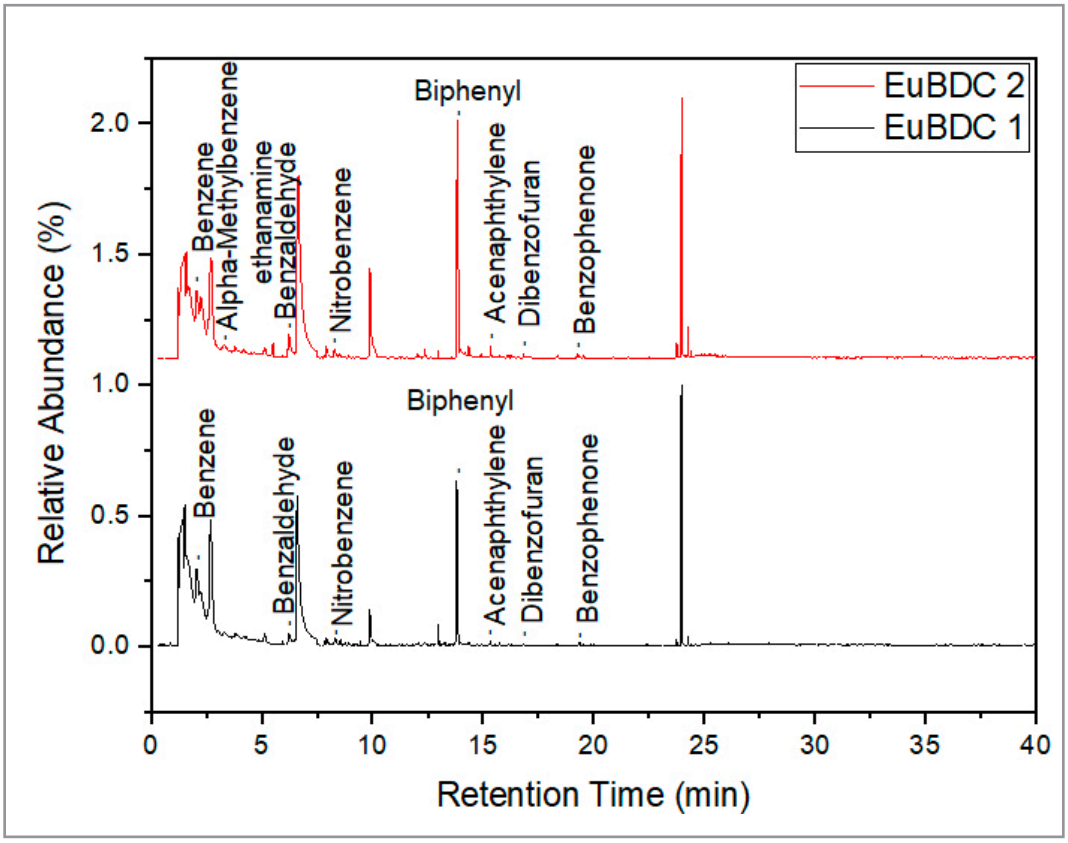


Figure 7. Chromatograms obtained from as-fired cartridge of NTA marked with EuBDC.

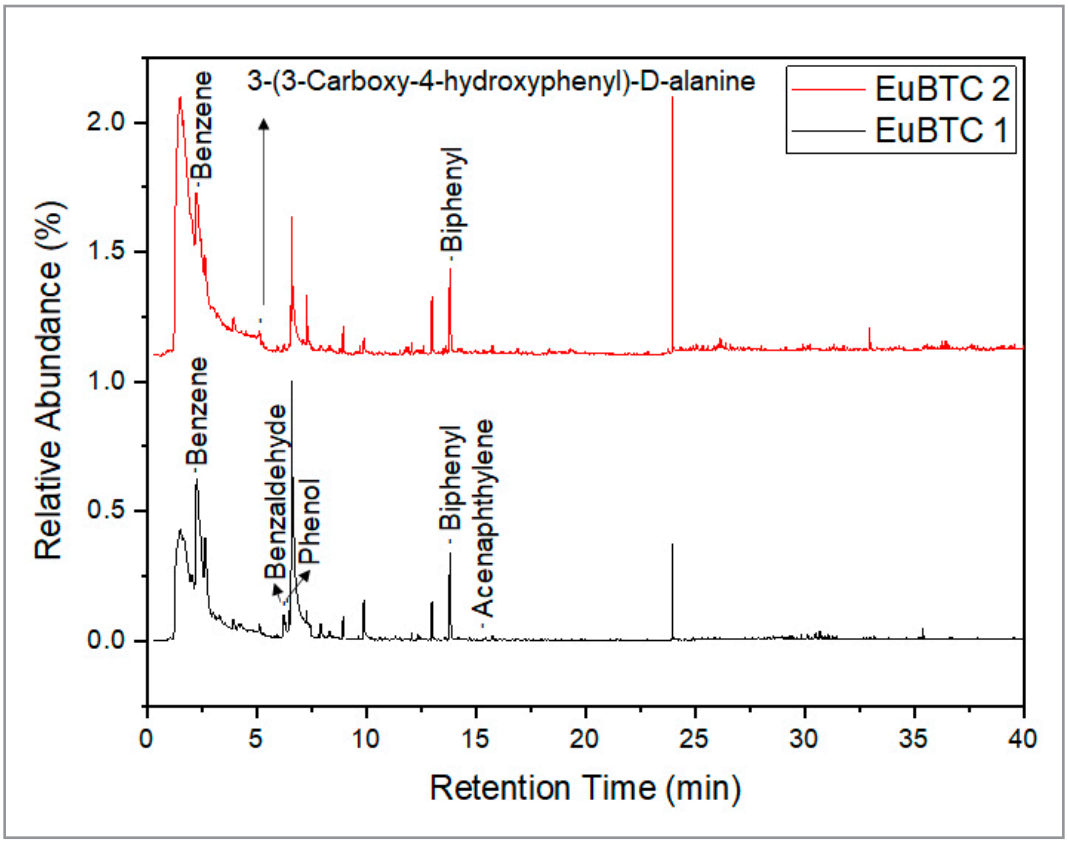


Figure 8. Chromatograms obtained from as-fired cartridge of NTA marked with EuBTC.

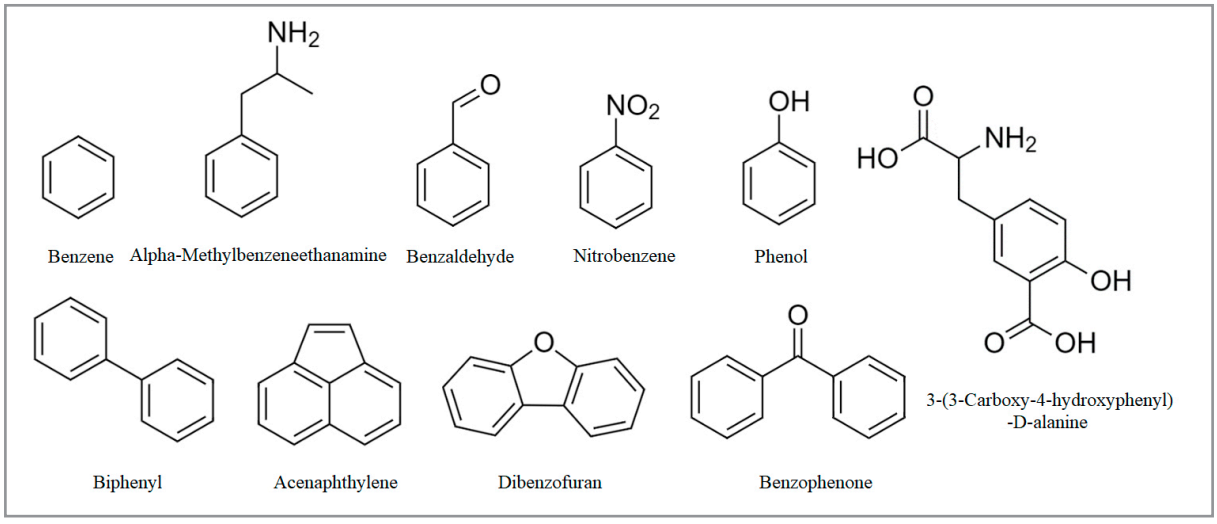


Figure 9. Structures of compounds found in GSR-NTA marked with EuBDC or EuBTC.

Among the listed compounds, pyridine and benzene are related to degradation of the markers, while the other compounds, despite not appearing in the chromatograms of pure fired NTA, may be byproducts of pure ammunition or result of a reaction between the marker and the gunpowder during the shot. For example, compounds such as phenol, biphenyl, benzaldehyde and nitrobenzene (found only in the marked samples) have already been described in the literature as byproducts of fired NTA [31,32]. Of course, they also can be due to the degradation of the markers.

This result indicates that the structure of the LMOFs generates at least two compounds (benzene and pyrene) which can be related to the addition of the marker to the gunpowder. These compounds may pose some risk to the health of a frequent shooter who suffers chronic exposure for a prolonged period.

According to IARC [38], an acute pyridine intoxication can cause several effects on the central nervous system resulting in dizziness, headache, nausea and anorexia. In addition, inhaling the material may cause necrotic damage of the nasal epithelium. Jori *et al.* [39] reported a study in which workers were exposed to pyridine vapors, in a concentration of about 125 ppm, for a period of 4 h per day, during one to two weeks; they found the workers presented symptoms such as headache, dizziness, insomnia, nausea and anorexia.

NIOSH [40] considers benzene a potential human carcinogen; exposure should be reduced and controlled in the work environment. Acute intoxication, in humans, can led to nerve inflammation, central nervous system depression and cardiac sensitization. Moreover, concentrations above 3000 ppm can lead to eye irritation, nose and respiratory tract. On the other hand, chronic exposure may lead to irreversible problems in the blood-forming organs and can cause leukemia [40].

Regarding the quantity of the byproducts found, previous studies have shown that a significant portion of the LMOF remains unchanged in the firing process, this fact can be visually identified because the LGSR particles deposited on the gun, hands or at the firing site remain luminescent after the shot (Figure 10). This fact has been corroborated by previous studies [21,24] which prove that the Raman and luminescence spectral profiles also remain unchanged, showing that only a small part of the marker has been degraded.

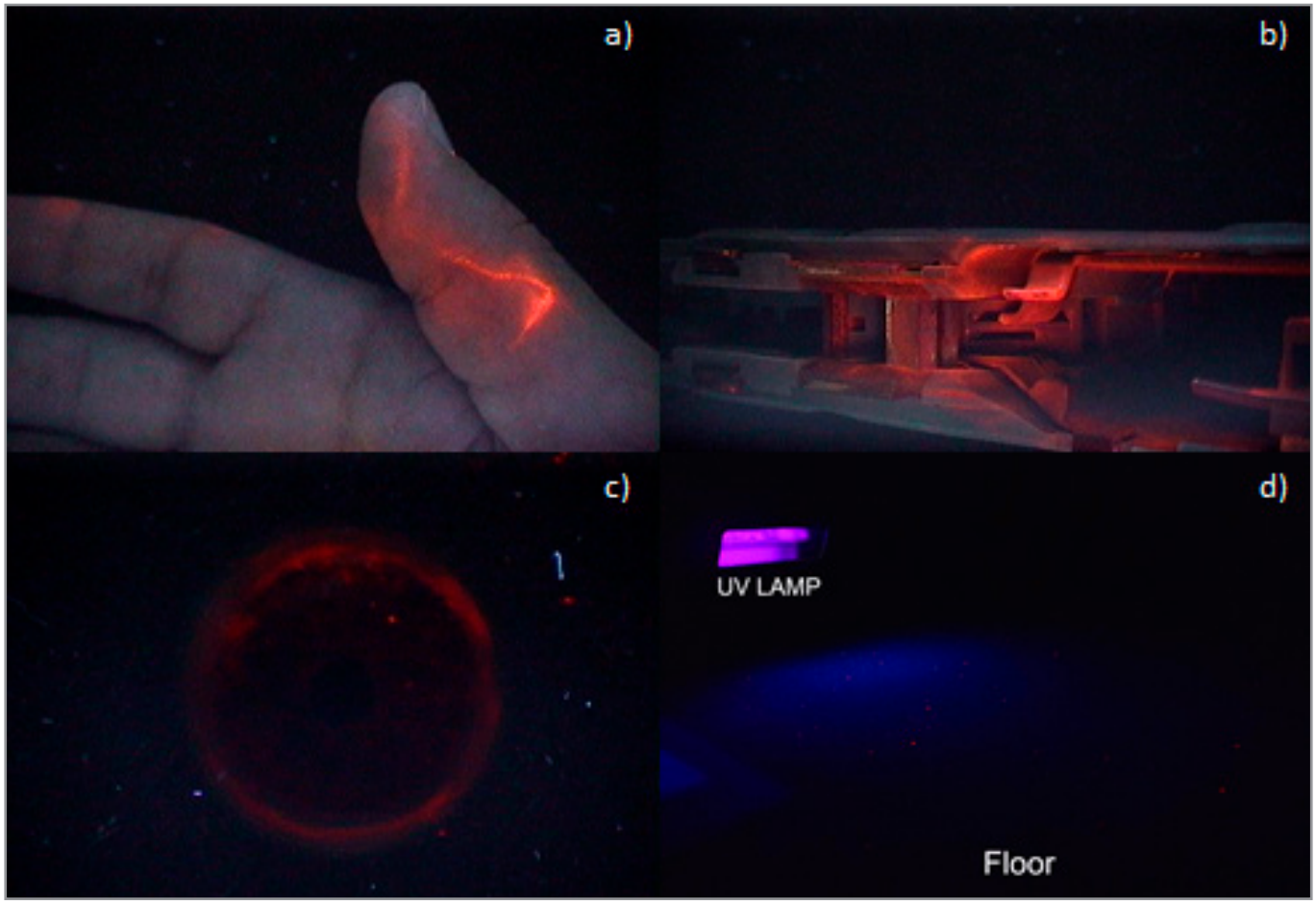


Figure 10. LGSR particles deposited on the (a) hands of the shooter, (b) gun, (c) cartridge and (d) at the shooting site irradiated with an UV lamp (λ = 254 nm).

It is possible to estimate the number of rounds that are needed to produce an acute toxic effect regarding the degradation of the markers. In each ammunition, 40 mg of the marker was added to 400 mg of gunpowder. Supposing that about 30% of the marker undergoes degradation (based on experience, we believe that this value is overestimated), one shot of the ammunition marked with EuBTC would produce 2.69 mg of benzene. Considering that shot was carried out in a closed space (200 m^3), the amount of marker needed to saturate the environment and achieve a concentration above 3000 ppm, that cause some acute toxicity effect [40], it would be necessary to fire more than 220 thousand marked ammunition with EuBTC marker. In real-life situations, this is an unrealistic scenario. Furthermore, the amount of marker added to the ammunition can be reduced up to 2% wt, still making it possible to visualize the LGSR produced, as described in a previous work [19]. With this change, the level of security for the use of the markers can be increased.

So, although the markers generate some toxic byproducts, the acute toxicity of NTA, as understood in our study, is not significantly altered when the LMOF is added, considering the small amount of material formed. However, the long-term exposure needs to be evaluated for both pure and marked GSR-NTA, to define a safety margin for frequent shooters.

CONCLUSIONS

The gunshot residue of an NTA was analyzed with a GC-MS analysis, using SPME as the extraction method to identify byproducts formed, and relate its toxicity to a possible toxicity of the ammunition. Many studies have been carried out on the toxicity of inorganic GSR, however, none has been carried out on the toxic effect of organic GSR. The presence of organic compounds with known toxicity can indicate a possible toxic effect of the ammunition, these compounds are known to be dangerous and still imply that, since the amount used is small, the acute effect can be disregarded, nevertheless the chronic one can be quite dangerous. In fact, for NTA ammunition studies are needed to assess the chronic toxicity of the organic part of the GSR, just as done for the inorganic one.

The toxicity effect of the NTA marked with the LMOFs $_{\infty}$[Eu(DPA)(HDPA)], $_{\infty}$[$Eu_2(BDC)_3(H_2O)_2$]$_n$ and $_{\infty}$[Eu(BTC)] was also evaluated. The two target compounds found in fired marked cartridges were pyridine and benzene. Other compounds like phenol, biphenyl, and benzaldehyde were also identified. Although these compounds have a certain toxicity, the toxicity of pure NTA ammunition remained mainly unaltered, considering that some of those compounds could also be found in the firing of pure NTA. Furthermore, the amount of marker added to the gunpowder is too small to produce toxic effect.

Conflicts of interest

This work was supported by CNPq, CAPES PROFORENSE NEQUIFOR 3509/2014.

Acknowledgements

We thank the Brazilian Federal Police and experts Eduardo Makoto Sato, Ronei Maia Salvatori, André Lima Lograd o and Elvio Dias Botelho for research collaboration. This work was supported by CNPq, CAPES PROFORENSE NEQUIFOR 3509/2014.

The English text of this paper has been revised by Sidney Pratt, Canadian, MAT (The Johns Hopkins University), RSAdip - TESL (Cambridge University).

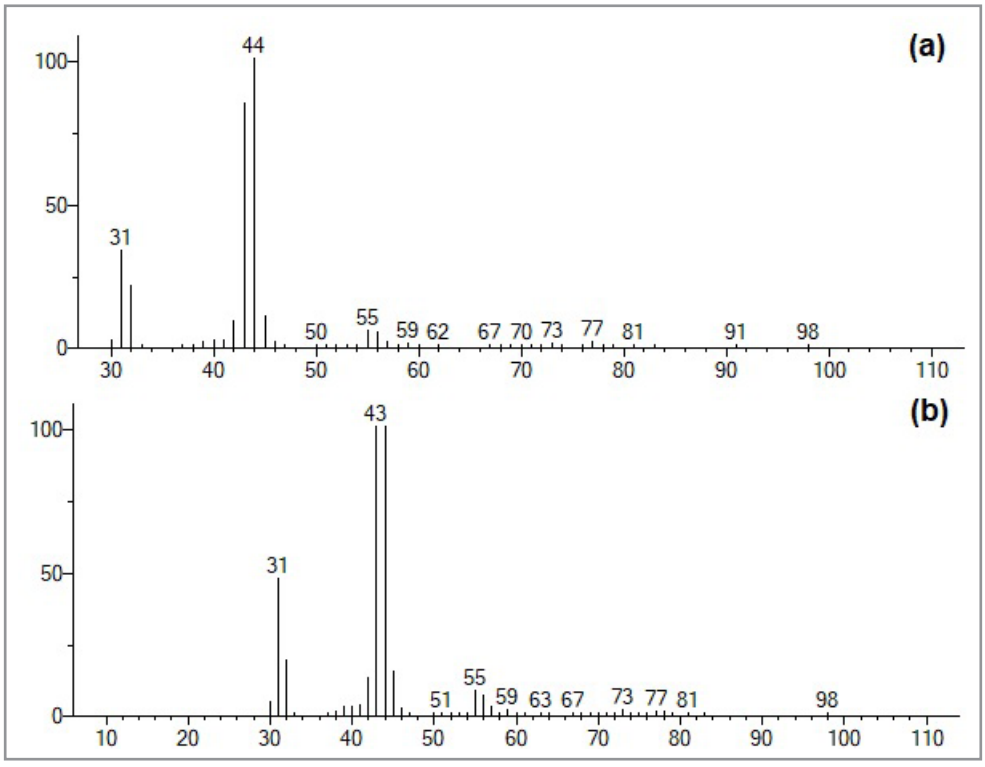
REFERENCES

1. National Institute for Occupational Safety and Health (NIOSH). Center for Disease Control (CDC). *Lead exposure and design considerations for indoor firing ranges*, **1975**.
2. National Institute for Occupational Safety and Health (NIOSH). Center for Disease Control (CDC). *Preventing occupational exposures to lead and noise at indoor firing ranges*, **2009**, Volume 139.
3. Rocha, E. D.; Sarkis, J. E. S.; Carvalho, M. D. F. H.; Dos Santos, G. V.; Canesso, C. *Int. J. Hyg. Environ. Health*, **2014**, *217* (6), pp 702–704 (https://doi.org/10.1016/j.ijheh.2013.12.004).

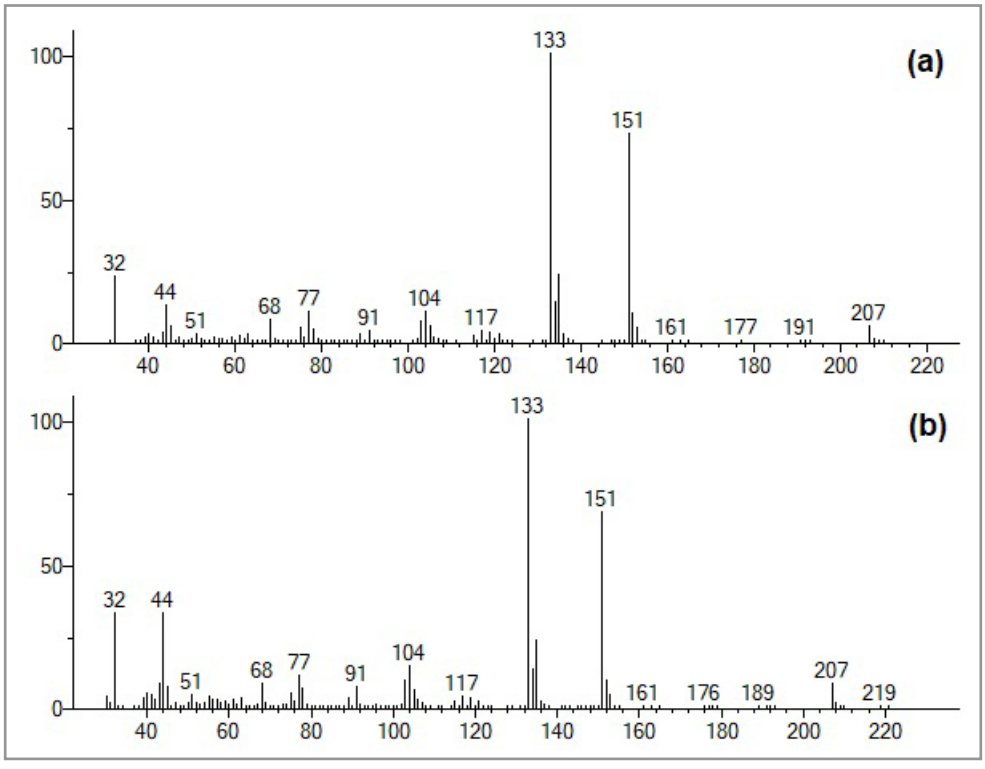
4. National Institute for Occupational Safety and Health (NIOSH). *Reducing Exposure to Lead and Noise at Outdoor Firing Ranges*, **2012**.
5. National Institute for Occupational Safety and Health (NIOSH). Center for Disease Control (CDC). *Reducing Exposure to Lead and Noise at Indoor Firing Ranges*, **2010**.
6. National Institute for Occupational Safety (NIOSH). *Preventing Occupational Exposures to Lead and Noise at Indoor Firing Ranges*, **2009**.
7. Martiny, A.; Campos, A. P. C.; Sader, M. S.; Pinto, M. A. L. *Forensic Sci. Int.*, **2008**, *177* (1), pp e9–e17 (https://doi.org/10.1016/j.forsciint.2007.07.005).
8. Brandone, A.; De Ferrari, F.; Pelizza, P.; Signori, M. *Forensic Sci. Int.*, **1990**, *47* (3), pp 289–295 (https://doi.org/10.1016/0379-0738(90)90298-D).
9. Niewoehner, L.; Buchholz, N.; Merkel, J. *The Journal of Scanning Microscopies*, *Proceedings of SCANNING 2005 Monterey, CA*, **2005**, *27*, p 69.
10. Lucena, M. A. M.; De Sá, G. F.; Rodrigues, M. O.; Júnior, S. A.; Talhavini, M.; Weber, I. T. *Anal. Methods*, **2013**, *5* (3), pp 705–709 (https://doi.org/10.1039/c2ay25535a).
11. Destefani, C.; Motta, L. C.; Vanini, G.; Souza, L. M.; Allochio Filho, J. F.; Macrino, C. J.; Silva, E. M.; Greco, S. J.; Endringer, D. C.; Romão, W. *Microchem. J.*, **2014**, *116*, pp 216–224 (https://doi.org/10.1016/j.microc.2014.05.009).
12. Weber, I. T.; Geber de Melo, A. J.; Lucena, M. A. M.; Rodrigues, M. O.; Junior, S. A. *Anal. Chem*., **2011**, *83*, pp 4720–4723 (https://doi.org/10.1021/ac200680a).
13. Weber, I. T.; Terra, I. A. A.; de Melo, A. J. G.; Lucena, M. A. M.; Wanderley, K. A.; Paiva-Santos, C. O.; Antônio, S. G.; Nunes, L. A. O.; Paz, F. A. A.; de Sá, G. F.; Júnior, S. A.; Rodrigues, M. O. *RSC Adv.* **2012**, *2* (7), pp 3083–3087 (https://doi.org/10.1039/c2ra01214f).
14. Lucena, M. A. M.; Rodrigues, M. O.; Gatto, C. C.; Talhavini, M.; Maldaner, A. O.; Alves Jr, S.; Weber, I. T. *J. Lumin*., **2016**, *170*, pp 697–700 (https://doi.org/10.1016/j.jlumin.2015.04.010).
15. Serwy, I. B.; Wanderley, K. A.; Lucena, M. A. M.; Maldaner, A. O.; Talhavini, M.; Rodrigues, M. O.; Weber, I. T. *J. Lumin*., **2018**, *200*, pp 24–29 (https://doi.org/10.1016/j.jlumin.2018.02.039).
16. Venturini Filho, E.; de Sousa Filho, P. C.; Serra, O. A.; Weber, I. T.; Lucena, M. A. M.; Luz, P. P. *J. Lumin*., **2018**, *202*, pp 89–96 (https://doi.org/10.1016/j.jlumin.2018.05.012).
17. Júnior, J. C. A.; dos Santos, G. L.; Colaço, M. V.; Barroso, R. C.; Ferreira, F. F.; dos Santos, M. V.; de Campos, N. R.; Marinho, M. V.; Jesus, L. T.; Freire, R. O.; Marques, L. F. *J. Phys. Chem. C*, **2020**, *124* (18), pp 9996–10006 (https://doi.org/10.1021/acs.jpcc.0c01374).
18. Silva, M. A.; de Campos, N. R.; Ferreira, L. A.; Flores, L. S.; Júnior, J. C. A.; dos Santos, G. L.; Corrêa, C. C.; dos Santos, T. C.; Ronconi, C. M.; Colaço, M. V.; et al. *Inorganica Chim. Acta*, **2019**, *495*, 118967 (https://doi.org/10.1016/j.ica.2019.118967).
19. Weber, I. T.; Melo, A. J. G.; Lucena, M. A. M.; Consoli, E. F.; Rodrigues, M. O.; de Sá, G. F.; Maldaner, A. O.; Talhavini, M.; Alves, S. J. *Forensic Sci. Int*., **2014**, *244*, pp 276–284 (https://doi.org/10.1016/j.forsciint.2014.09.001).
20. Arouca, A. M.; Lucena, M. A. M.; Rossiter, R. J.; Talhavini, M.; Weber, I. T. *Forensic Sci. Int*., **2017**, *281*, pp 161–170 (https://doi.org/10.1016/j.forsciint.2017.09.022).
21. Lucena, M. A. M.; Ordoñez, C.; Weber, I. T.; Torre, M.; García-Ruiz, C.; López-López, M. *Forensic Sci. Int*., **2017**, *280*, pp 95–102 (https://doi.org/10.1016/j.forsciint.2017.09.013).
22. Arouca, A. M.; Lucena, M. A. M.; Rossiter, R. J.; Talhavini, M.; Weber, I. T. *J. Forensic Sci*., **2020**, *65* (1), pp 67-72 (https://doi.org/10.1111/1556-4029.14143).
23. Lucena, M. A. M.; Arouca, A. M.; Talhavini, M.; Alves-Júnior, S.; Weber, I. T. *Microchem. J*., **2019**, *145*, pp 539–546 (https://doi.org/10.1016/j.microc.2018.09.013).
24. Carneiro, C. R.; Silva, C. S.; De Carvalho, M. A.; Pimentel, M. F.; Talhavini, M.; Weber, I. T. *Anal. Chem*., **2019**, *91* (19), pp 12444–12452 (https://doi.org/10.1021/acs.analchem.9b03079).
25. Lucena, M. A. M.; Oliveira, M. F. L.; Arouca, A. M.; Talhavini, M.; Ferreira, E. A.; Alves, S.; Veiga-Souza, F. H.; Weber, I. T. *ACS Appl. Mater. Interfaces*, **2017**, *9* (5), pp 4684–4691 (https://doi.org/10.1021/acsami.6b13474).

26. Talhari, A. L. R.; Lucena, M. A. M.; Mauricio, F. G. M.; Oliveira, M. F. L.; Veiga-Souza, F. H.; Alves Junior, S.; Weber, I. T. *ACS Appl. Bio Mater*., **2020**, *3* (5), pp 3049-3056 (https://doi.org/10.1021/acsabm.0c00107).
27. Fernandes, A.; Jaud, J.; Dexpert-Ghys, J.; Brouca-Cabarrecq, C. *Polyhedron*, **2001**, *20* (18), pp 2385–2391 (https://doi.org/10.1016/S0277-5387(01)00841-5).
28. Qu, Y.; Ke, Y.; Lu, S.; Fan, R.; Pan, G.; Li, J. *J. Mol. Struct*., **2005**, *734* (1–3), pp 7–13 (https://doi.org/10.1016/j.molstruc.2004.03.035).
29. Serre, C.; Millange, F.; Thouvenot, C.; Gardant, N.; Pelle, F.; Férey, G. *J. Mater. Chem*., **2004**, *14* (10), pp 1540–1543 (https://doi.org/10.1039/B312425H).
30. Taudte, R. V.; Beavis, A.; Blanes, L.; Cole, N.; Doble, P.; Roux, C. *Biomed Res. Int*., **2014**, *2014* (https://doi.org/10.1155/2014/965403).
31. Weyermann, C.; Belaud, V.; Riva, F.; Romolo, F. S.; *Forensic Sci. Int*., **2009**, *186* (1–3), pp 29–35 (https://doi.org/10.1016/j.forsciint.2009.01.005).
32. Goudsmits, E.; Sharples, G. P.; Birkett, J. W. *TrAC Trends Anal. Chem*., **2015**, *74*, pp 46–57 (https://doi.org/10.1016/j.trac.2015.05.010).
33. Agency for Toxic Substances and Diseasw Registry (ATSDR). Toxicological *Profile for Naphthalene, 1-Methylnaphthalene and 2-Methylnaphthalen.* Available at: http://www.atsdr.cdc.gov/toxprofiles/tp67.pdf [Accessed 08 October 2016].
34. International Agency for Research on Cancer (IARC). *IARC Monographs on the Evalution of Carcinogenic Risks to Humans: Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene*, **2002**, Volume 82.
35. Shopp, G. M.; White, K. L.; Holsapple, M. P.; Barnes, D. W.; Duke, S. S.; Anderson, A. C.; Condie, L. W.; Hayes, J. R.; Borzelleca, J. F. *Fundam. Appl. Toxicol*., **1984**, *4*, pp 406–419 (https://doi.org/10.1016/0272-0590(84)90198-2).
36. National Library of Medicine (NLM). *Hazardous Substances Data Bank (HSDB)*, **2003**.
37. Tanii, H.; Hashimoto, K. *Arch. Toxicol*., **1984**, *55*, pp 47–54.
38. International Agency for Research on Cancer (IARC). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **2000**, Volume 77.
39. Jori, A.; Calamari, D.; Cattabeni, F.; Di Domenico, A.; Galli, C. L.; Galli, E.; Silano, V. *Ecotoxicol. Environ. Saf*., **1983**, *7* (3), pp 251–275 (https://doi.org/10.1016/0147-6513(83)90071-4).
40. Nacional Institute for Occupational Safety and Health (NIOSH). Center for Disease Control (CDC). *Occupational Health and Safety Guideline for Benzene*, **1988**.

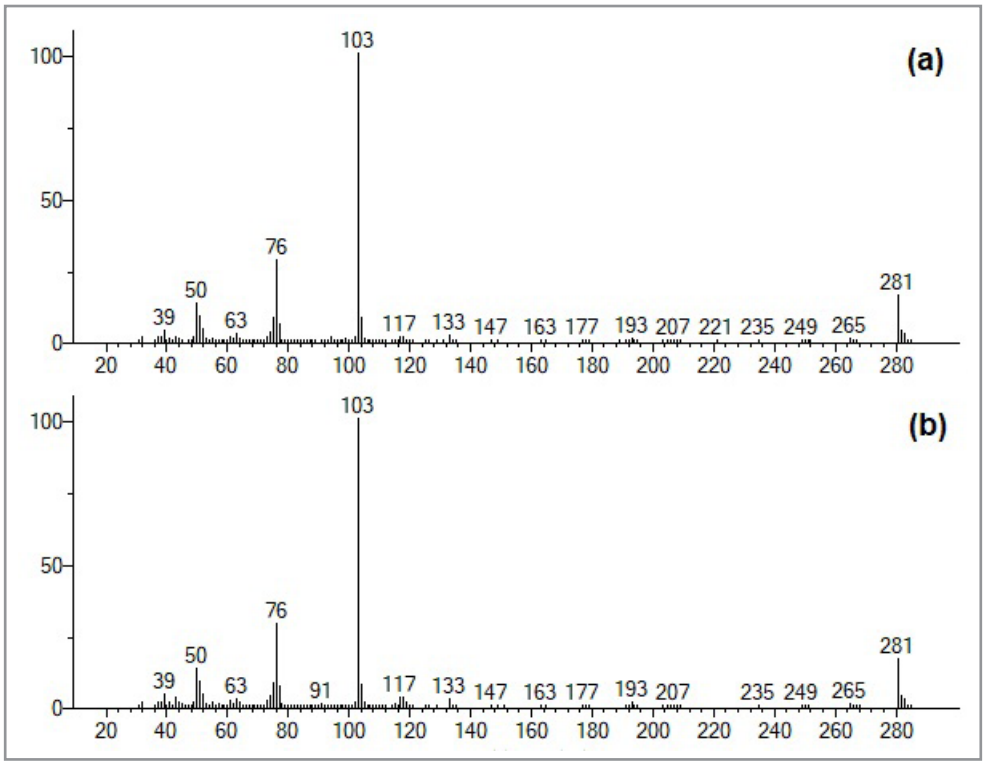
Supplementary Material



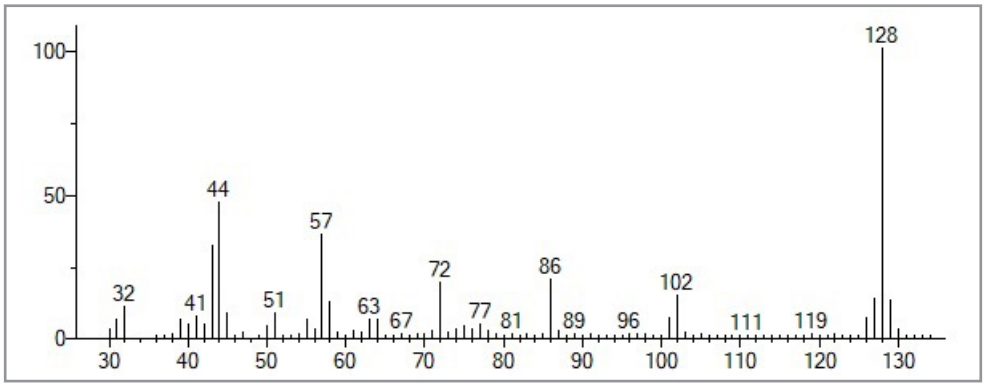
GC-MS spectra of Glycidol (retention time 2.720–2.727 min in samples (a) NTA 1 and (b) NTA 2.



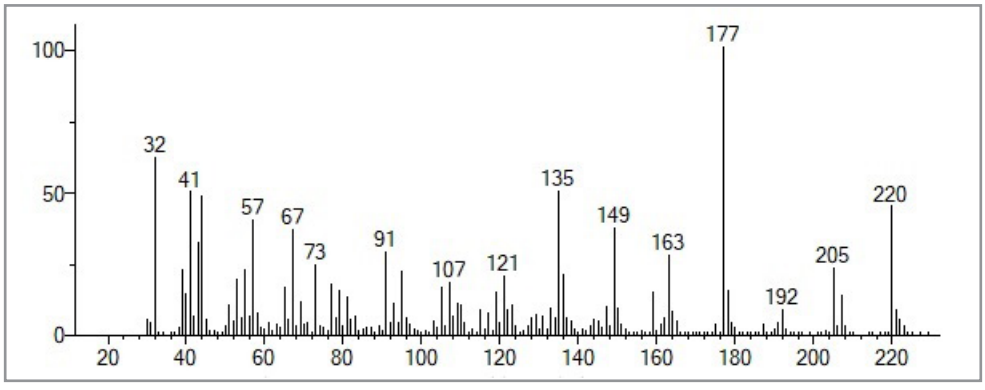
GC-MS spectra of oxime-methoxy-phenyl (retention time 5.191 min) in samples (a) NTA 1 and (b) NTA 2.



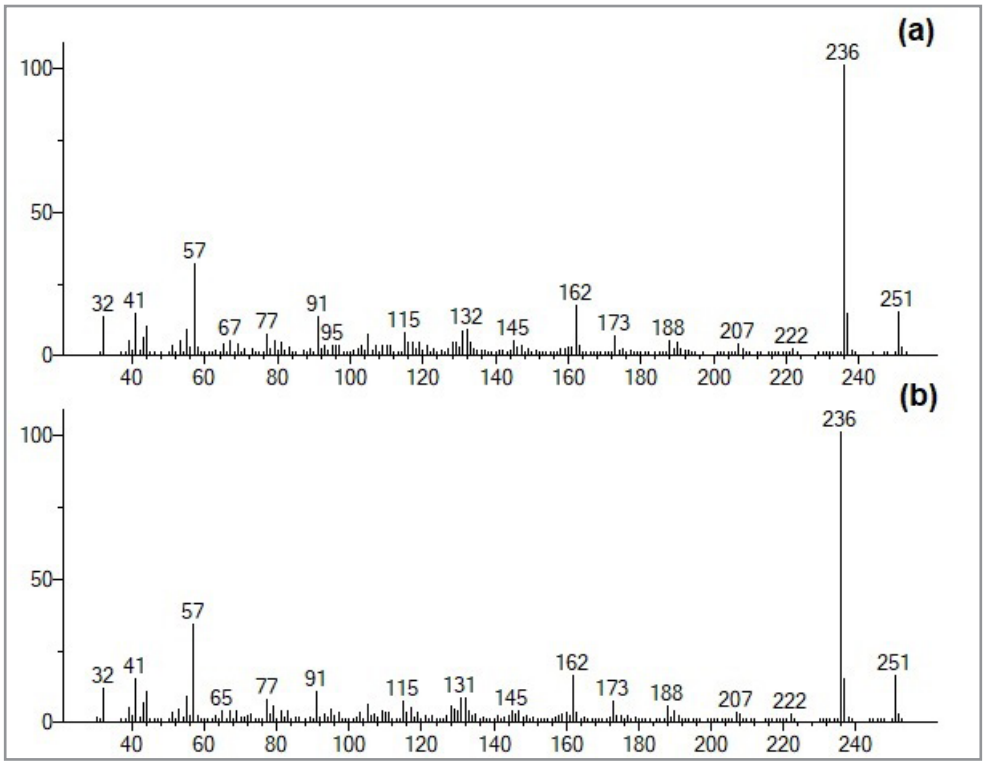
GC-MS spectra of benzonitrile (retention time 6.668 min) in samples (a) NTA 1 and (b) NTA 2.



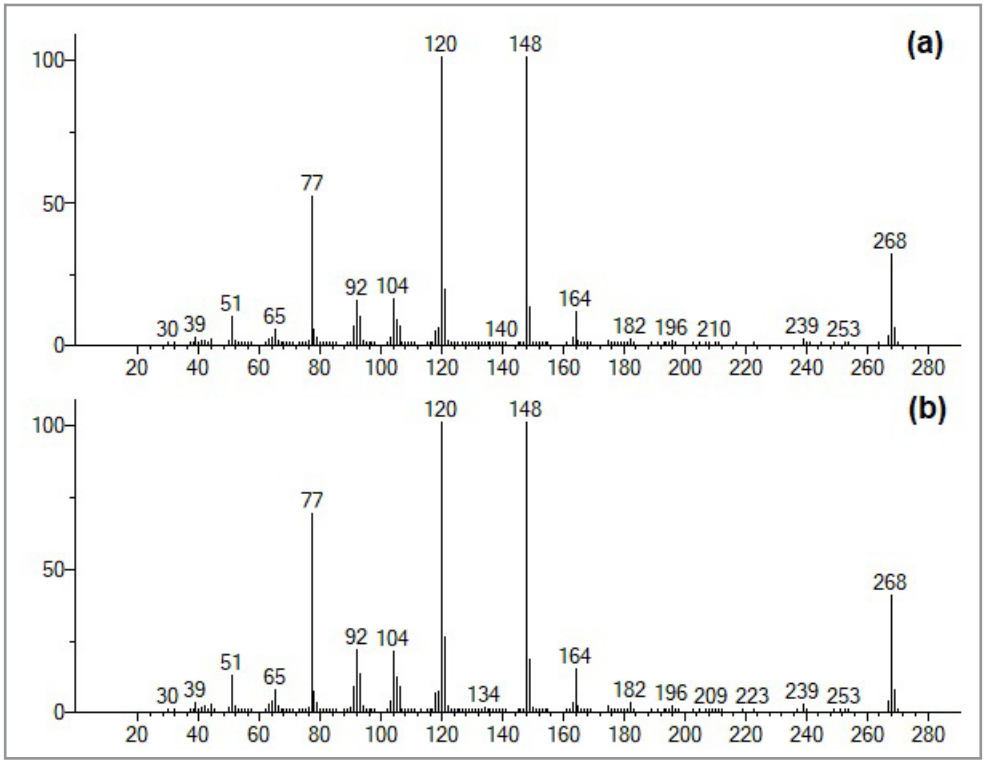
GC-MS spectra of naphthalene (retention time 9.992 min) in sample NTA 1.



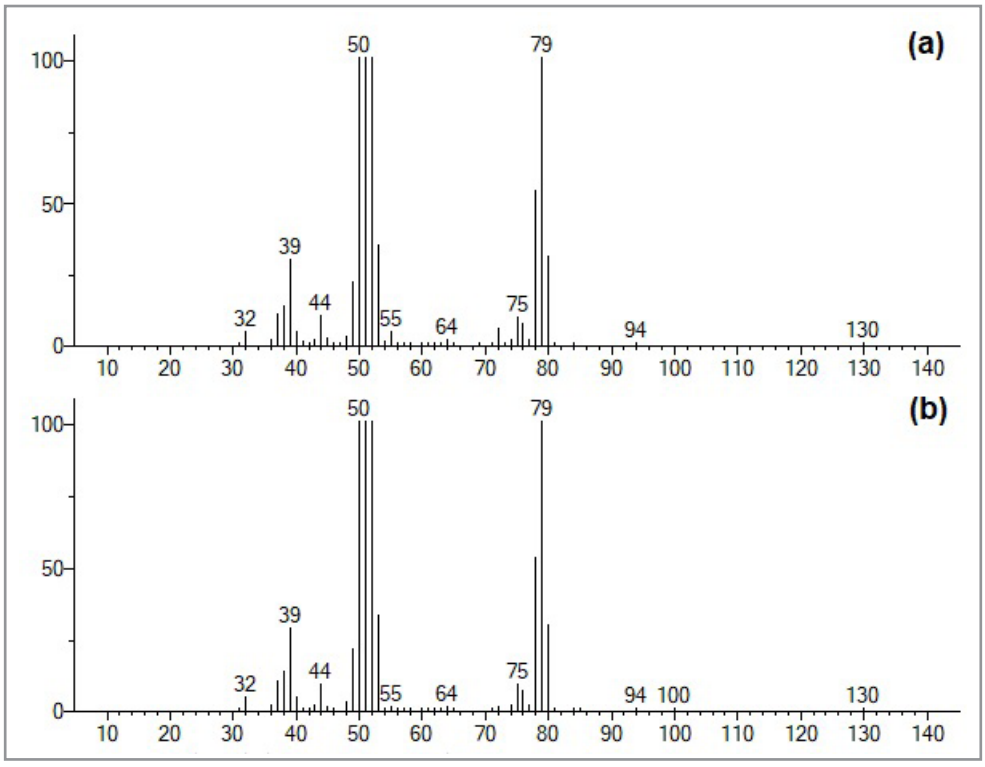
GC-MS spectra of 2,6-di-tert-butylbenzoquinone (retention time 15.703 min) in sample NTA 2.



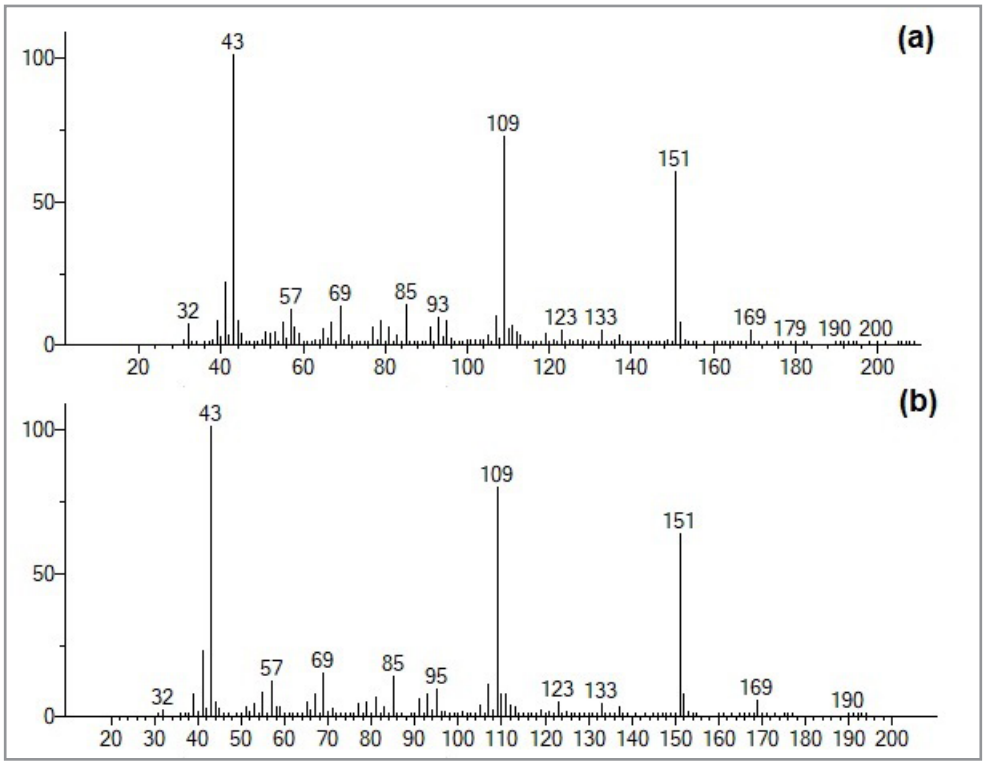
GC-MS spectra of 2,4-ditert-butyl-6-nitrophenol (retention time 20.058–20.445 min) in samples (a) NTA 1 and (b) NTA 2.



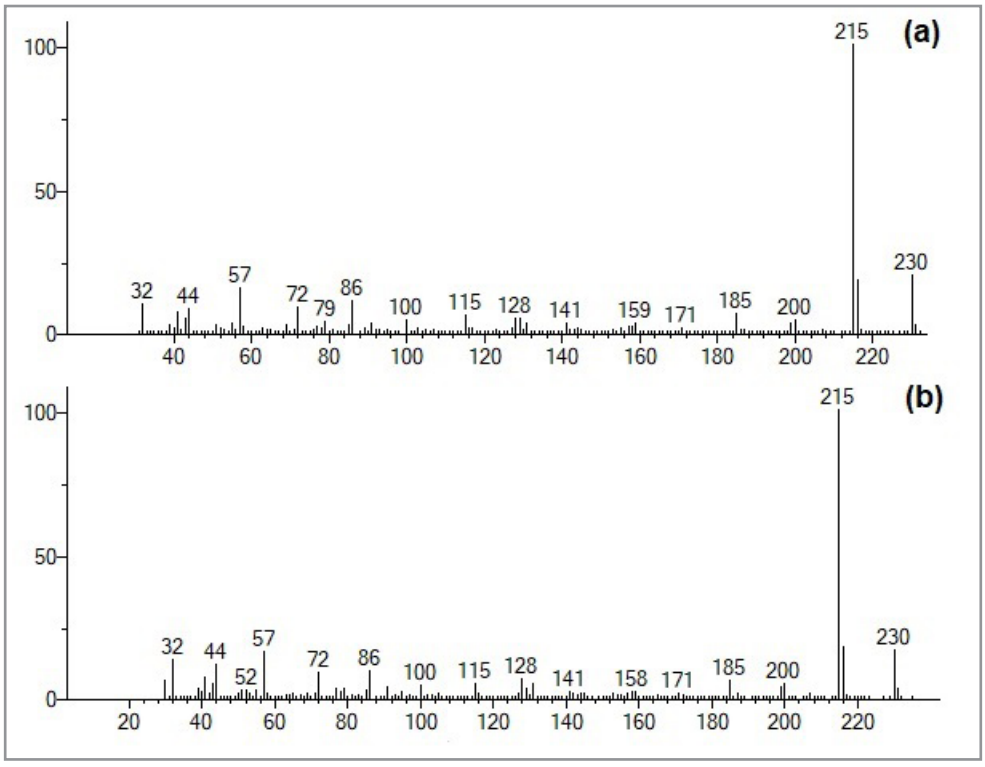
GC-MS spectra of N,N'-diethyl-N,N'-diphenylurea (retention time 24.059–24.452 min) in samples (a) NTA 1 and (b) NTA 2.



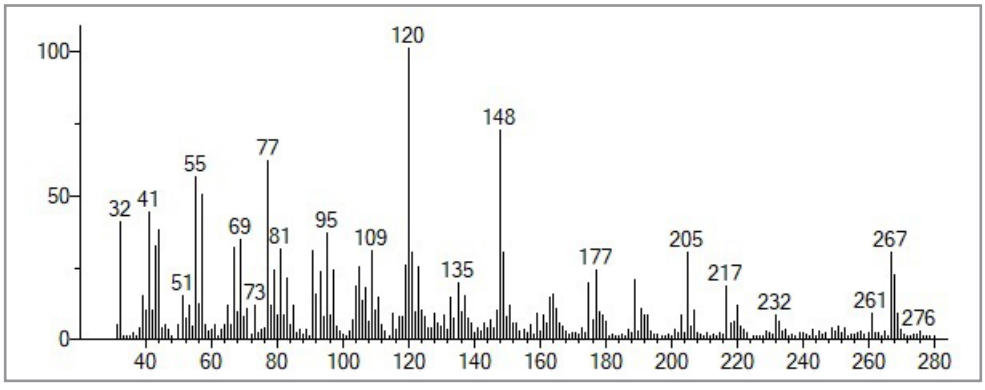
GC-MS spectra of pyridine (retention time 3.002–3.015 min) in samples (a) DPA 1 and (b) DPA 2.



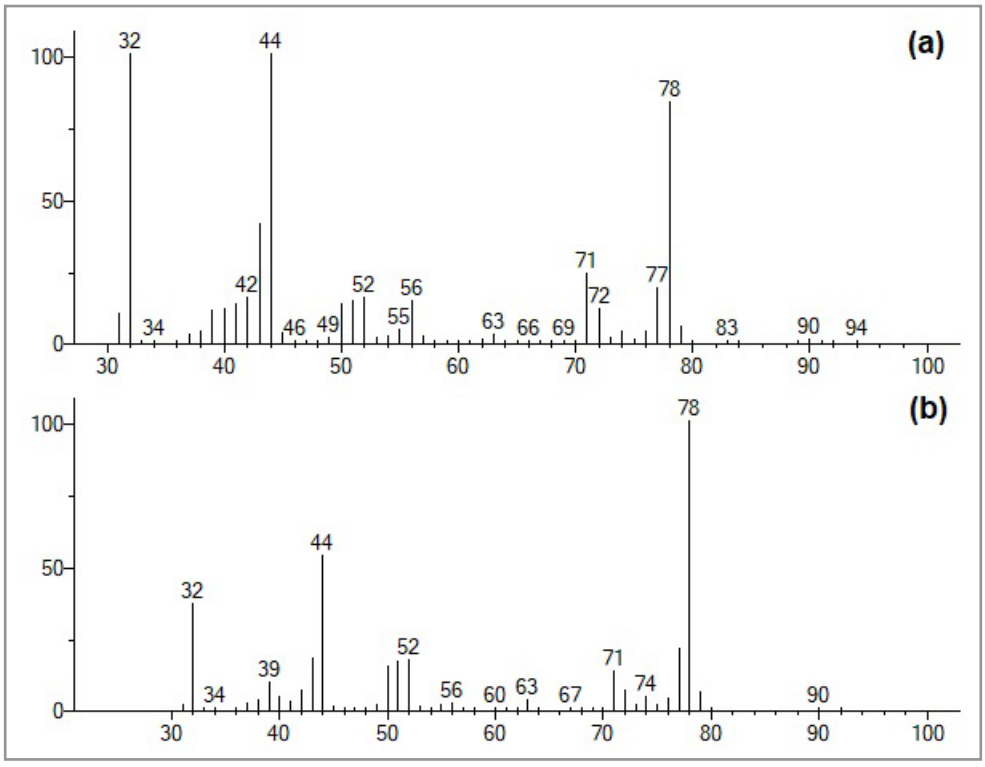
GC-MS spectra of 2,4,7,9-tetramethyl-5-decyne-4,7-diol (retention time 14.357–14.362 min) in samples (a) DPA 1 and (b) DPA 2.



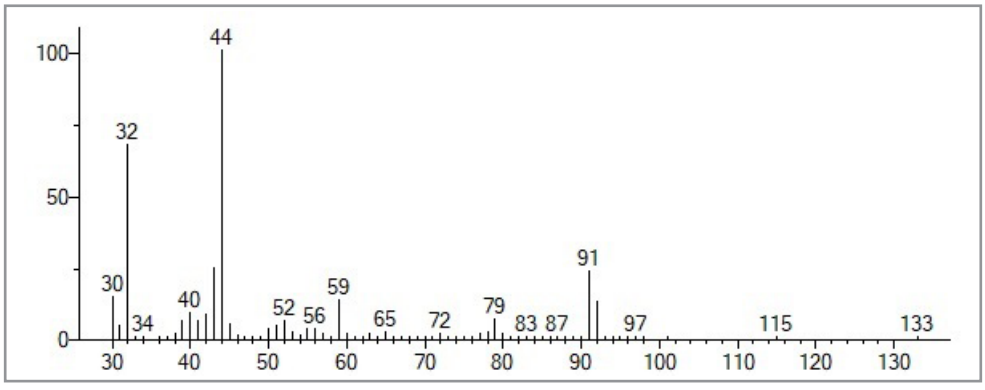
GC-MS spectra of 6-tert-butyl-4-ethyl-1,1-dimethylindan (retention time 19.155–19.156 min) in samples (a) DPA 1 and (b) DPA 2.



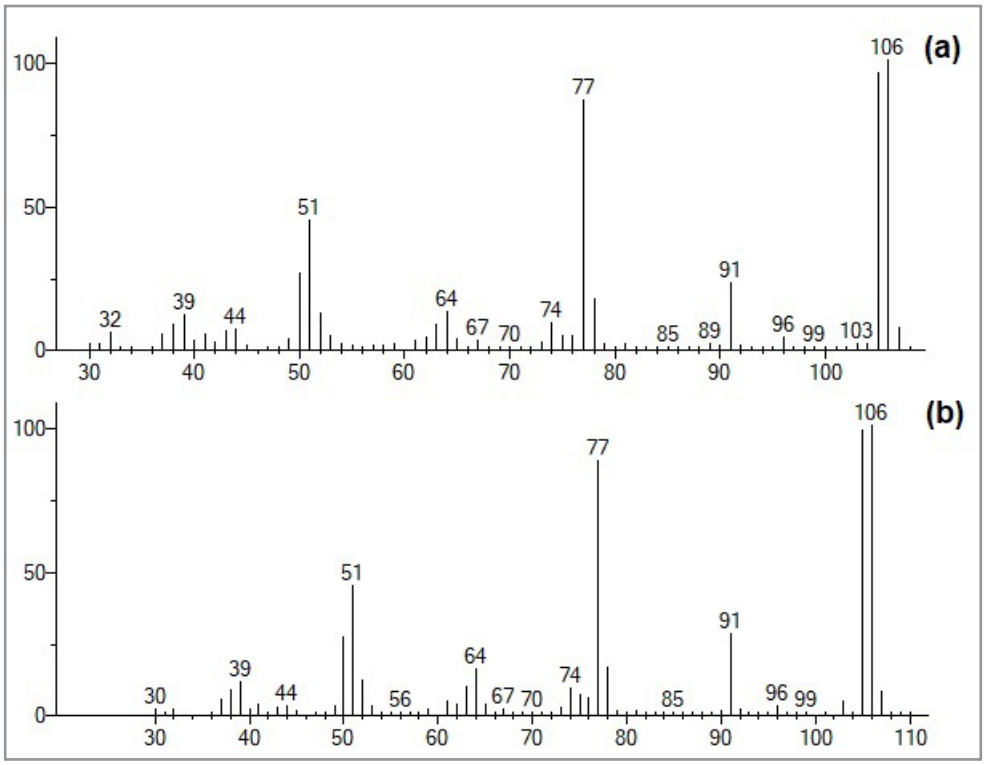
GC-MS spectra of 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (retention time 24.104 min) in sample DPA 1.



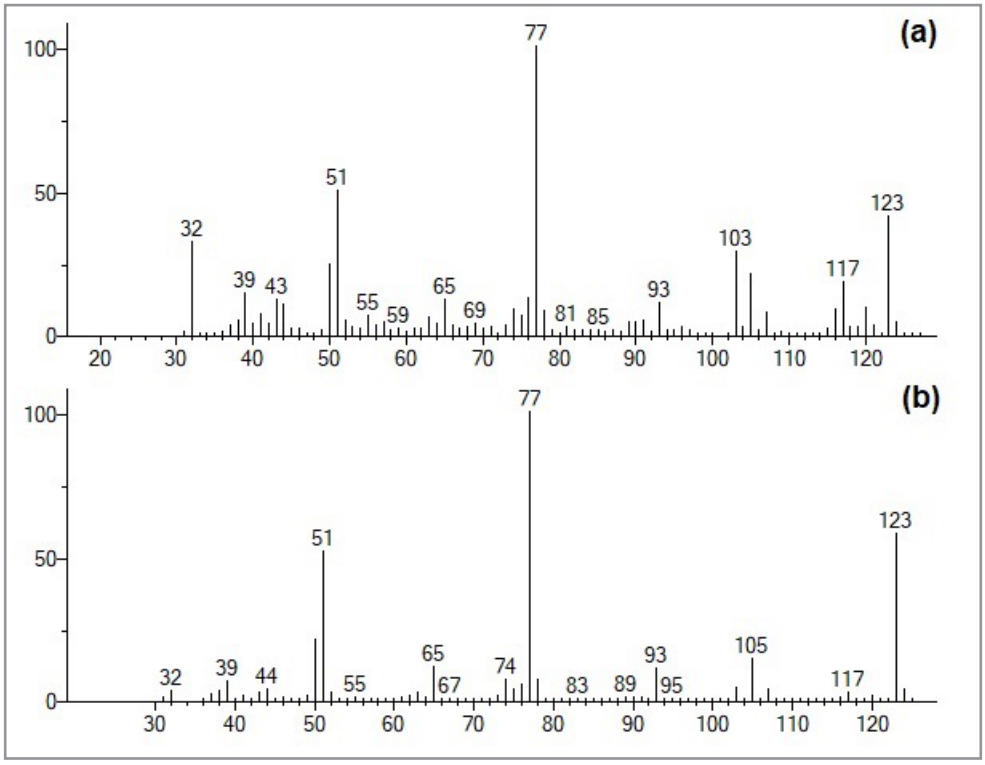
GC-MS spectra of benzene (retention time 2.231–2.248 min) in samples (a) BDC 1 and (b) BDC 2.



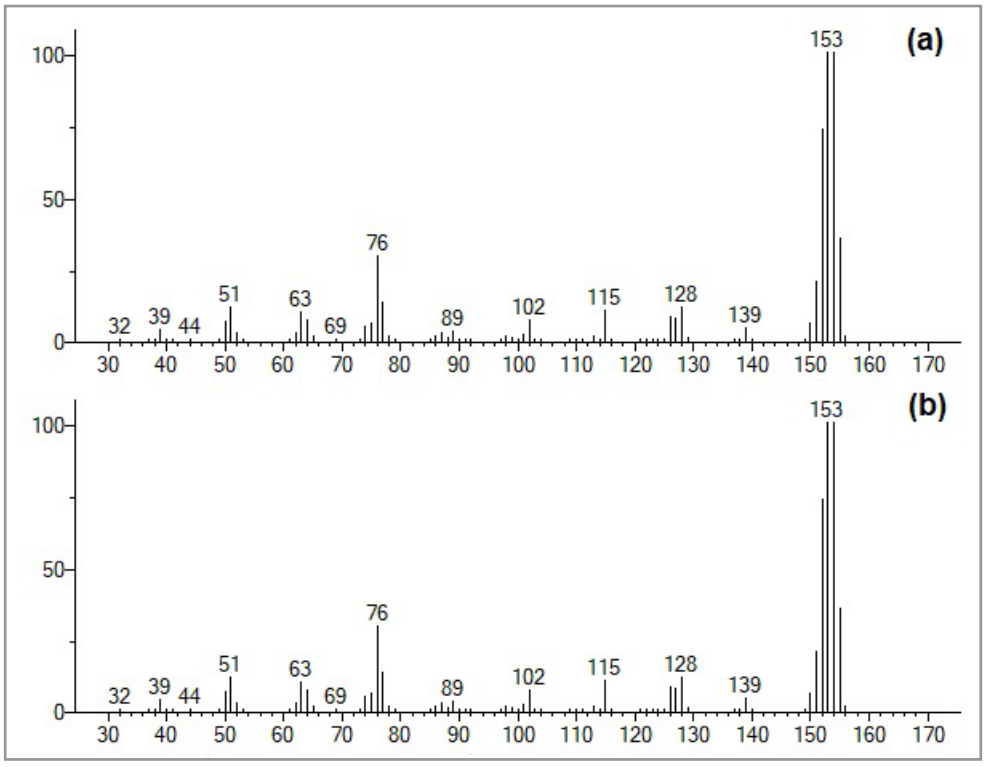
GC-MS spectra of alfa-methylbenzeneethanamine (retention time 3.298 min) in sample BDC 2.



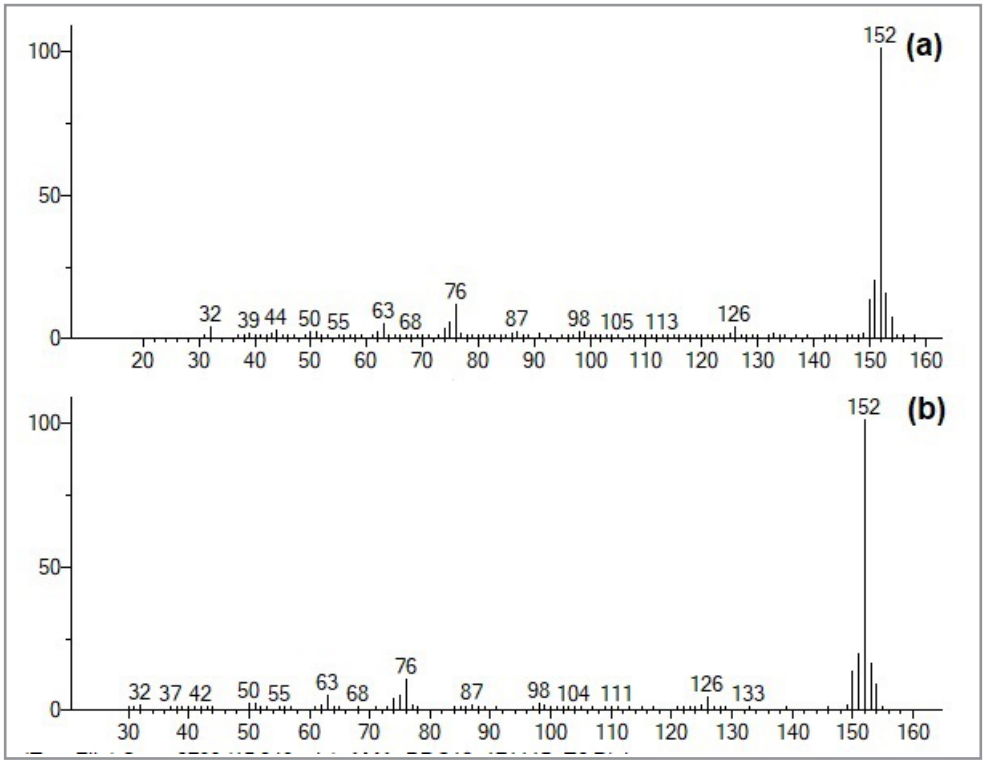
GC-MS spectra of benzaldehyde (retention time 6.235 min) in samples (a) BDC 1 and (b) BDC 2.



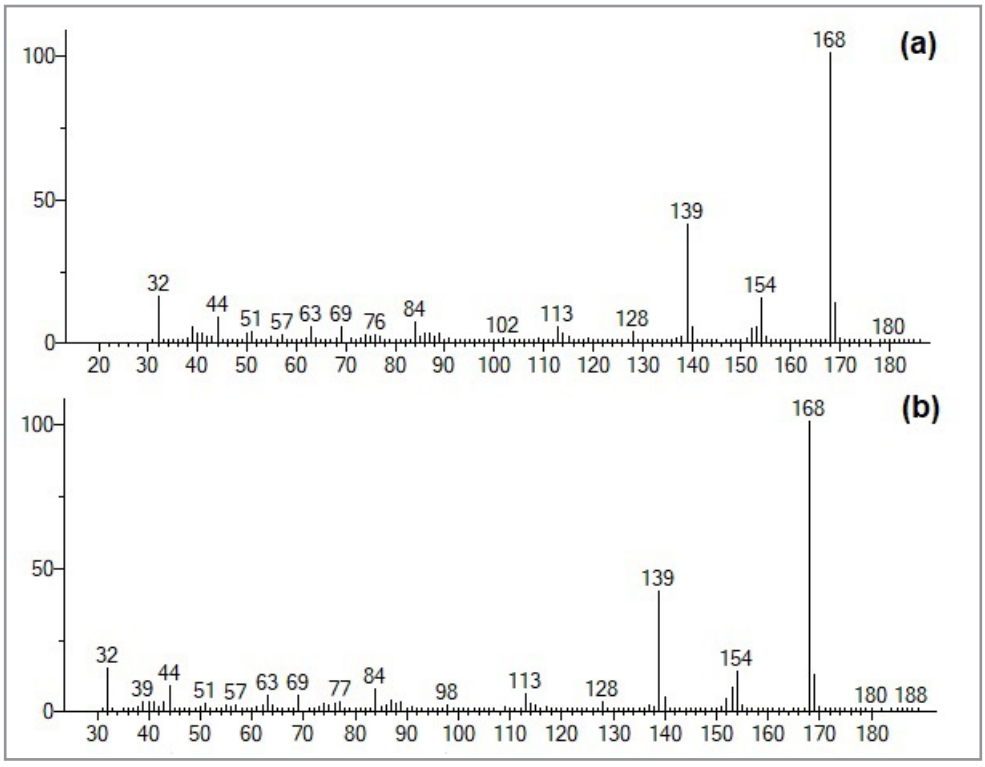
GC-MS spectra of nitrobenzene (retention time 8.244–8.249 min) in samples (a) BDC 1 and (b) BDC 2.



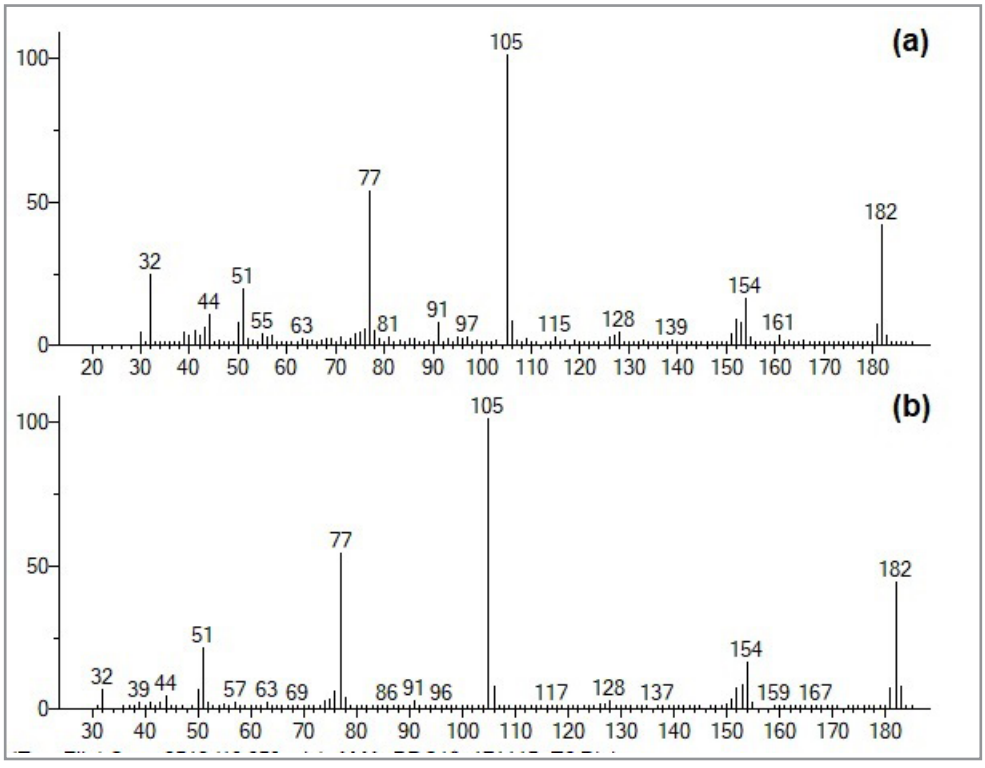
GC-MS spectra of biphenyl (retention time 13.820–13.872 min) in samples (a) BDC 1 and (b) BDC 2.



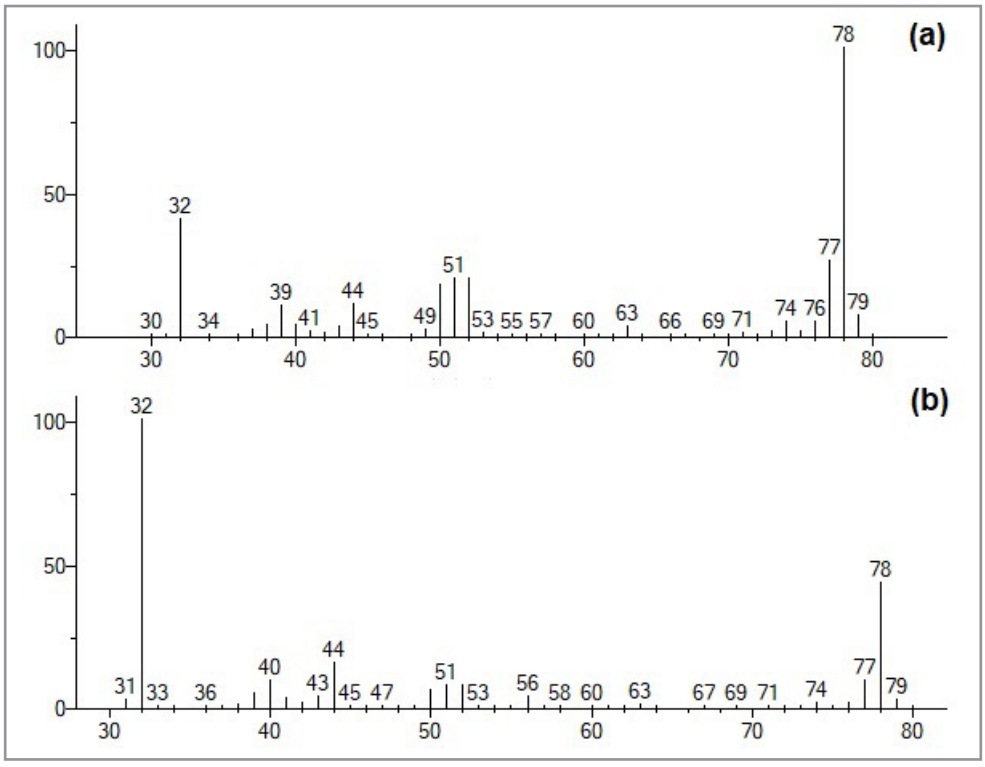
GC-MS spectra of acenaphthylene (retention time 15.346–15.347 min) in samples (a) BDC 1 and (b) BDC 2.



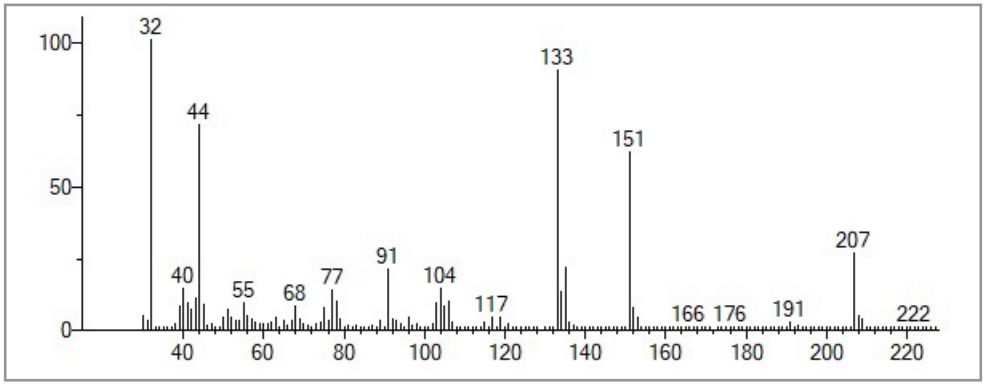
GC-MS spectra of dibenzofuran (retention time 16.833–16.834 min) in samples (a) BDC 1 and (b) BDC 2.



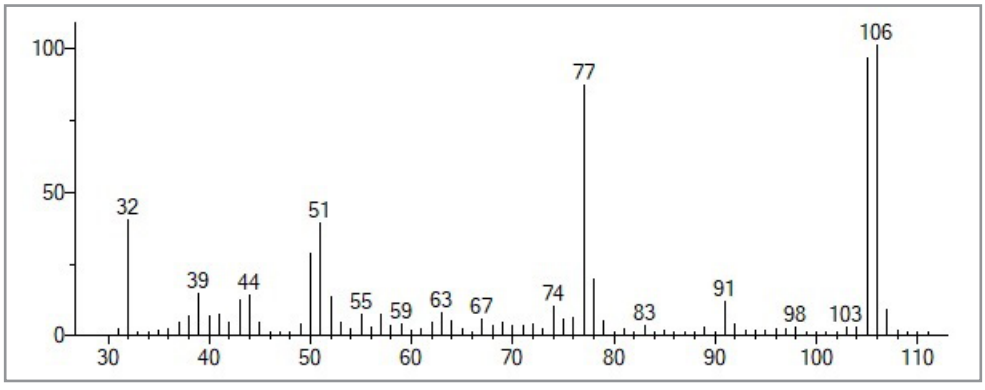
GC-MS spectra of benzophenone (retention time 19.255–19.258 min) in samples (a) BDC 1 and (b) BDC 2.



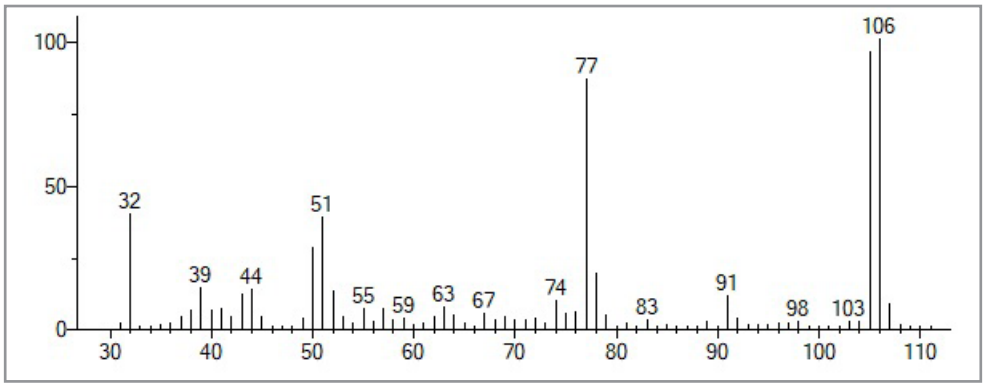
GC-MS spectra of benzene (retention time 2.231–2.248 min) in samples (a) BTC 1 and (b) BTC 2.



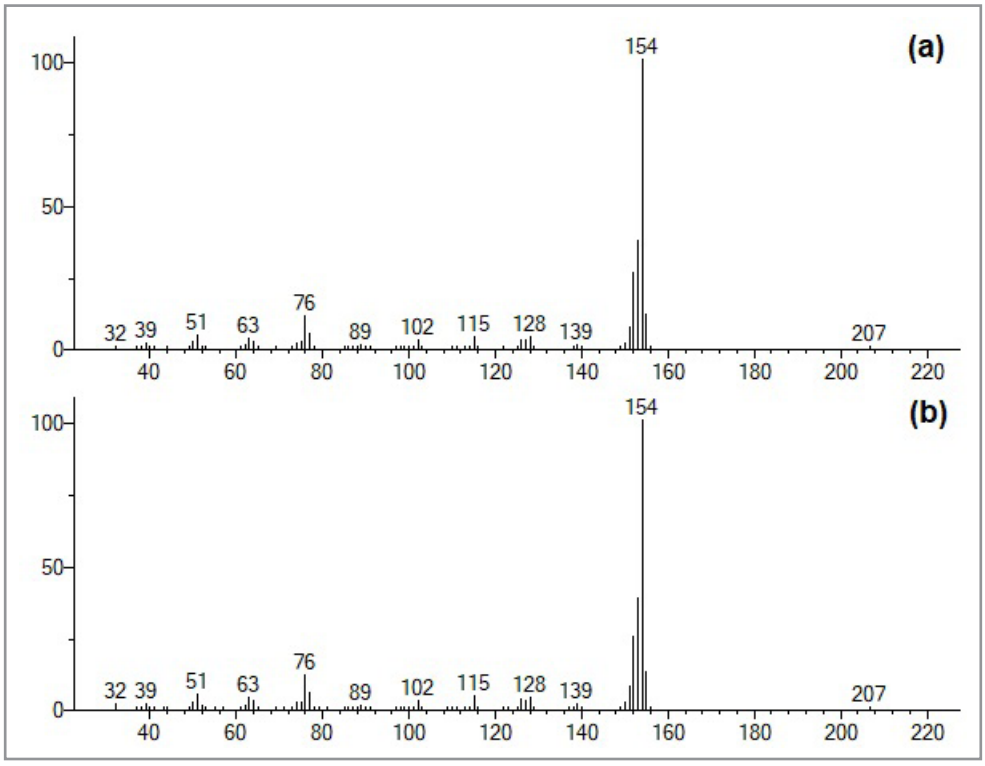
GC-MS spectra of 3-(3-Carboxy-4-hydroxyphenyl)-d-alanine (retention time 5.121 min) in sample BTC 2.



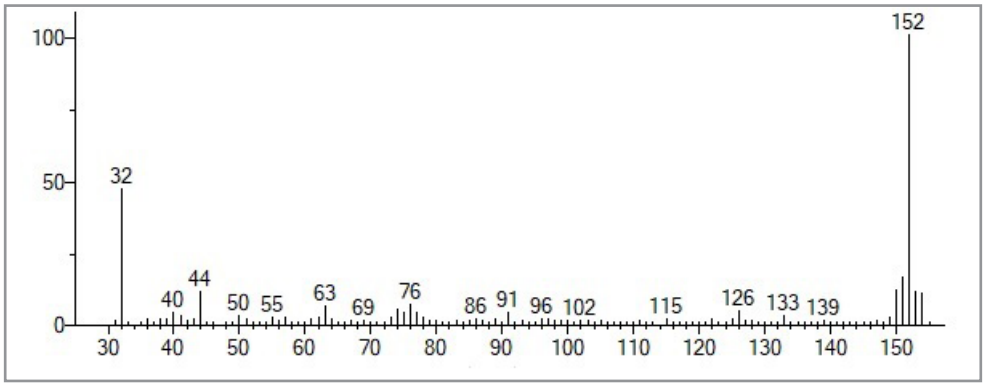
GC-MS spectra of benzaldehyde (retention time 6.237 min) in sample (a) BTC 1.



GC-MS spectra of phenol (retention time 6.460 min) in sample BTC 1.



GC-MS spectra of biphenyl (retention time 13.809–13.813 min) in samples (a) BTC 1 and (b) BTC 2.



GC-MS spectra of acenaphthylene (retention time 15.347 min) in sample BTC 1.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 162-176
doi: 10.30744/brjac.2179-3425.AR-42-2021

BrJAC

ARTICLE

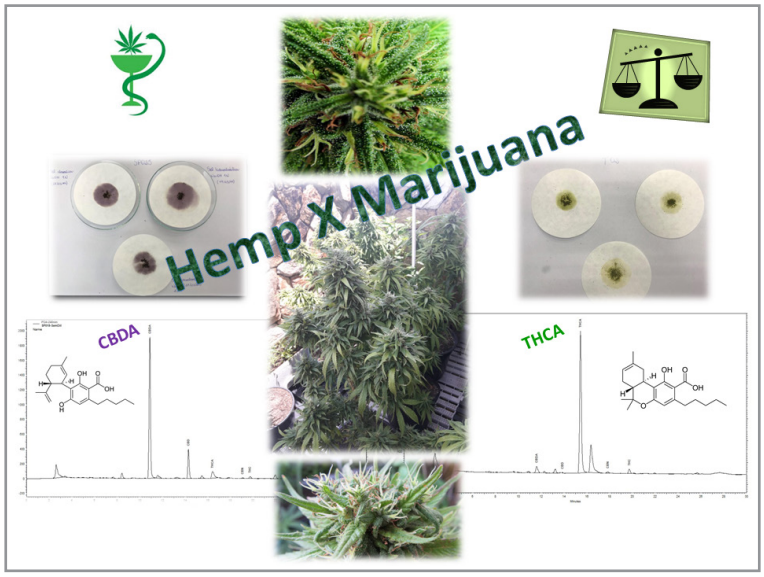
Facing the Forensic Challenge of Cannabis Regulation: A Methodology for the Differentiation between Hemp and Marijuana Samples

Presumptive and confirmatory methods for hemp and marijuana analysis

Virgínia Martins Carvalho[1]*, Fernando Gomes de Almeida[1,2], Andrey Fabiano Lourenço de Aguiar[1], Fernanda Cardoso dos Santos Pereira[1], Ernesto Díaz Rocha[1]

[1]*Laboratório de Análises Toxicológicas, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Av. Clementino Fraga Filho, 373, Ilha do Fundão, Cidade Universitária, 21941-902, Rio de Janeiro, RJ, Brazil*

[2]*Laboratório Geral de Perícias Químicas, Instituto de Criminalística Carlos Éboli, R. Pedro I, 28, 20060-050, Rio de Janeiro, RJ, Brazil*



Access to medical and recreational cannabis has been regulated in several countries. Cannabinoids are secondary metabolites used as chemical markers of *Cannabis* genus plants. Tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most abundant cannabinoids and their proportion is used to differentiate hemp (THC/CBD < 1) from marijuana (THC/CBD > 1) varieties. Brazilian sanitary regulations permit prescription, importation and sale of cannabis-derived products by pharmaceuticals distributors and drugstores, even though their crops are still prohibited, and the current scenario is characterized by marijuana trafficking, legal trade of medical cannabis products, and cultivation and production of cannabis-derived products by patients and non-governmental organizations (NGOs), with or without judicial authorizations. Medical cannabis regulation is in progress and there is an urgent need to implement analytical methods for monitoring the chemical profile of cannabis crops. Thus, the goal of the present study was to propose a methodology based on presumptive and confirmatory methods to differentiate the two principal chemovars of *Cannabis* genus plants, i.e., CBD-rich (hemp) and THC-rich (marijuana). A color test and a validated high-performance liquid chromatography (HPLC) method were applied to six cannabis samples cultivated by patients with judicial authorization. The methodology proved

Cite: Carvalho, V. M.; de Almeida, F. G.; de Aguiar, A. F. L.; Pereira, F. C. S.; Rocha, E. D. Facing the Forensic Challenge of Cannabis Regulation: A Methodology for the Differentiation between Hemp and Marijuana Samples – *Presumptive and confirmatory methods for hemp and marijuana analysis*. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 162-176. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-42-2021

Submitted 23 March 2021, Resubmitted 25 May 2021, Accepted 03 June 2021, Available online 23 June 2021.

to be useful for forensic purposes and for quality control of medical cannabis. Plants cultivated by patients showed three profiles, classified as THC-rich, CBD-rich and approximately 1:1 THC/CBD ratio.

Keywords: cannabinoids, hemp, marijuana, medical cannabis, forensic methodology

INTRODUCTION

Cannabis access has been regulated in several countries and many of them have adopted two different framework regulations for the medical and recreational market [1,2]. Before the revolution in its regulation, cannabis fibers had already been legalized in United States of America, Canada and many European countries. Botanically, plants of the genus *Cannabis* are considered to be either monospecific with several subspecies, such as *Cannabis sativa* subsp. *sativa*, *Cannabis sativa* subsp. *indica*, *Cannabis sativa* subsp. *ruderalis*, *Cannabis sativa* subsp. *spontanea*, *Cannabis sativa* subsp. *Kafiristanca* [3], or a multispecies genus, such as *Cannabis sativa* and *Cannabis indica* [4,5]. Although the taxonomic organization is still debatable, all *Cannabis* genus plants contain cannabinoids (terpenephenolic compounds) as chemical markers. More than one hundred cannabinoids have been described, but the most abundant are tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), both derived from cannabigerolic acid (CBGA). CBGA is converted to THCA, CBDA and cannabicromenic acid (CBCA) by THCA, CBDA and CBCA synthases [6].

Acid forms can be converted to decarboxylated derivatives, such as the neutral cannabinoids THC, CBD and cannabinol (CBN), which is a by-product from heat-induced THC degradation and heat-combustion, or accumulated as a result of plant-aging. Although neutral forms are the most pharmacologically active due to their role as modulators of several components of the endocannabinoid system, acid forms have also been associated with therapeutic properties [7].

The proportion between THC and CBD content in plants is used to differentiate chemovars types. According to the United Nations Office on Drugs and Crimes (UNODC), plants are classified based on the concentration of their main phytocannabinoids. For forensic purposes, in a gas chromatographic analysis, if the peak area ratio of [THC+CBN]/[CBD] is <1, then the cannabis plant is considered to be a fiber-type (hemp). If the ratio is >1, it is considered drug-type (marijuana) [3]. US and Canadian regulations only permit hemp cultivars containing less than 0.3% THC to be grown for textile, cosmetics and food supplement markets [1].

Unlike countries in North America and Europe, Brazil has never permitted the cultivation of cannabis fiber-type (hemp) crops. Considering that the Brazilian Federal Drug Law No. 11343/2006 does not define which compounds and plant chemotypes are illegal within its territory, the Brazilian Health Regulatory Agency on Sanitary Surveillance (*Agência Nacional de Vigilância Sanitária*-ANVISA) regulates cannabis and cannabinoid utilization by "*Ordinance No. 344/98"* and other specific sanitary bills. At the present, the use of medical cannabis is permitted only in the form of pharmaceutically formulated products and CBD-rich extracts containing no more than 0.2% THC can be used for any pathological conditions. Cannabis-derived products containing more than 0.2% THC are restricted to palliative care, when either other therapeutic alternatives fail to improve patients' welfare, or in the presence of irreversible or terminal clinical conditions.

Although cannabis crops are still prohibited, some patients and NGOs have been turning to the courts to obtain permission to cultivate cannabis plants and produce medicinal extracts. Thus, *Habeas corpus* (the mechanism of urgent protection against arbitrary detentions) has been granted as a preventive measure for protection of cannabis cultivation. Currently, dozens of patients are being granted *Habeas corpus* and some NGOs have obtained legal permission to cultivate, produce and supply cannabis extracts to hundreds of people with different disorders, with anecdotal claims that they are serving thousands of patients. In this unusual scenario, a project called *Farmacannabis* has been created at the Faculty of Pharmacy of the Federal University of Rio de Janeiro in order to ensure pharmaceutical support for patients who cultivate cannabis under *Habeas corpus*, and to monitor cannabis-based therapies for children with

intractable epilepsy, patients with cancer, chronic pain, Alzheimer's, Parkinson's and other diseases [8]. Within the context of the *Farmacannabis* project, a methodology consisting of a presumptive color test and a confirmatory HPLC method has been developed, and the latter was validated, in order to differentiate CBD-rich and THC-rich cannabis samples.

Medical cannabis regulation requires improvement in forensic analytical methodologies. Thus, the present study proposes the use of this methodology as a tool to clarify cases related to drug trafficking, individual cultivation for medical purposes and sanitary crimes, such as the sale of products that do not obey sanitary specifications.

MATERIALS AND METHODS

Chemicals and reagents

All certified reference standards (CRS) for CBDA, CBD, THCA, THC and CBN were purchased from Cerilliant® (Texas, USA) and stored at -20 °C. Methanol, ultrapure water and n-hexane at HPLC grade were purchased from Scharlau Chemicals® (Barcelona, Spain) and ethanol absolute (≥99.5%, reagent grade) was obtained from Tedia Company® (Ohio, USA). Ammonium formate and sodium hydroxide, which were used for preparation of mobile phase and presumptive test, respectively, were both purchased from Loba Chemie Pvt. Ltd. (Mumbai, India).

Sample collection

Dried flowers (n=5) and leaves (n=1) of cannabis plants cultivated by patients enrolled in a safety monitoring project [8] approved by Ethics Committee of Clementino Fraga Filho Hospital, Number 2021817.0.00005257, were donated for this study between 2018 and 2019. Patients received pharmaceutical support from Farmacannabis project to monitor the crops cultivated and preparation of medical cannabis extracts. Cannabis plants were cultivated by clonal propagation, except Medikit CBD strain, which was germinated from a seed. Clonal propagation explants were obtained by cutting and rooting either in rockwool or jiffies at high humidity and light conditions. After root growth, the young plants were transplanted to pots assembled with coconut fiber, perlite, humus and substrate. The plants were maintained under light and moderate humidity at all times until the flowering period begins in indoor (Cinderella, Harle-Tsu^{a} and Harle-Tsu^{b}) and outdoor (Amnesia Haze, Tolomelli and Medikit CBD) cultivation. After completed the vegetative period plants were maintained in a 12-hour light/dark cycle to estimulate the flowering. The flowers were harvested when trichomes showed brown color, except for Harle-Tsu^{b} specimen, which was harvested in the beginning of flowering. The total period between plant rooting and flower harvesting ranged from four to six months. After harvesting, flowers and leaves were separated by grower and maintained in dark and dry conditions. Harle-Tsu^{a} and Harle-Tsu^{b} strains were transported to the laboratory still fresh, one day after their harvest. Tolomelli was transferred to lab after ten days and Cinderella, Amnesia Haze and Medikit CBD were taken to lab thirty days after harvest.

In the laboratory, samples were dried in a forced air oven (40 °C/15 hours) and stored at -20 °C until analysis.

Confirmatory method

Sample preparation

The efficiency between dynamic maceration (DM) and ultrasonic bath (US) as extraction procedures, besides absolute ethanol and methanol:n-hexane 9:1 v/v as extraction solvents, was compared. Dried inflorescences of a hemp strain were grinded and homogenized by mixing. The masses of 50, 100 and 200 mg were extracted by proposed techniques.

The sample to be extracted by DM was transferred to a beaker glass and 10 mL of extraction solvent was added to be stirred with a magnetic bar for 10 min. The extract was centrifuged at 2007 *g* and the supernatant collected. In the extraction by US, samples were transferred to polypropylene tubes and added 10 mL of extraction solvent, followed by bath in the Elma Schmidbauer® (Singen, Germany)

Elmasonic E30H during 10 min and followed by centrifugation at 2007 *g*. In both extraction techniques, the supernatants were collected into 25.00 mL volumetric flasks and the procedure was repeated twice with the same sample, using 10 and 5 mL of solvent each time. The extracts were filtered through a 0.22 µm membrane and analyzed by HPLC. In order to assess the recovery of extraction in the first-step procedure, the residual sample left after collection of supernatants was fully extracted again two more times and analyzed by HPLC.

HPLC-PDA analysis

A quantification method by HPLC was developed and validated for five cannabinoids, THCA, THC, CBDA, CBD and CBN. The separation and identification were performed using a Thermo Fisher Scientific® (Breda, The Netherlands) HPLC-PDA equipped with a quaternary pump model 600, type Rheos 5600, an Accela autosampler and a 20 Hz PDA Accela detector. Data were processed with ChromQuest 5.0 software. The HPLC separation was based on a method previously published [9], after modifications. A C-18 column (250 mm × 4.6 mm i.d., 5.0 µm) purchased from Advanced Chromatography Technologies Ltd. (Aberdeen, UK) was kept at a constant temperature of 30 °C. The mobile phase consisted of (A) 50 mM ammonium formate buffer, pH 5.19, and (B) methanol at flow rate of 1.0 mL min^{-1}. A gradient elution program was conducted as follows: a linear increase of 68%-85% B (0-10 min) and 85%-92% B (10-20 min) and finally 95% B over 5 min. After 25 min, the column was set to the initial condition and re-equilibrated for 5 min. The total processing time was 30 min. Full spectra were recorded in the range 200-400 nm and quantification was performed at 240 nm. Spectral conditions to calculate the peak purity were wavelength step 1, scan threshold 5 mAU and peak coverage 95%.

Method validation and preparation of secondary reference material

The method was validated according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q2(R1) [10], the guideline for sanitary regulation of herbal medicines [11] and ANVISA's guideline for analytical method validation, RDC Nº 166/2017 [12]. The parameters considered for validation were as follows: selectivity, linearity, limit of detection and quantification (LD and LQ, respectively), repeatability (within-day precision) and intermediate repeatability (between-day precision), and accuracy.

The calibration curves were constructed with CRSs. CBDA, CBD, THCA, THC and CBN CRSs were diluted in methanol to obtain a stock solution at concentration 200 µg mL^{-1}. The stock solution was diluted to obtain six calibrators whose concentrations were established at 2.00, 5.00, 10.00, 25.00, 50.00 and 110.00 µg mL^{-1}. Each calibrator was analyzed in triplicate and calibration curves were constructed by linear regression and the coefficient of determination (r^2) was calculated. The calibration curves were used to quantify the five cannabinoids found in plant samples and to determine their concentration in the secondary reference material (SRM) produced by supercritical fluid extraction (SFE) and used in the repeatability, intermediate repeatability and accuracy assay.

The SRM was prepared from cannabis flowers by supercritical fluid extraction (SFE) in a Top Industrie® (Vaux le Penil, France) 100 mL model extractor (unpublished data). Dry CBD-rich and THC-rich cannabis flowers previously analyzed by HPLC method were grinded, mixed, inserted into the SFE cell and extracted with CO_2 in supercritical state. Methanol was then added to the equipment's separator in order to obtain a methanolic cannabinoid extract. The extract was stored overnight at -20 °C, filtered through a 0.45 µm membrane and quantified according to the calibration standard curves constructed with CBDA, CBD, THCA, THC and CBN CRSs. The SRM was diluted in methanol in order to obtain a solution with approximate concentrations of 2.0 mg mL^{-1} for THCA and THC, 1.0 mg mL^{-1} for CBDA and CBD, and 0.5 mg mL^{-1} for CBN. The final extract was analyzed by HPLC in eight replicates and the mean concentration of each cannabinoid was measured. The final concentrations were THCA=1.75 ± 0.06, THC=1.64 ± 0.05, CBDA=1.14 ± 0.05, CBD=1.25 ± 0.05, and CBN=0.53 ± 0.02 mg mL^{-1}. The SRM was diluted in methanol in order to obtain the quality controls (QCs) used for precision and accuracy assays as shown in Table I.

Table I. Concentrations of quality controls (QCs) used in precision and accuracy assays

Analyte	Concentration [µg mL^{-1}]		
	QC_1	QC_2	QC_3
THCA	18.0	52.0	105.0
THC	16.0	49.0	98.0
CBDA	11.0	34.0	68.0
CBD	13.0	37.0	75.0
CBN	5.0	16.0	32.0

For precision and accuracy assays, QCs were transferred to polypropylene tubes containing vegetal matrix to mimic the extraction conditions in a real situation. As there is no cannabinoid-free cannabis strain, the cannabis vegetal waste (CVW), which was obtained after cannabinoid extraction by SFE of plants cultivated by patients accompanied by Farmacannabis project for preparation of medicinal extracts, was used as vegetal matrix in precision and accuracy assays. CVWs were collected and analyzed eight times by HPLC to determine the average peak areas for cannabinoids, which were subsequently subtracted from the areas of cannabinoid peaks measured in QCs analysis.

The LD was defined as the lowest concentration of each cannabinoid CRS that yielded a signal greater than noise and a good resolution of peak shape. The lowest concentrations were analyzed in duplicate for three runs and those which reached RSD≤10% were adopted as LD. The LQ was defined as the lowest point of the calibration curve constructed with CRS in the linear interval from 2.00 to 110.00 µg mL^{-1}.

The selectivity was determined by the total peak purity and by analysis of nine non-*cannabis* species supplied by the Laboratory of Pharmacobotany (Faculty of Pharmacy, Federal University of Rio de Janeiro). The plant species were rhizomes of *Glycyrrhiza glabra* L. (*alcaçuz*), dry leaves of *Artemisia annua*, *Atropa belladonna* L., *Brugmansia suaveolens*, *Erythroxylum coca*, *Digitalis purpurea*, *Mentha spicata*, seeds of *Paullinia cupana* (*guarana*) and fruits of *Cola nitida* (cola nut). The non-cannabis species were selected based on availability of botanically characterized species. Considering that illicit cannabis can be diluted with several non-cannabis specimens, species containing psychoactive compounds, such as alkaloids and xanthenes, were also included.

Presumptive method

Beam test is based on an alkaline solution that reacts with CBD, resulting in a purple violet color [13,14]. The Beam reagent was prepared by 98% dilution of sodium hydroxide (NaOH) in absolute ethanol or in a hydroalcoholic solution (absolute ethanol:ultrapurified water, 1:1 v/v) to obtain three solutions as follows: (1) 1 mol L^{-1} NaOH alcoholic solution; (2) 1 mol L^{-1} NaOH hydroalcoholic solution and (3) 2.5 mol L^{-1} NaOH hydroalcoholic solution.

Dry flowers (10 mg) from same cannabis samples previously analyzed by HPLC were grinded and transferred to the center of a circular filter paper weight 80 g m^{-2}, 70 mm diameter. Beam modified reagent was dripped on the sample with a Pasteur pipette, 3 drops in the center of sample. The color was observed after 5 minutes.

Data analysis

Microsoft excel and GraphPad Prism 8.4.3 software were used for table plot and statistical analysis, respectively. The data of extraction optimization were compared by the Kruskal-Wallis test, followed by the Dunn's post hoc test at the 0.5% level of significance.

RESULTS AND DISCUSSION

HPLC method

HPLC is the best choice in situations where it is required to differentiate cannabinoids in acid and neutral forms because the analysis does not require heating, in contrast to gas chromatography methods that favors decarboxylation [9,15]. The quality of raw medical cannabis material needs to be determined in order to differentiate acid and neutral forms and to clarify the conditions of cannabis storage that can be also useful for police intelligence. For instance, higher concentrations of acid forms found in fresh flowers can indicate recent harvesting or mild storage temperatures.

The cannabinoid extraction technique provides clean chromatograms with minimal interferences. Extraction with organic solvents is simple compared to other techniques, such as solid-phase extraction. Besides, methanol:chloroform 9:1 v/v, methanol:n-hexane 9:1 v/v and ethanol-based extractions are often described in HPLC methods [6,9,16–18]. Extraction with methanol:n-hexane 9:1 v/v mixture over a period of 10 to 30 minutes shows good cannabinoid yields in a single step [6].

In the extraction optimization assays, we compared the efficiency of extraction using different sample weights. The concentration of CBD extracted from dried plants was affected by the amount of sample analyzed ($H = 7.200$, $Df = 2$, p-value = 0.003), with 50 mg samples showing a slightly increased CBD concentration than 100 mg samples (p-value=0.021). CBDA and THCA extractions were not affected by initial weight of plant material ($H = 5.067$, $Df = 2$, p-value = 0.085 for CBDA; and $H = 5.422$, $Df = 2$, p-value = 0.071 for THCA; Figure 1.A). Furthermore, masses of 50, 100 and 200 mg showed a CBDA yield of more than 99% in the first extraction and CBDA concentrations near the detection limit for the second and third extraction (Figure 1.B). Next, we evaluated the impact of different extraction techniques on the total concentration of cannabinoids. Even though there was a significant difference between medians obtained for CBD and THCA ($H = 9.359$, $Df = 2$, p-value = 0.002 for CBD; and $H = 7.423$, $Df = 2$, p-value = 0.029 for THCA; Figure 2), follow-up analysis by Dunn's post hoc test did not show differences that reached the significance threshold established in our protocol ($\alpha = 5\%$) for any groups. Such difference was not identified for CBDA ($H = 6.897$, $Df = 2$, p-value = 0.050; Figure 2). It was selected the extraction technique based on 100 mg of vegetal mass in methanol:n-hexane 9:1 v/v because this same solvent is used to extract cannabis oily medicinal extracts in our laboratory [19] and 100 mg is the mass used to analyze terpenes by a non-destructive GC-MS method [20].

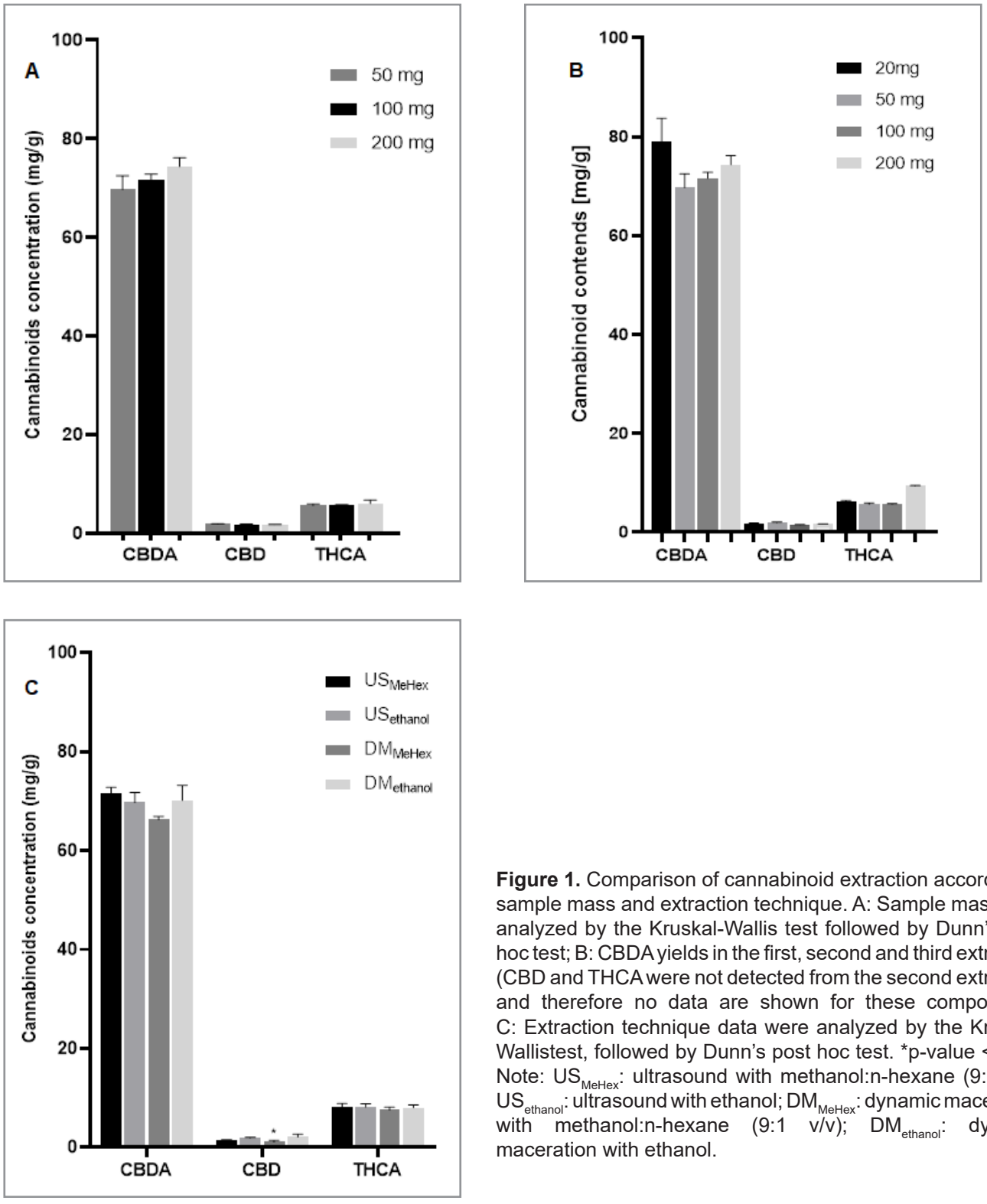


Figure 1. Comparison of cannabinoid extraction according to sample mass and extraction technique. A: Sample mass data analyzed by the Kruskal-Wallis test followed by Dunn's post hoc test; B: CBDA yields in the first, second and third extraction (CBD and THCA were not detected from the second extraction and therefore no data are shown for these compounds); C: Extraction technique data were analyzed by the Kruskal-Wallistest, followed by Dunn's post hoc test. *p-value < 0.05. Note: US_{MeHex}: ultrasound with methanol:n-hexane (9:1 v/v); $US_{ethanol}$: ultrasound with ethanol; DM_{MeHex}: dynamic maceration with methanol:n-hexane (9:1 v/v); $DM_{ethanol}$: dynamic maceration with ethanol.

Typical HPLC cannabis chromatograms (Figures 2 and 3) differed from those of other plant species such as *Atropa belladonna* (Figure 4), even in comparison with the CVW (Figure 5). The chromatographic conditions were selective in the separation of five cannabinoids, the peaks showed good shape and good resolution (Figure 6), with minimal interferences.

Plant analysis is a forensic challenge because the chemical profile varies according to genotype and cultivation conditions (temperature, soil quality, humidity, light conditions, etc.) and a blank is not available since a plant is not expected to be free of typical secondary metabolites [21]. However, the chromatographic profiles of authentic samples were typical and can be combined with the spectra of cannabinoid reference standards to confirm the *Cannabis* genus.

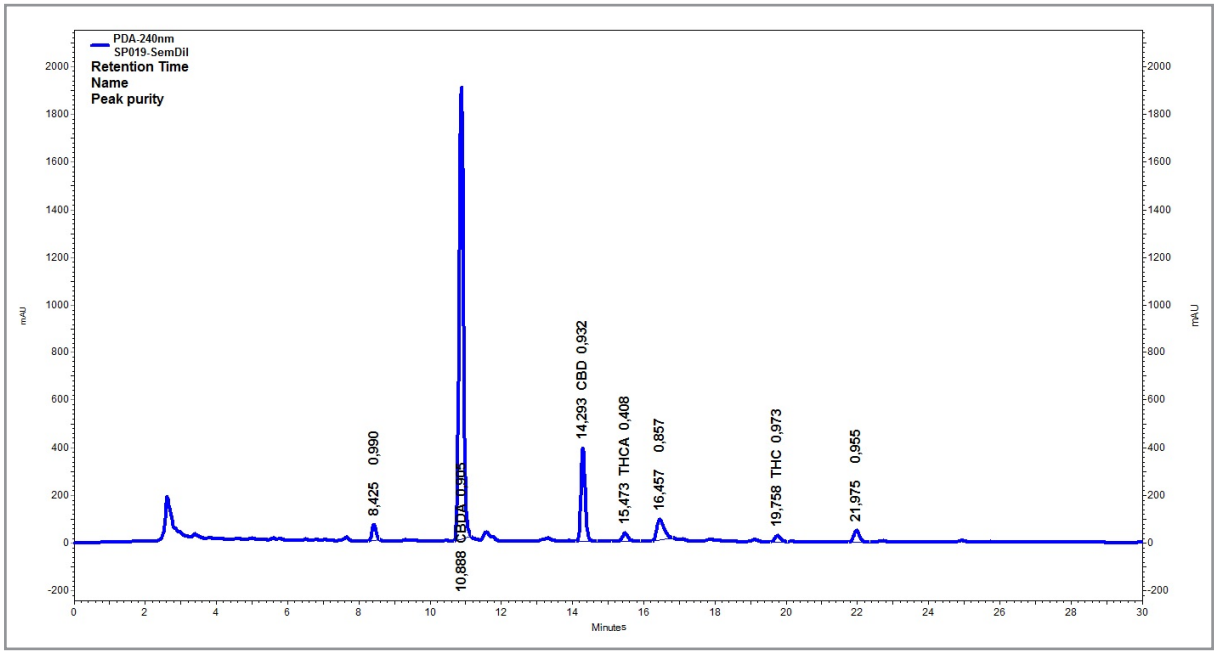


Figure 2. Chromatographic profile of the Harle-Tsu strain of cannabis.

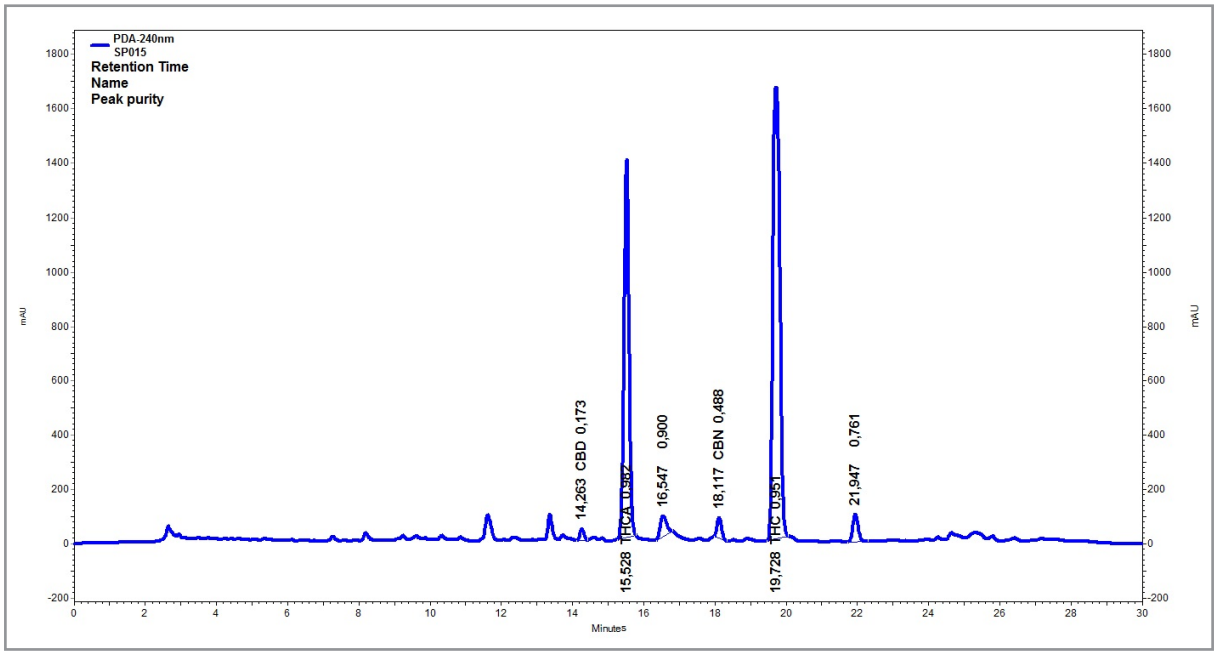


Figure 3. Chromatographic profile of the Amnesia Haze strain of cannabis.

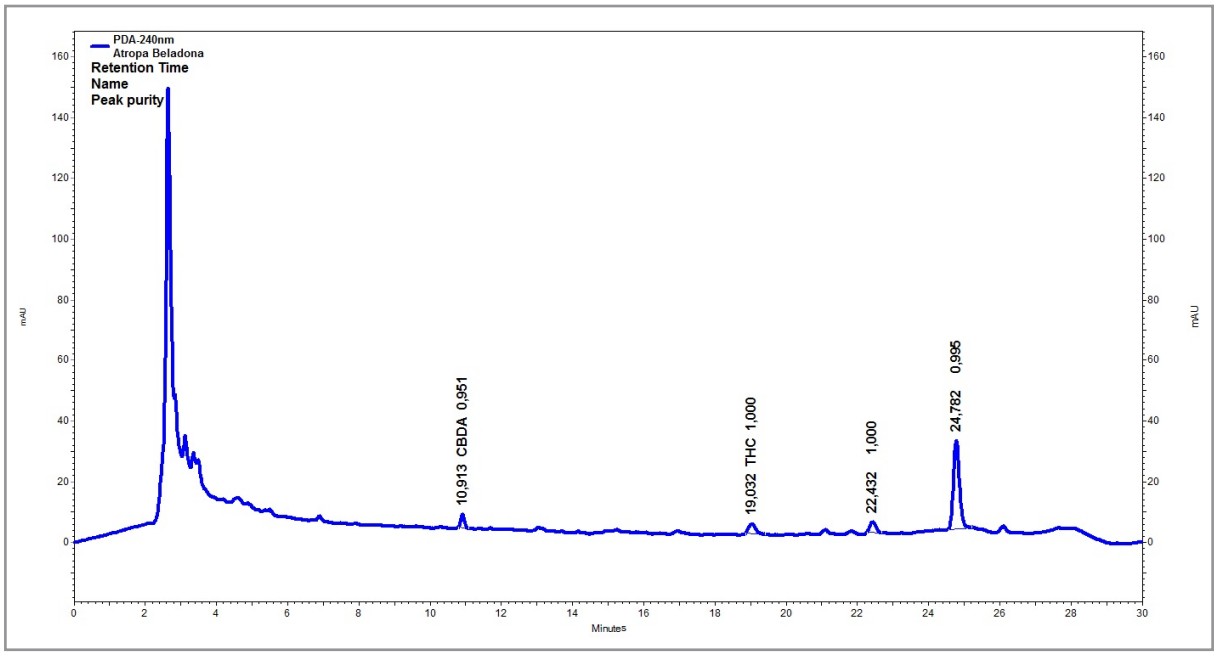


Figure 4. Chromatographic profile of the *Atropa belladonna* leaves.

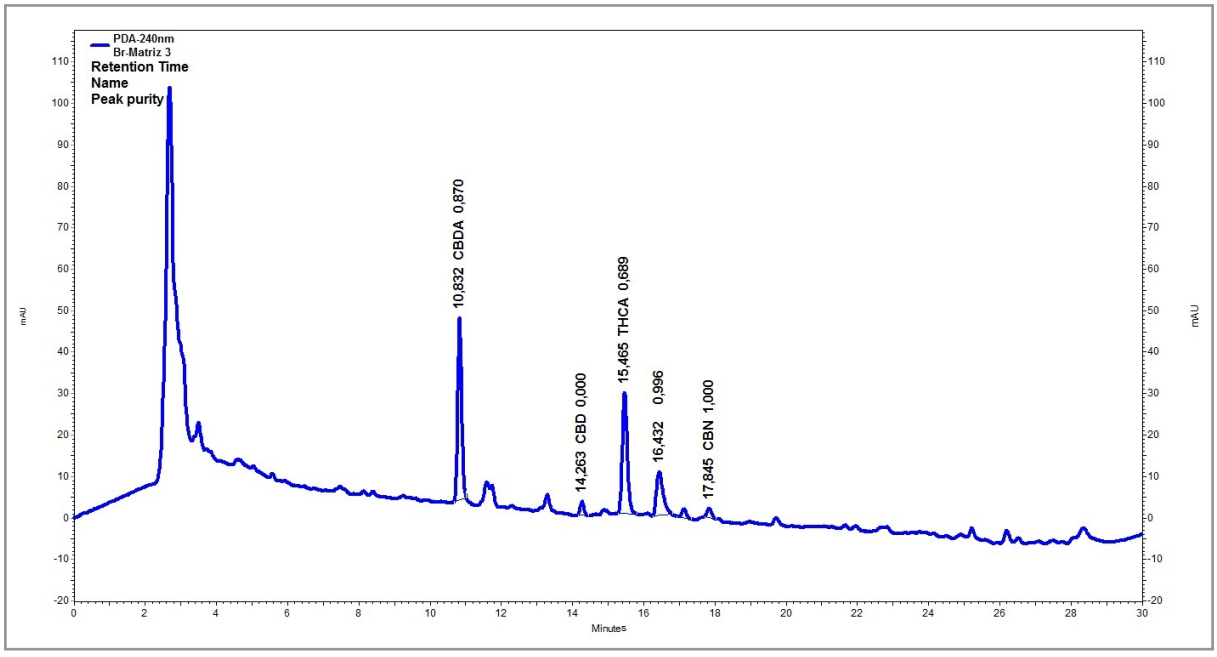


Figure 5. Chromatographic profile of the cannabis vegetable waste after extraction by SFE.

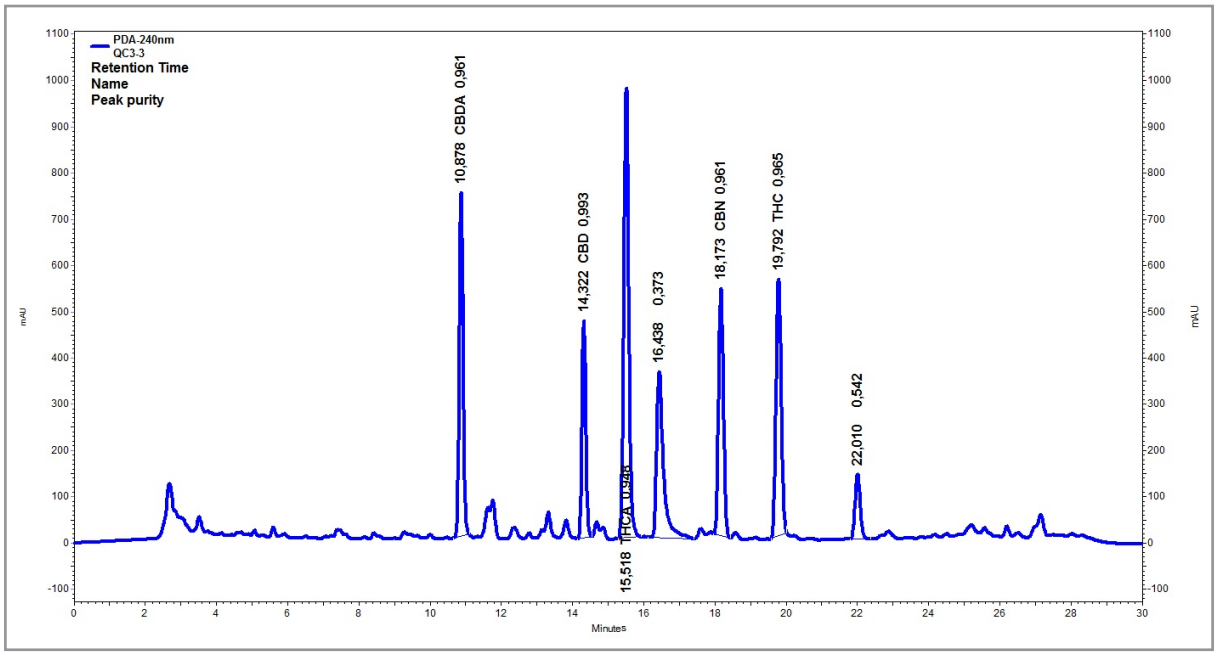


Figure 6. Chromatographic profile of QC3 solution.
Note: Cannabinoid concentrations: CBDA=68.0, CBD=75.0, THCA=105.0, CBN=32.0, THC=98.0 μg mL^{-1}.

Unlike most countries in North America and Europe, the purchase of cannabinoid CRS in Brazil is an obstacle for method validation. There are no national suppliers and importation has become more difficult with the COVID-19 pandemic. Due to the lack of reference standards from plant-derived drugs, Brazilian regulation accepts secondary reference standards (SRS) to validate analytical methods applied to herbal medicines [11,22]. Thus, a methanolic cannabinoid extract prepared by SFE was used as SRS.

A blank plant matrix is not often available because secondary metabolites will always be present in plants. Thus, a cannabis vegetable waste after extraction by SFE (Figure 5) was used as reference matrix for precision and accuracy assay by a spiked technique. The validation results (Table II) satisfied the criteria recommended for herbal medicines [11,22]. LD and LQ were below the cannabinoid levels previously quantified in cannabis leaves (between 1.10% and 2.10%) and inflorescences (between 15.77% and 20.37%) [6].

Table II. Validation results obtained by HPLC for the quantification of cannabinoids in cannabis samples

Parameter	THCA	THC	CBDA	CBD	CBN
LD (μg mL^{-1})	0.40	0.70	0.60	0.80	0.50
LD (mg g^{-1})	0.10	0.18	0.15	0.20	0.12
LQ (μg mL^{-1})	2.00	2.00	2.00	2.00	2.00
LQ (mg g^{-1})	0.50	0.50	0.50	0.50	0.50
Linearity 2.00 to 110 μg mL^{-1} (r^2)	0.999898	0.999995	0.999708	0.999991	0.999992

Table II. Validation results obtained by HPLC for the quantification of cannabinoids in cannabis samples (Cont)

Parameter	THCA	THC	CBDA	CBD	CBN
Repeatability (%RSD)					
QC1	3.18	3.33	1.69	3.20	1.93
QC2	0.32	1.44	0.38	0.40	0.31
QC3	1.60	1.58	1.42	1.46	1.51
Intermediate repeatability (%RSD)					
QC1	4.27	3.86	3.33	2.66	3.82
QC2	1.47	2.02	2.98	2.23	1.79
QC3	1.83	1.99	2.16	1.16	2.14
Recovery (%RSD) – Accuracy					
QC1	76.92	109.76	88.32	94.77	114.82
QC2	82.07	119.28	94.71	99.45	116.85
QC3	79.95	115.06	91.52	96.07	113.21

LD=limit of detection; LQ=limit of quantification; LD and LQ were determined for cannabinoid CRSs (μg mL^{-1}) and estimated for vegetal samples (mg g^{-1}); QC=quality control in μg mL^{-1} is shown in Table I; RSD=relative standard deviation.

The proposed method was applied to five samples of cannabis inflorescences and one sample of cannabis leaves and tree different profiles were obtained: CBD-rich, THC-rich and approximately 1:1 THC:CBD ratio (Table III). Total CBD and total THC values were calculated by the acid form plus the neutral form multiplied by the factor 0.88, which was used to adjust for the different molecular weights of the cannabinoid and carboxylic conjugative components: 314.46/358.47 for THC/THCA and 314.46/358.47 for CBD/CBDA [23].

Concentrations of neutral and acid THC and CBD forms are used to identify marijuana and hemp plants types [6,24]. In this study, five cannabinoids (CBDA, CBD, THCA, THC and CBN) were present in most samples, except for sample with immature flowers (Harle-Tsu[b]) containing 1.6% CBD and THC-free.

Table III. Cannabinoid content in six authentic samples analyzed by HPLC method

	Concentration [mg g^{-1}]							%		Ratio	Classification
Variety	**CBDA**	**CBD**	**THCA**	**THC**	**CBN**	**CBD_{total}**	**THC_{total}**	**CBD**	**THC**	**THC/CBD**	
Cinderella	4.23	1.06	12.76	0.11	0.33	4.78	11.34	0.48	1.13	0.42	marijuana
Amnesia Haze	3.28	1.80	33.57	170.24	1.70	4.68	199.78	0.47	19.98	0.02	marijuana
Tolomelli	37.83	4.52	26.71	12.29	1.06	37.81	35.79	3.78	3.58	1.06	intermediate
Harle-Tsu[a]	60.04	6.30	6.29	1.91	nd	59.14	7.45	5.91	0.74	7.94	hemp
Harle-Tsu[b]	15.55	2.51	nd	nd	nd	16.19	0,00	1.62	0.00	THC free	hemp
Medikit CBD	75.88	100.31	4.84	5.67	1.55	167.08	9.93	16.71	0.99	16.83	hemp

Note: CBD_{total}=CBDA*0.88+CBD; THC_{total}=THCA*0.88+THC=; 0.88 factor: CO_2 lost in the decarboxylation; nd: not detected.

Presumptive color test

Six cannabis samples analyzed by HPLC (Table III) were used to standardize the presumptive color test proposed to differentiate CBD-rich and THC-rich cannabis strains.

The Beam reagent was referred as a specific color test for CBD with positive result characterized by purple violet color [13,14]. The presumptive test was developed with a NaOH solution dripped on a 10 mg of pulverized dry sample, placed in the center of a circular filter paper. The color test has shown positive results in a presumed CBD concentration approximately above 2% (Figure 7). CBD-rich samples showed purple violet color based on CBD content. Considering the intensity of color reaction, the purple violet color was more intense for Medikit CBD, Harle-Tsu^{a} and Harle-Tsu^{b}, suggesting a possible correlation between CBD concentrations and color intensity. THC-rich samples, such as Cinderella and Amnesia Haze strains, showed no color change and samples with CBD and THC contents of approximately 1:1 w/w were positive (purple color). The Beam reagent was proposed in the last century as a color test reagent for the identification of cannabis samples. It was described as a 5% of potassium hydroxide solution in ethanol [25] and a 2% potassium hydroxide solution in ethanol or acidic medium (95% ethanol saturated with HCl) [14], but its use is no longer accepted by forensic laboratories because seized cannabis material has failed to yield a positive reaction with either reagent. The test was later shown to be specific for CBD, while no reaction is led by THC content [14]. In our study, acid solutions (HCl 1 mol L^{-1} alcoholic and HCl 1 mol L^{-1} hydroalcoholic solutions) were tested on the six cannabis samples and the results were negative in all assays (no color change).

Nowadays it is important to identify CBD-rich and THC-rich cannabis species for the medical and forensic areas. In the present study, a reagent consisting of a 1 to 2.5 mol L^{-1} NaOH alcoholic or hydroalcoholic solutions was able to identify CBD levels above 16 mg g^{-1} in dry samples. THC-rich samples with CBD levels below 5 mg g^{-1} showed negative results characterized by no color change.

The 1 mol L^{-1} NaOH alcoholic solution showed more intense colorations and provided the best visualization of colors, especially for Harle-Tsu^{b} and a CBD level 16 mg g^{-1} which is suggested to be the LD.

Many patients enrolled in the *Farmacannabis* project reported buying Beam reagent on the internet, where it is sold as a rapid simple test for the identification of CBD in plants and cannabis extracts. However, the test does not identify THC-rich plants, besides THC/CBD ratio of approximately 1:1 w/w also result in purple violet color. The test is not useful to discriminate medical cannabis strains when THC represents a risk for treatment. For instance, epileptic children under treatment with CBD-rich cannabis may suffer increased seizure frequency if their extracts are prepared with a cannabis strain containing a THC:CBD ratio of approximately 1:1 w/w.

The color test proved to be useful for forensic laboratories as a presumptive rapid test to discriminate hemp and marijuana cannabis strains and can be combined with botanic analysis and other classical color tests (Fast Blue B and Duquenois-Levine) to identify high-THC cannabis (marijuana or drug-type). For instance, a negative result in modified Beam color test and positive result in Fast Blue or Duquenois combined with a positive result in botanic analysis suggest a marijuana strain.

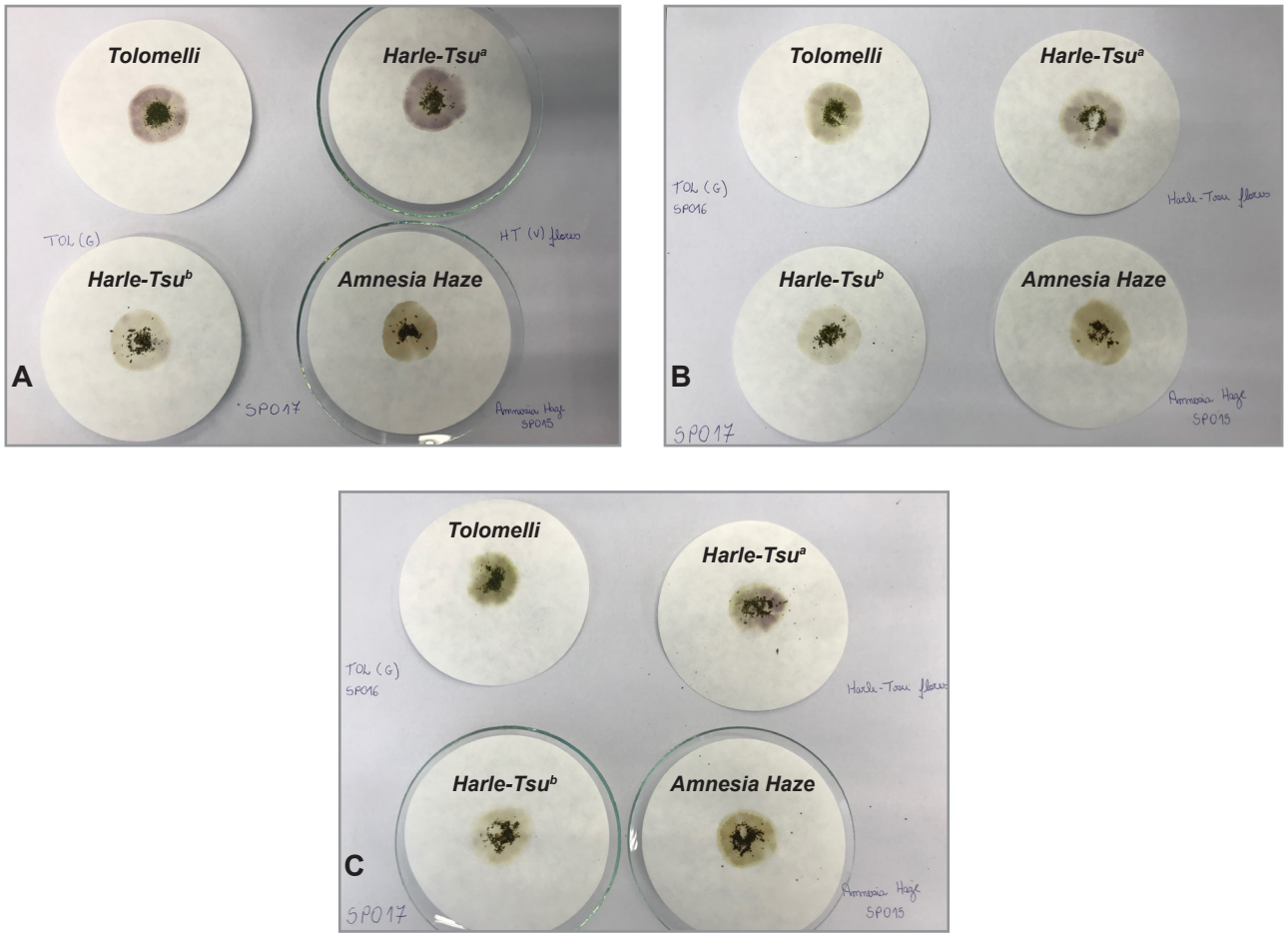


Figure 7. Modified Beam color test applied to cannabis strains. [A] NaOH alcoholic 1 mol L^{-1}; [B] NaOH hydroalcoholic 1 mol L^{-1}; [C] NaOH hydroalcoholic 2.5 mol L^{-1}.

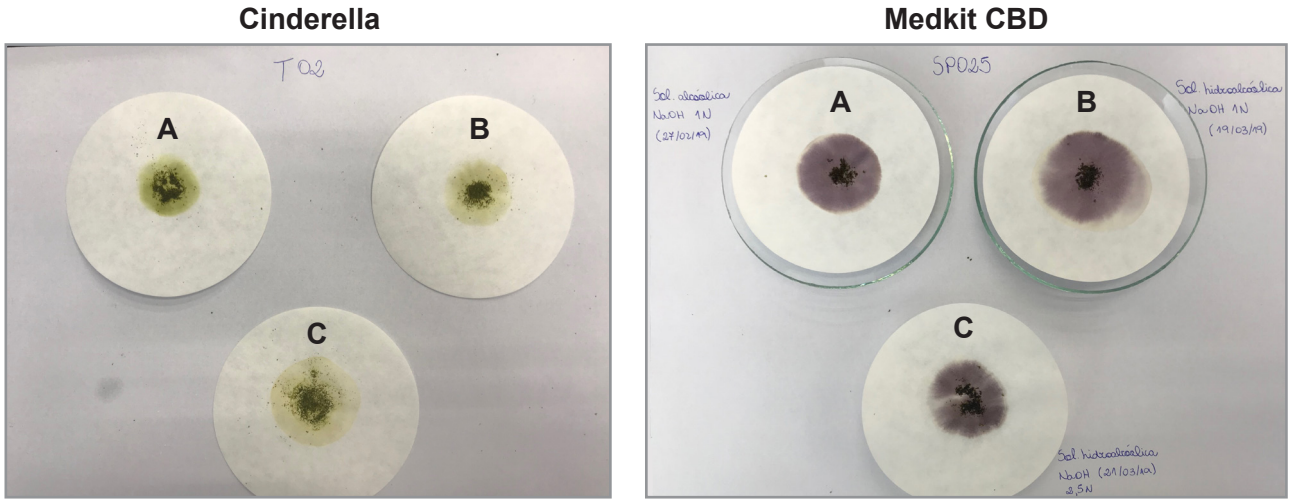


Figure 8. Modified Beam color test applied to cannabis strains. [A] NaOH alcoholic solution 1 mol L^{-1}; [B] NaOH hydroalcoholic solution 1 mol L^{-1}; [C] NaOH hydroalcoholic solution 2.5 mol L^{-1}.

CONCLUSIONS

The proposed methodology, consisting of presumptive and confirmatory methods, is simple and less expensive compared to mass spectrometry-based methods, which could lead to its implementation in most Brazilian laboratories either for forensic purposes or sanitary inspections.

The proposed presumptive color test detects CBD at concentrations above 16 mg g^{-1} in minimal sample quantities (10 mg) and is useful as a presumptive test to differentiate marijuana from hemp-types, but not from intermediate types (THC:CBD ratio of approximately 1:1 w/w).

The cannabinoid profile of the cannabis samples donated by patients indicates three cannabis types, i.e., marijuana, hemp and intermediate types. The medical cannabis profile used in Brazil should be better investigated after increasing the sample size.

Conflicts of interest

We wish confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

The authors gratefully acknowledge financial support from the Serrapilheira Institute [grant number Serra-1709-18891], “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPQ), Brazil [Process Number-426406/2018-4], from the “Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro” [Process Numbers E26 010.000230/2017 and E26/210.260/2018] and scholarship awarded for Fernanda Cardosos dos Santos Pereira [Process Number E-26/202.977/2018].

The authors gratefully acknowledge technical support from the Prof. Dr. Antônio Jorge Ribeiro da Silva, “Instituto de Pesquisas de Produtos Naturais Walter Mors”, UFRJ, for use of supercritical fluid extraction equipment.

REFERENCES

1. Mead, A. *Epilepsy Behav*., **2017**, *70*, pp 288-291 (https://doi.org/10.1016/j.yebeh.2016.11.021).
2. Cox, C. *Health Policy,* **2021**,*125* (1), pp 12–16 (https://doi.org/10.1016/j.healthpol.2020.10.016).
3. United Nations Office on Drugs and Crime. *Recommended methods for the identification and analysis of cannabis and cannabis products*. New York, **2012**.
4. Hillig, K. W.; Mahlberg, P. G. *Am. J. Bot.*, **2004**, *91* (6), pp 966-975 (https://doi.org/10.3732/ajb.91.6.966).
5. Sawler, J.; Stout, J. M.; Gardner, K. M.; Hudson, D.; Vidmar, J.; Butler, L.; Page, J. E.; Myles, S. *PLoS One*, **2015**, *10* (8), e0133292 (https://doi.org/10.1371/journal.pone.0133292).
6. Jin, D.; Dai, K.; Xie, Z.; Chen, J. *Sci. Rep.*, **2020,** *10* (1), pp 1-14 (https://doi.org/10.1038/s41598-020-60172-6).
7. Romero, P.; Peris, A.; Vergara, K.; Matus, J. T. *Plant. Sci*., **2020**, *298*, 110571 (https://doi.org/10.1016/j.plantsci.2020.110571).
8. Carvalho, V. M. *Braz. J. Anal. Chem.,* **2017***, 4 (*16*),* pp 44-49.
9. De Backer, B.; Debrus, B.; Lebrun, P.; Theunis, L.; Dubois, N.; Decock, L.; Verstraete, A.; Hubert, P.; Charlier, C. *J. Chromatogr. B*, **2009**, *877* (32), pp 4115-4124 (https://doi.org/10.1016/j.jchromb.2009.11.004).
10. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. ICH Q2(R1) guideline. *Validation of analytical procedures: Text and Methodology,* **2005**.
11. Agência Nacional de Vigilância Sanitária, ANVISA. Instrução Normativa nº 4 – *Guia de orientação para registro de Medicamento Fitoterápico e registro e notificação de Produto Tradicional Fitoterápico*, Brasília, **2014**.
12. Agência Nacional de Vigilância Sanitária, ANVISA. *Resolução da Diretoria Colegiada - RDC nº 166*, Brasília, **2017.**

13. Thornton, J. I.; Nakamura, G. R. *J. Forensic. Sci. Soc*., **1972**, *12* (3), pp 461-519 (https://doi.org/10.1016/s0015-7368(72)70716-1).
14. Spiro, B. US4771005A, September 13, **1988**, Erez Forensic Technology Ltd., Israel.
15. Patel, B.; Wene, D.; Fan, Z. T. *J. Pharm. Biomed. Anal*., **2017**, *146*, pp 15-23 (https://doi.org/10.1016/j.jpba.2017.07.021).
16. Ambach, L.; Penitschka, F.; Broillet, A.; König, S.; Weinmann, W.; Bernhard, W. *Forensic Sci. Int*., **2014**, *243*, pp 107-111 (https://doi.org/10.1016/j.forsciint.2014.06.008).
17. Gul, W.; Gul, S. W.; Radwan, M. M.; Wanas, A. S.; Mehmedic, Z.; Khan, I. I.; Sharaf, M. H.; ElSohly, M. A. *J. AOAC Int*., **2015**, *98* (6), pp 1523-1528 (https://doi.org/10.5740/jaoacint.15-095).
18. Berman, P.; Futoran, K.; Lewitus, G. M.; Mukha, D.; Benami, M.; Shlomi, T.; Meiri, D. *Sci. Rep.*, **2018**, *8* (1), pp 1-15 (https://doi.org/10.1038/s41598-018-32651-4).
19. Carvalho, V. M.; Aguiar, A. F.; Baratto, L. C.; Souza, F. L.; Rocha, E. D. *Quim. Nova*, **2020**, *43* (1), pp 90-97 (https://doi.org/10.21577/0100-4042.20170457).
20. Rocha, E. D.; Silva, V. E.; Pereira, F.; Jean, V. M.; Souza, F. L. C.; Baratto, L. C.; Vieira, A.; Carvalho, V. M. *Rodriguésia*, **2020**, *71*, e01192019 (https://doi.org/10.1590/2175-7860202071040).
21. Vickery, C. R.; La Clair, J. J.; Burkart, M. D.; Noel, J. P. *Curr. Opin. Chem. Biol*., **2016**, *31*, pp 66-73.
22. Swift, W.; Wong, A.; Li, K. M.; Arnold, J. C.; McGregor, I. S. *PLoS One*, **2013**, *8* (7), e70052 (https://doi.org/10.1371/journal.pone.0070052).
23. Richins, R. D.; Rodriguez-Uribe, L.; Lowe, K.; Ferral, R.; O'Connell, M. A. *PLoS One*, **2018**, *13* (7), e0201119 (https://doi.org/10.1371/journal.pone.0201119).
24. Lau-Cam, J.; McDonnell, C. A. *Bull. Narcotics*, **1978**, *30*, pp 63–68. Available at: https://www.unodc.org/unodc/en/data-and-analysis/bulletin/bulletin_1978-01-01_2_page008.html [Accessed 02 March 2021].

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 177-187
doi: 10.30744/brjac.2179-3425.AR-57-2021

BrJAC

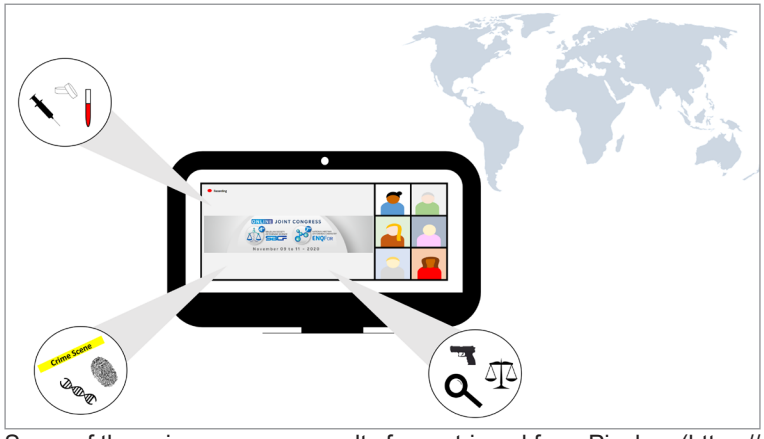
ARTICLE

Trends in scientific communication and continuing education in Forensic Sciences during the pandemic of COVID-19: The role of virtual conferences and experiences of the 2020 Online Congress of the Brazilian Society of Forensic Sciences

Eduardo Geraldo de Campos[1], Daniel Junqueira Dorta[2], Bruno Spinosa De Martinis[2]*

[1]*Departamento de Análises Clínicas, Toxicológicas e Bromatológicas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, Ribeirão Preto, 14040-903, São Paulo, Brazil*

[2]*Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, Ribeirão Preto, 14040-901, São Paulo, Brazil*



Some of these images were royalty free retrieved from Pixabay (https://pixabay.com/pt/)

The pandemic of COVID-19 has changed the landscape of scientific meetings in 2020, leading to the cancellation, postponement or change of format in many conferences. The latter type of change involved the conversion of in-person to virtual meetings. The decision as well as the logistics involved in these changes are not easy. However, the benefits and gains provided by virtual scientific conferences through 2020 in many disciplines have shown that this format can overcome many challenges and promote the diversity in Forensic Sciences conferences as well. In this paper, the benefits and potentials of virtual conferences in Forensic Sciences, including Forensic Chemistry and Toxicology, in promoting scientific communication, accessibility, diversity and continuing education is discussed, in the light of the experiences of the Brazilian Society of Forensic Sciences with the 2020 SBCF Online Congress. The experiences obtained with virtual scientific meetings in 2020 have shown that these formats are very promising and important, and should be increasingly incorporated in future scientific meetings, as an alternative strategy to promote scientific communication, reaching large audiences, without travel limitations and at reduced costs for the attendees.

Cite: Campos, E. G.; Dorta, D. J.; De Martinis, B. S. Trends in scientific communication and continuing education in Forensic Sciences during the pandemic of COVID-19: The role of virtual conferences and experiences of the 2020 Online Congress of the Brazilian Society of Forensic Sciences. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 177-187. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-57-2021

Submitted 07 April 2021, Resubmitted 21 June 2021, Accepted 16 August 2021, Available online 03 September 2021.

However, this approach should not replace in-person meetings, which have many benefits that cannot be replaced by virtual meetings. Therefore, the combination of online and in-person meetings can be very useful strategies.

Keywords: Virtual conferences, scientific communication, continuing education, Forensic Chemistry, Forensic Sciences

INTRODUCTION

The pandemic of COVID-19 has been affecting many people and sectors of our societies in 2020, with high death tolls, elevated rates of unemployment and many other problems. The research and development of vaccines for immunization against the SARS-CoV-2 has been growing in many countries but there is some uncertainty about when the vaccines will become highly available for the majority of the population, mainly in low-income countries. Therefore, for now, the World Health Organization (WHO) still recommends that mask use and social distancing are the best tools for protection against the COVID-19 and for reducing the virus spread [1]. In order to help the spread of the virus following these recommendations, many activities involving a high number of people have been postponed or cancelled in 2020, which include scientific conferences.

Scientific conferences have been directly affected by the pandemic of COVID-19, leading to an impact on other related sectors as well. These conferences are of great importance in academia, industry, public service and more as they serve as an important way for scientific communication and exchange [2], building and fostering partnerships and collaborations, meeting colleagues and promotion of career/job opportunities [3]. In addition, the effects of the pandemic on the mental health of young people and adults due to social distance are quite worrying. The increase in the number of individuals with symptoms of depression, anxiety, distress, use and abuse of licit and illicit drugs during the pandemic is significant, and often leading to suicide attempts and consummation. Maintaining and increasing the promotion of scientific / social activities with the ultimate goal of promoting continuing education, even if it is in a virtual format, can be a way of easing the effects of the serious and sad situation that the world is facing. More specifically, in Forensic Sciences, including Forensic Chemistry and Toxicology, these conferences are a great opportunity for students and professionals from academia, crime laboratories, law enforcement and public health agencies, private laboratories to reunite and discuss the latest advancements within their fields, to present their research, to foster collaborations and to interact with peers. However, in these unprecedented times, when these conferences cannot be held in-person, these opportunities are missed.

In 2020, many important conferences have been cancelled or postponed [3]. The decision of cancelling such important and big events is not easy and involves many factors, especially considering the time lapse of just a few months to analyze the landscape and decide about the congress, as recently highlighted by some scientific societies organizing these meetings (e.g. [4,5]). On the other hand, with the pandemic of COVID-19 in 2020, many opportunities to organize and promote online conferences have arisen. Consequently, a high number of conferences and scientific meetings has been hosted online [2,6], respecting the social distancing recommendations demanded to control the pandemic. In the field of Forensic Sciences many events were hosted during the pandemic in 2020 and early 2021: the inaugural meeting of the International Alliance of Clinical and Forensic Toxicologist (IACFT) [7], the 2020 and 2021 Online Forensic Symposium Series [8–10], The International Association of Forensic Toxicologists (TIAFT) Online Educational Symposium [11], 73rd American Academy of Forensic Sciences Annual Scientific Meeting (United States) [12], which also include the meeting of the Brazilian Society of Forensic Sciences (SBCF) [13].

HOSTING A VIRTUAL CONFERENCE IN 2020: EXPERIENCES OF THE BRAZILIAN SOCIETY OF FORENSIC SCIENCES (SBCF)

The Brazilian Society of Forensic Sciences (SBCF) is "a non-profit association whose objective is to promote research and teaching in Forensic Sciences, to stimulate contact between professionals in the area and to promote the progress of Forensic Sciences in Brazil" [14]. SBCF was founded in 2013 by professors of the BSc in Chemistry with a Major in Forensic Chemistry Program of the Department of Chemistry at University of São Paulo, in Ribeirão Preto, Sao Paulo State, Brazil, and since then has been hosting biannual in-person meetings in Brazil, jointly with the National Meeting of Forensic Chemistry (ENQFor). In 2020, the SBCF Online Joint Congress – 4th Meeting of the SBCF and the 7th National Meeting of Forensic Chemistry (ENQFor) was scheduled to be held in August 2020. However, due to the pandemic, the Congress was postponed to November 2020 and changed to an online format [13].

The SBCF Online Joint Congress (Meeting of the SBCF and the National Meeting of Forensic Chemistry) is already a traditional and expected Conference in Forensic Sciences communities in Brazil. The National Meeting of Forensic Chemistry has been hosted in Brazil since 2008 whereas the first meeting of the SBCF was hosted in 2014. The Conference is an invaluable opportunity to reunite students and professionals from different sectors to discuss the advances and challenges of Forensic Sciences in Brazil and abroad. Many of the students enrolled in the B.S. in Chemistry with a Major in Forensic Chemistry program, offered by the University of São Paulo in Brazil, attend the conference. It is a traditional conference on Forensic Chemistry in Brazil and a good opportunity to meet important professors and researchers in the field as well as to present research results, which may be developed during their internships.

Based on all these aspects, the decision regarding hosting or not the Congress in 2020 was very difficult for the Board of Directors of the SBCF and the 2020 Organizing Committee. In the early stages of the pandemic in March 2020, this decision was put on hold, since the events were still unfolding and there were no clear indications about the world situation by the second semester of 2020. At that point, the Congress already had early bird registrations completed. However, with the spread of the COVID-19 and the recommendations of health agencies and governments, an in-person edition of the Congress was definitely dismissed. From that moment, the Organizing Committee started to consider cancelling the Congress in 2020, to postpone the edition to 2021 or to host the Congress in 2020 in a virtual platform. Considering the importance of guaranteeing the safety of all the participants, the importance and impact of the Congress and the importance of providing continuing education for the forensic community in a year so greatly affected with the cancellation of several meetings, the Organizing Committee decided to host the Congress in 2020 in an online platform [13].

The 2020 SBCF Online Joint Congress was a three-day meeting, with workshops, thematic sessions and conferences. The conference was hosted online using Zoom® platform (https://zoom.us/meetings). Ten workshops were offered in the Congress first day: Evidences interpretation, Infringing conduct in adolescence, Ante mortem and Postmortem Forensic Toxicology, Validation of Analytical Methods in Forensic Chemistry, Stable Isotopes in Forensic Sciences, Investigation in the Dark Web, Portable Methods in Forensic Chemistry and Scientific Methods in Crime Scene Investigation [13]. During the Conference second and third days, twelve virtual thematic sessions were hosted simultaneously: Crime Scene Investigation and Forensic Ballistics, Legal Medicine, Legal Odontology and Forensic Anthropology, Environmental Crime Scene Investigation, Computer Forensics and Investigation in Audiovisuals and Electronics, Documentoscopy, Forensic Chemistry and Toxicology, Criminology and Forensic Psychology, Forensic Biometrics, Forensic Genetics, Errors in Forensic Science and the role of the Technical Assistant, Teaching in Forensic Sciences and Criminalistics in Latin America [13]. The session of Forensic Chemistry and Toxicology was the 7th National Meeting of Forensic Chemistry (ENQFor). Each one of these twelve sessions hosted four or more talks with experts from Brazil and abroad. In addition to these sessions, four panels were held in the Congress, to discuss interdisciplinary, forensic topics of interest. These conferences discussed: the access to trends and perspectives of Forensic Sciences; expertise/investigation in Brazil; organized crime in Brazil and; the interface between investigative journalism and crime investigation [13].

The scientific program also included a special lecture on fake news and oral presentations sessions. Furthermore, during the online meeting, the fourth edition of the traditional ceremony of the "Prêmio Destaque Forense" award, granted by the SBCF during each biannual meeting since 2014, was also held [15]. The number of participants (including attendees and guest speakers) was 637, with 72 guest speakers from Brazil and 30 guest speakers from 13 countries (Switzerland, Canada, South Korea, Portugal, USA, Spain, Mexico, Australia, Chile, Argentina and Colombia) [13]. In the previous edition of the meeting, held in-person in 2018, the number of participants (including guest speakers) was of 364, with 53 guest speakers. Among the attendees, there were undergraduate and graduate students, postdoctoral researchers, professors, experts with State and Federal Police and other forensic professionals [13]. The number of abstracts submitted and approved was 49, being 42 in the format of expanded abstracts and 7 in the format of platforms [13]. The organizing committee of the event adopted the model of "expanded abstracts", consisting in abstracts with the possibility of longer length and inclusion of figures and tables, with the latter being components that are not normally accepted in regular abstracts. Regarding abstract submission there was a great decrease compared to previous editions of the meeting that were held in-person: in 2020, 49 abstracts were accepted in contrast to 126 abstracts accepted in the edition of 2018. However, some reasons associated with this reduced number of works submitted for presentation might be the short timeframe for submission, due to the change from in-person to online event, and the restrictions imposed by the pandemic, suspending or restricting researches in many laboratories.

INCREASING THE ACCESS AND SCIENTIFIC COMMUNICATION THROUGH ONLINE CONFERENCES

An online conference has a high potential to promote national and international access to the event, with increased accessibility. Participants and speakers that would not have access to the in-person meeting have more chances to attend it online. Since there is no need to travel, no costs with transportation and accommodation and no need to have visas or passports to attend, the participation can be easier and limitations may be overcome for an increased number of attendees, including people from other regions in the world or with disabilities or vulnerabilities, for example [2,16]. Moreover, it is also a great opportunity to invite speakers from different countries, overcoming the physical distance, travelling issues and potential conflicts of agenda with other appointments. As an example, in the 2020 SBCF Online Congress, guest speakers and/or attendees from North America, South America, Asia, Europe and Oceania participated in the conference, which might not have been possible for an in-person conference.

A virtual conference in Forensic Sciences, such as in other Sciences, is also a great opportunity to foster international collaboration and rapid scientific communication. In Forensic Chemistry and Toxicology, there is a need for rapid communication and notification especially in some fields, such as in the notification of new synthetic drugs in forensic casework. This is of great interest for researchers, forensic chemists and toxicologists, law enforcement and public health agents and communities involved in this field. Therefore, online conferences can be a very useful tool for this purpose, making it possible hosting conferences and symposiums more frequently throughout the year and increasing the target audience. For example, biannual meetings could be hosted annually, alternating between online and in-person meetings. The potential to increase the target audience is also very beneficial, to promote the internationalization of these meetings and the exchange of information between forensic professionals. These professionals may be facing similar challenges in different countries or learn different strategies from other countries that could work in their casework, for example.

OPPORTUNITIES FOR CONTINUING EDUCATION IN VIRTUAL CONFERENCES

Continuing education is highly important for students and professionals involved with Forensic Sciences in order to promote the advance of the field [17]. In this case, continuing education opportunities in conferences, workshops and symposiums are an excellent way to keep updated or gain knowledge in a specific topic or theme. In addition, these opportunities can be even more interesting for those that

were not able to attend an undergraduate or graduate program in Forensic Sciences but are interested in pursuing a career in this field. Educational programs (undergraduate and graduate) in Forensic Sciences are increasingly being designed and offered in many countries, but these specific programs may still be limited in some areas. Furthermore, in Forensic Sciences, certifying organizations may require continuing education and professional development for maintaining a certification of those certified professionals, which may include attending courses, workshops and conferences [18,19], such as the American Board of Toxicology [20] and the American Board of Forensic Toxicology (ABFT) [21] for example. During the scientific meetings promoted by associations partnered with these professional organizations, some activities grant continuing education credits, such as the ABFT, which accepts continuing education credits for the presentation of workshops, posters or platforms, for example, during the meetings of the American Academy of Forensic Sciences (AAFS), Society of Forensic Toxicology (SOFT) and The International Association of Forensic Toxicologists (TIAFT) [21]. In case these meetings are not offered by any reason or the professional is not able to attend the meeting, an invaluable opportunity for continuing education is missed.

Virtual conferences may offer the opportunity for participants to access recorded lectures, workshops and other activities hosted in the event, in alternative schedules or even after the end of the event. In the 2020 SBCF online meeting, the attendees had the possibility of access all the content of the event (which was previously authorized by the speakers and recorded) for up to 30 days after the end of the event (if member of the SBCF) or up to 10 days after the event (if non-member of the SBCF) [13].

THE BENEFITS AND REQUIREMENTS OF VIRTUAL PLATFORMS VIRTUAL CONFERENCES

Virtual conferences are hosted in online platforms, which can allow a number of benefits. For example, through virtual platforms, visualization and sharing of content and data can be easier and occur still during the conference [2]. The content of the presentations is displayed directly on the computer, tablet or smartphone screen. The use of the platform can also lead to a more rapid interaction between speakers, sessions chairs and audience, which can occur through chat or microphone/audio. The session chairs have more control of the questions and can organize the questions and answers sessions in a more effective way, grouping similar questions and optimizing time for more interactions. However, it is important to consider that each type of virtual platform has its own features and allows different types of interaction [6].

The use of virtual platforms also makes authorized recording of sessions easier, and the content can be offered for the attendees to watch or rewatch it on-demand after the conference [16]. During in-person meetings, in general, sessions are not recorded and the participants need to choose to watch one presentation over another when they are occurring simultaneously, while in the online conference, they can watch one session through live streaming and the other one on-demand after the conference. This is particularly interesting in the field of Forensic Sciences. As multidisciplinary events, Forensic Sciences meetings usually host a number of different thematic sessions. In the 2020 SBCF Online Congress, the majority of the guest speakers authorized recording their lectures in thematic sessions or workshops, which were made available for the attendees. However, the guest speakers because of ethical issues regarding images, videos or data, which is not unusual in Forensic Sciences, may not authorize recording their lectures, and some of the lectures may not be offered on-demand to the attendees. For this reason, it is very interesting to inform the participants in advance which sessions are not being recorded and which ones will.

It should be noted that despite the benefits, there are some requirements in regards to the online platform. Some aspects should be considered when designing an online event: potential audience size, duration of the event, interactivity, recording options and technical support. First, if the goal of the conference is to reach large audiences and have no limit of public, the organizing committee should search and use platforms with no public limitation or with high capacity, in order to avoid any issues or limitations in registrations. In regards to the duration of the meeting, a similar approach should be used and platforms with no time limitation are recommended, especially considering that delays may occur and alter the

duration of each planned activity in the event. In addition to these topics, technical support is very useful, even for more experienced users of specific and on-line meeting platforms. It is recommended to have at least one technical staff in each virtual room of the conference, to support session chairs, guest speakers and attendees in case of any issue or to provide technical orientations. Although online meetings and classes are becoming more common nowadays, some people may not be familiar with new or less known platforms and having technical support is paramount. It is also a good strategy to keep a direct contact line between chairs of the sessions and technical staff, in case the chair of the session loses internet connection or similar issues. In this case, the session chair or moderator can easily communicate with the technical staff to communicate attendees and guest speakers. An interesting strategy adopted by the organizing committee of the 2020 SBCF Online Congress was to host a non-mandatory "training session" for all interested guest speakers, which were offered in different time-slots throughout two different days, in order to provide them with training in how to access and use the platform. Some of the speakers had interest in attending it and could train how to share their screen, see the attendees and more. The organizing committee organized two virtual rooms with a technical staff present and also provided the speakers with a guide and a tutorial to access the platform and use common tools such as microphone, video, screen share and chat. These approaches may be particularly interesting if a non-public available platform (e.g., an institutional platform) will be used for the conference.

There are several platforms available online, in free and paid versions. However, in general, most of these platforms have limited availability in free versions, limiting the use of it for large group meetings, during unlimited time and/or with technical support. For these reasons, it is more recommended to adopt paid versions of these platforms or to use alternative platforms that have no limitations on number of participants and time. In this context, it is important to find a platform that has these features.

THE IMPACT OF MULTIPLE LANGUAGES IN ONLINE CONFERENCES

The promotion of diversity and inclusion is very important in Science. There is a need for searching strategies and approaches to promote access for very diverse audiences. This is surely very beneficial in any field, including the Forensic Sciences, because it can increase the access to scientific advances and, at the same time, it can promote the discussion, exchange of information in a very open and diverse environment. Therefore, as mentioned before, hosting an international online conference is a powerful tool to promote these actions in Forensic Sciences. However, the language barrier can be a challenge to overcome.

A virtual conference is a great opportunity to foster international exchange of information and collaboration, including in Forensic Sciences. Offering translation and captioning during the presentations is a very useful and powerful tool to increase the access of people from different nationalities and/or with hearing or visual disabilities to these conferences [2]. This strategy applies not only to the attendees in general but also to guest speakers, and a diverse number of specialists from different nationalities can be invited to present and share their experiences. For example, one of the main characteristics of the SBCF biannual congresses is to offer translation of lectures and courses in English to Portuguese, to promote the inclusion and access to all the attendees who are not fluent in English, since the official language in Brazil is Portuguese. During previous in-person meetings, this service was offered and used by many attendees, who can attend the lectures of renowned specialists even without speaking the speaker's language. In the 2020 SBCF Online Congress, the translation services were offered for almost all panels, lectures and workshops presented in English or French. The sessions in Spanish were not translated because there is more similarity between Spanish and Portuguese languages. In virtual conferences, this service is easier to use, since no accessory is needed (such as the use of headphones for hearing the interpreter rather than the speaker during in-person meetings) and there are specific features in the platform that allow selecting the language.

It is important to note that the translation in an online conference involves the platform features, the technical staff and the interpreter. For this reason, previous tests before the conferences are useful to

coordinate these actions, especially if the speaker or the interpreter have no experience with the platform used. In the 2020 SBCF Online Congress, the Zoom® platform was used for the conference and the attendee had the option to choose between listening to the guest speaker's audio or the interpreter's audio. The experience was positive and worked well for all the sessions. However, the translation increases the access to all sessions and, overall, to the Congress and, for example, in Brazil, this service is costly, especially for highly-trained professionals. Therefore, this needs to be considered when these services are required. On the other hand, with the development of new technologies, translation apps and softwares might be more available nowadays, which allow the reduction of the translation-related costs. In the 2020 SBCF Online Congress, based on the experiences of previous meetings with the presence of the interpreter and the lack of knowledge on translation apps, the interpreter was present for the translation, but it is important to highlight that there may be some alternatives to this approach. Overall, although there may be some limitation regarding costs with these services, the possibility of translation in the online conference has important benefits, increasing the access to all the sessions in a language other than the attendee's language and supporting the goal of reaching larger audiences, and therefore, the internationalization. This can lead to more diverse audiences and events that are more inclusive.

POSTERS AND PLATFORMS PRESENTATIONS IN VIRTUAL CONFERENCES

Posters and platform presentations are core sessions of many scientific conferences. In online formats, these sessions are different and may not offer the same level of interaction. On the other hand, during virtual conferences, abstracts, platforms and posters may be more easily accessible and be available longer, making it possible to attend oral and poster presentations that are occurring simultaneously [2]. For example, in the 2020 SBCF Online Congress, the organizing committee offered two options for submitting abstracts for presentation: expanded abstracts and recorded oral presentations. Expanded abstracts were available by the beginning of the conference in the abstracts book and oral presentations were exhibited during the event for all the attendees. The experience with these two formats were both successful and led to an important part of the congress. However, the direct interaction between presenters and attendees for questions and comments was missed.

In online conferences, it may be interesting to explore alternatives that allow the contact between presenters and audience conferences for questions and comments. In the event of the SBCF, due to the short time frame for restructuring the event, it was not possible to explore and search for different platforms that would allow the exhibition of posters and to host a session of oral presentations with time for questions (the oral presentations were prerecorded). For example, the presentations (poster or platforms) can be performed online, in which the attendees can see the poster or watch the presentation and ask questions to the presenter, who will be online in a specific period, such as in parallel virtual rooms [22]. For example, building a virtual exhibit hall for the event can be a strategy for fostering sponsorship opportunities [22]. In addition, if possible, this can also be a good strategy to exhibit posters submitted for presentation. For example, a virtual exhibit hall was implemented during the virtual event of the AAFS held in 2021 [12]. However, these strategies lead back to the discussion of which platform to select for the conference and it is important to consider the availability of technical resources for this. Although the level of interaction may not be the same when comparing online and in-person meetings, there are online tools that can provide a good level of interaction between presenters and attendees.

PLANNING OF COSTS AND REGISTRATIONS IN VIRTUAL CONFERENCES

In a fully online conference, there are no costs with venue and air tickets and hotels for guest speakers and organizers, which significantly reduce the overall costs of the event. In this sense, online conferences are usually less costly than in-person conferences for the organizing institutions [2]. However, as mentioned before, the "online facilities" have costs as well, including the platform and technical assistants. In addition, translation services, organizing companies and design and marketing services are still required and have costs as well.

There are two options for online conferences in regards to the registrations: free or paid registrations. In the 2020 SBCF Online Congress, the organizing committee has decided to charge for the registrations but at a much smaller fee in comparison to an in-person meeting. This needs to be rationalized considering attendees will not be offered a "physical structure" for the event and will not have the social part of the conference, which demands costs as well. However, a free-of-charge conference may be more accessible and increase the number of attendees. In this case, the support from sponsors and from the organizing society are invaluable sources of resources to host the online conferences.

PERSPECTIVES OF VIRTUAL CONFERENCES IN FORENSIC SCIENCES

Distance education is a pedagogical method that has been in use for a long time and that has moved from correspondence-based to internet-based courses over the years [23]. This methodology is interesting in many situations such as for students with limited access to a university or time to attend an in-person course, for example [23,24]. With the pandemic of COVID-19, the inclusion of online learning in many sectors has been accelerated, including scientific conferences. These conferences are very important in many areas and are the way some scientific associations have to discuss the advancements in their fields. Although with the pandemic some of these conferences had to be cancelled or postponed, in some cases such as in the 2020 SBCF Congress, conferences were moved to a digital platform. The virtual format for conferences certainly has several benefits in regards to access to the conference, scientific communication and interactivity.

The promotion of diversity and inclusion in Sciences is paramount and has been increasingly discussed. The diversity in the scientific environment should be and will be increasingly focused and approached by researches and funding agencies [2]. In Forensic Sciences, diversity and inclusion should be increasingly promoted as well. This field has been attracting and fascinating many people over the years, in many countries, especially young people searching for a career and degrees. Unfortunately, not all regions in the globe have opportunities, programs and events in Forensic Sciences. However, the opportunity of virtual scientific meetings can change this landscape and offer alternative ways to attend these conferences and advance the knowledge in this field. These virtual conferences can reach larger audiences over the world and promote increased access to the Forensic Sciences. Reduced-cost or free registrations and translations are some of the strategies that certainly can make these virtual conferences more accessible to diverse and larger audiences. Ultimately, this can lead to increased accessibility and more diversity in the audience, and scientific communication to larger audiences.

The partnership with other scientific associations, institutions and sponsors in hosting online conferences can have benefits not only for the conference itself. As mentioned, when the conference is being hosted online, the extent of the conference is usually greater and more people can be reached. In this context, the publicizing of the organizing scientific societies and institutions is also increased. For example, when education institutions are sponsors of these conferences, there is an increased possibility of reaching potential students nationally and abroad. In addition, for scientific associations and certification associations, it is an increased opportunity to reach out for new members and to communicate the actions and projects of these institutions. For example, in the 2020 SBCF Online Congress, the SBCF offered special registration fees for members of other partner associations such as the Brazilian Society of Toxicology (SBTox) and the National Association of Federal Criminal Experts (APCF) [13].

Scientific communication and research collaborations can also be improved, reaching more people in the scientific community through virtual conferences. In the forensic community, these national and international collaborations are paramount, especially considering many aspects of the several disciplines within the Forensic Sciences. The need for effective and rapid notifications within the Forensic Sciences can help build a global network in several areas. Novel psychoactive substances, DNA analysis in human identification and sexual assault cases and cybercrimes are some examples of current topics of forensic interest that can reach more people and international stakeholders, benefiting the exchange of information and rapid notification. The potential of having presentations recorded can also benefit the

scientific discussion after the conference, promoting the collaboration between different research groups, institutions and agencies. Furthermore, online conferences help in reduction of carbon footprint [2].

For the success of the conference, good planning and best practices should be adopted and performed. Sessions chairs, guest speakers and attendees should be oriented regarding the functioning of the virtual platform, the ways of interaction available and best practices for the event. A good practice is to prepare a material with recommendations for the guest speakers, including orientations regarding accessing the virtual room, installing the platform, minimal requirements for internet connection and other recommendations such as environmental light and sounds. The chairs of the sessions should have a clear protocol to coordinate each session, including introducing the speakers and interacting with the audience as well as with the technical staff when needed. Technical support is a key factor in online conferences and it is recommended to have this support available during the live conference, and testing sessions with hosts and guest speakers before the conference are recommended as well [25]. Another important point to highlight is to consider several different strategies of marketing the conference, to reach large target audiences [25]. This step is an important part of the conference and can directly affect the number of participants. In order to promote internationalization and increase the access, using several ways to divulge the conference, including social media, and eventually marketing in different languages may increase the probability of reaching a larger audience. It is also useful to create mechanisms for both speakers and participants being able to communicate their participation in the conference, creating a different level of interaction [25]. Social media (e.g., Facebook, Twitter and Instagram) has been growingly used by scientific organizations to communicate their activities and share information. In this edition of the event of the SBCF, the event was publicized via official website [13], through the official pages of the SBCF on Facebook (https://www.facebook.com/SociedadeBrasileiraDeCienciasForenses/), Instagram (https://www.instagram.com/sbcf.oficial/) and LinkedIn (https://www.linkedin.com/company/sociedade-brasileira-de-ciencias-forenses/) and also through the official page of the event on Instagram (https://www.instagram.com/enqfor/).Therefore, this is a useful way to reach large audiences.

The experiences hosting online conferences in 2020 should not be completely replaced by in-person meetings in the near future, hopefully with the end of the pandemic of COVID-19. On the other hand, it is not the goal of this work to suggest the complete substitution of in-person conferences by virtual conferences. In-person conferences are invaluable sources of research communication and networking, with the unique possibility to host social activities to bring the forensic community together for more interactions. When the conference is held in-person, the attendees can meet their colleagues, friends and potential collaborators in the field. Another advantage is the possibility to interact directly with exhibitors in the booths, to discover new technologies and products offered by the company and to discuss potential collaborations. Some of these exhibitors usually bring instrumentations to the conference, demonstrating in-person how these instruments work, which allows a very helpful discussion for researchers and laboratories interested in those instrumentations. In addition, in-person conferences are a great way to promote the city/state/country hosting the conferences, in social, economic and cultural aspects, which is only possible when these conferences are hosted in-person. Therefore, in-person meetings cannot be completely substituted. However, the gains obtained by online education strategies adopted in 2020 should be continuously adopted in the future.

Considering the benefits and limitations of both virtual and in-person conferences, there are some potential ways to promote the combination of these models, taking advantage of the benefits offered by both models. There are some potential ways to promote this. For example, it is possible to develop a hybrid model, where the attendees and speakers have the chance to attend the conference in-person or online [26]. If one is not able to attend the in-person conference then this person can register for the online conference and watch it live from home, office or laboratory. Another possibility is to include an online-only edition of these conferences, eventually between in-person conferences, in order to promote higher access to those not able to travel and attend in-person.

In summary, the benefits of virtual conferences should be considered for future conferences in Forensic Sciences, as another mode of scientific communication and networking, with potential for increased accessibility and diversity. This format can be useful in fostering and supporting international and national partnerships and collaborations and delivering scientific content. These virtual models can also be combined with in-person models, bringing the advantages of both types of scientific conferences together and increasing the impact of the event.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to thank all members of the Organizing and Scientific Committees, participants, guest speakers, partners, sponsors and AGeventos® for their collaboration in the success of the 2020 SBCF Online Congress – 4th Meeting of SBCF and 7th ENQFor. The authors also thank Ms. Adriana Gediel, director of AGeventos®, for gathering and providing the statistics of the events.

REFERENCES

1. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance [Accessed 24 May 2021].
2. Sarabipour, S. *eLife*, **2020**, *9*, e62668 (https://doi.org/10.7554/eLife.62668).
3. Achakulvisut, T.; Ruangrong, T.; Bilgin, I.; Van Den Bossche, S.; Wyble, B.; Goodman, D. F.; Kording, K. P. *eLife*, **2020**, *9*, e57892 (https://doi.org/10.7554/eLife.57892).
4. https://tiaft2021.co.za/dear-tiaft-friends/ [Accessed 24 May 2021].
5. https://news.aafs.org/meeting-theme/73rd-aafs-annual-scientific-meeting-goes-virtual/ [Accessed 24 May 2021].
6. Fulcher, M. R.; Bolton, M. L.; Millican, M. D.; Michalska-Smith, M. J.; Dundore-Arias, J. P.; Handelsman, J.; Klassen, J. L.; Milligan-Myhre, K. C.; Shade, A.; Wolfe, B. E.; et al. *Trends Microbiol.*, **2020,** *28* (12), pp 949–952 (https://doi.org/10.1016/j.tim.2020.08.004).
7. https://www.iacft.online/programs-1 [Accessed 26 May 2021].
8. https://www.cfsre.org/continuing-education/2020-online-forensic-symposium-current-trends-in-forensic-trace-analysis-archival/ [Accessed 23 May 2021].
9. https://www.cfsre.org/continuing-education/2020-online-forensic-symposium-current-trends-in-forensic-toxicology-archival/ [Accessed 23 May 2021].
10. https://www.cfsre.org/continuing-education/2021-online-forensic-symposium-current-trends-in-seized-drug-analysis-archival/ [Accessed 23 May 2021].
11. http://www.tiaft.org/free-professional-development-symposium-1.html [Accessed 24 May 2021].
12. https://aafs.org/common/Uploadedfiles/Meetings/2021Meeting/2021MeetingProgram.pdf [Accessed 23 May 2021].
13. https://www.enqfor2020.sbcf.org.br/ [Accessed 23 May 2021].
14. https://sbcf.org.br/ [Accessed 24 May 2021].
15. https://www.sbcf.org.br/premio2020 [Accessed 21 June 2021].
16. Robinson, S.; Baumhammer, M.; Beiermann, L.; Belteki, D.; Chambers, A. C.; Gibbons, K.; Guimont, E.; Heffner, K.; Hill, E.-L.; Houghton, J.; et al. *The British Journal for the History of Science*, **2020**, *53* (4), pp 575–590 (https://doi.org/10.1017/S0007087420000497).
17. https://forensiceducation.cfsre.org/ [Accessed 23 May 2021].
18. Melbourn, H.; Smith, G.; McFarland, J.; Rogers, M.; Wieland, K.; DeWilde, D.; Lighthart, S.; Quinn, M.; Baxter, A.; Quarino, L. *Forensic Sci. Int. Synerg.*, **2019**, *1*, pp 161–169 (https://doi.org/10.1016/j.fsisyn.2019.08.001).

19. Cosbey, S.; Elliott, S.; Paterson, S. *Sci. Justice*, **2017,** *57* (1), pp 63–71 (https://doi.org/10.1016/j.scijus.2016.10.003).
20. Brock, W. J.; Adam, A. P.; Woolley, H.; Sugimoto, T. *Int. J. Toxicol.*, **2009,** *28* (3), pp 147–150 (https://doi.org/10.1177/1091581809337263).
21. https://abft.org/continuing-education/ [Accessed 1 April 2021].
22. Vervoort, D.; Ma, X.; Bookholane, H.; Nguyen, T. C. *Am. J. Surg.*, **2020,** *220* (6), pp 1539–1540 (https://doi.org/10.1016/j.amjsurg.2020.07.008).
23. Dietrich, N.; Kentheswaran, K.; Ahmadi, A.; Teychené, J.; Bessière, Y.; Alfenore, S.; Laborie, S.; Bastoul, D.; Loubière, K.; Guigui, C.; et al. *J. Chem. Educ.*, **2020,** *97* (9), pp 2448–2457 (https://doi.org/10.1021/acs.jchemed.0c00717).
24. Casanova, R. S.; Civelli, J. L.; Kimbrough, D. R.; Heath, B. P.; Reeves, J. H. *J. Chem. Educ.*, **2006,** *83* (3), pp 501-507 (https://doi.org/10.1021/ed083p501).
25. Rubinger, L.; Gazendam, A.; Ekhtiari, S.; Nucci, N.; Payne, A.; Johal, H.; Khanduja, V.; Bhandari, M. *Int. Orthop.*, **2020,** *44* (8), pp 1461–1466 (https://doi.org/10.1007/s00264-020-04615-9).
26. Hanaei, S.; Takian, A.; Majdzadeh, R.; Maboloc, C. R.; Grossmann, I.; Gomes, O.; Milosevic, M.; Gupta, M.; Shamshirsaz, A. A.; Harbi, A.; et al. *Disaster Med. Public Health Prep.*, **2020,** pp 1–6 (https://doi.org/10.1017/dmp.2020.406).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 188-197
doi: 10.30744/brjac.2179-3425.AR-59-2021

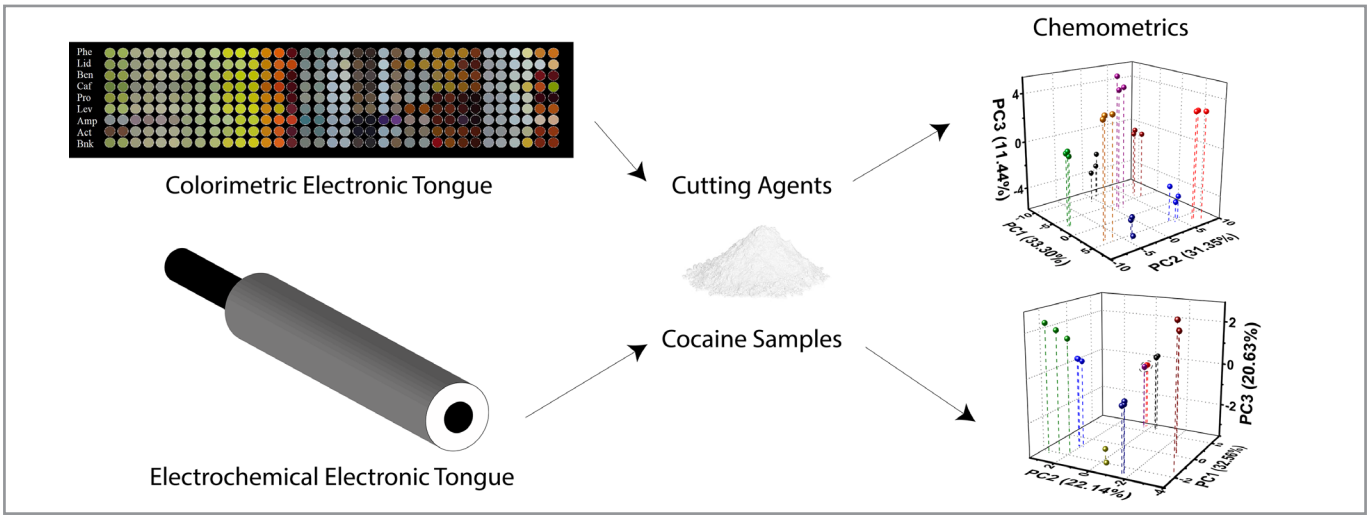
BrJAC

ARTICLE

Development and Evaluation of Two Different Electronic Tongues Aiming to the Discrimination of Cutting Agents Found in Cocaine Seized Samples

Thalita Guedes Silva, Thiago Regis Longo Cesar da Paixão*

Instituto de Química, Universidade de São Paulo, Av. Lineu Prestes, 748, Butantan, CEP 05508-000, São Paulo, SP, Brazil



The screening and impurity profiling of drugs, like cocaine, is essential information that provides chemical and/or physical characterization to assist police agencies in understanding the trafficking and identifying drug origin. This work proposes to show the development and applications of two different electronic tongues (e-tongues) on the profiling study of cocaine seized samples. The developed intelligent devices' primary objective is the simple, quick, and remote cocaine classification samples based on the individual cutting agents added. The paper-based colorimetric sensor was fabricated in the lab using chromatographic paper as a substrate, wax printing to produce spot zones of reactions, a smartphone as image detection, and an editing image software to extract the chemical information through RBG values. The voltammetric e-tongue applied a boron-dopped diamond electrode to extract the cutting agents' electrochemical information through the square wave voltammetry (SWV) technique. In any case, both described sensors were coupled to chemometric tools for data analysis to construct the discrimination model. According to the objective, the unsupervised pattern recognition technique, Principal Component Analysis (PCA), was applied to test the capability of the device on individually discriminating the most common cutting agents of cocaine.

Cite: Silva, T. G.; Paixão, T. R. L. C. Development and Evaluation of Two Different Electronic Tongues Aiming to the Discrimination of Cutting Agents Found in Cocaine Seized Samples. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 188-197. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-59-2021

Submitted 12 April 2021, Resubmitted 14 June 2021, 2nd time Resubmitted 20 July 2021, Accepted 30 July 2021, Available online 25 August 2021.

Keywords: electrochemical e-tongue, paper-based colorimetric e-tongue, chemometrics, forensic analysis, chemical profiling

INTRODUCTION

The ever-growing problem of drug consumption requires an increasing need for measurements to identify links and trafficking routes to assist drug intelligence agencies in containing drug markets' expansion. According to World Drug Report (WDR) published on United Nations Office on Drug and Crime (UNODC) in 2020 [1], the COVID-19 pandemic may reflect in the evolution of drug markets as a consequence mainly of the lesser control by the authorities of illicit crop cultivation and the economic crisis which turns more people to illegal activities. The seized drug characterization and impurity profiling are scientific tools to provide helpful information for drug law enforcement investigative work, e.g., classifying materials of related samples and identifying origin and distribution networks. Therefore, drug profiling may create a databank for pattern recognition that supplies a guide for identifying new illicit laboratories and new manufacturing methods, providing great help to the contention process [2,3]. Once the drug market and use situations change rapidly, studies developing techniques to analyze must also be quick and adaptable to the new circumstances.

Among the well-known drugs, cocaine is still one of the most widely illicit substances consumed worldwide [1], affecting mainly the Central Nervous (CNS) and Cardiovascular Systems. Cocaine is a tropane alkaloid and was commonly used as a local anesthetic due to its blocking the sodium channels depolarization, inhibiting impulse transmission and, therefore, the pain stimulus. This drug is a potent stimulant of CNS, inhibiting the catecholamines recapture, promoting euphoria and chemical dependence [4]. Nowadays, cocaine is frequently commercialized on the illicit market with a large variability of chemical composition due to clandestine laboratories and manufacturers' poor conditions [3]. Generally, pharmacological substances with similar properties, called cutting agents, are included in the manufacturing process to enhance or mimic the drug effects, increasing its profits by selling less cocaine [5]. Within this situation, this drug's impurity profiling could be a chemical signature assigned to every drug sample, providing a complete history of the sample as background support to the intelligent agencies [3].

In this context, Electronic Tongues and Noses are powerful tools to provide screening and chemical characterization with a high-quality and quick outcome. The classification of e-devices is related to the sample tested by the device used, like its mammalian analogs (tongue and nose). Liquid samples are evaluated by the e-tongue and gas samples by e-noses. Those devices are a kind of bionic detection approach similar to the mammalian recognition system. In another way, this device consists of a high-stability sensor with high cross-selectivity [6], which provides complex information about the sample. Such complex analytic data must be processed by using multivariate data analysis to obtain the expected answer. Seeking to establish the pattern and fingerprinting information to discriminate and classify samples, the e-tongues have been widely used in the research for quality control [7], beverages adulteration and tracking [8,9], environmental monitoring [10], pharmaceutical analysis [11], and medical diagnoses [12]. Several types of e-tongues have been developed based on different principles [11,13,14]. Electrochemical and paper-based colorimetric e-tongues systems have received particular attention due to their quick response, flexible application, low cost, and portability to remote analysis [8,12,15].

Commonly, the cutting agent's discrimination in cocaine samples is reported using Raman Spectroscopy [16] and gas chromatography-mass spectrometry [17] associated with multivariate analysis. This technique for detection is time-consuming, needs well-trained persons, and does not show possibilities for in-field applications by the police. Hence, this report presents the development and evaluation of two in-field different electronic tongues, voltammetric and paper-based colorimetric, to identify and discriminate, using non-supervised pattern recognition, the eight most common cutting agents found in cocaine seized samples [18]. All contaminants' individual chemical information was registered and extracted using the electrochemical sensor in the voltammetric technique and a smartphone associated with an image extraction program to develop the colorimetric device. The pattern output was achieved using chemometric analysis.

MATERIALS AND METHODS

Reagents

The cutting agents benzocaine, caffeine, procaine chloride, levamisole chloride, aminopyrine, acetaminophen, and phenacetin were obtained from Sigma-Aldrich (Steinheim, Germany). Each cutting agent's stock solution was prepared in a mixture (1:1 v/v) of water/acetonitrile from Merk (Darmstadt, Germany). To the electrochemical procedures, all stock solutions were diluted in phosphate buffer 0.1 mol L^{-1} (pH 6.8) purchased Synth (São Paulo, Brazil). The reagents iron(III) chloride, N-(1-Naphthyl) ethylenediamine dihydrochloride, iodine, potassium iodide, potassium ferricyanide, cobalt sulfate II, silver nitrate, sodium nitrite, potassium meta periodate, sodium carbonate, methyl orange, ammonium metavanadate, sodium benzoate, and sulfanilic acid were obtained from Sigma-Aldrich (Missouri, EUA), Merk (Darmstadt, Germany), Nuclear (São Paulo, Brazil) and Synth (São Paulo, Brazil). The chromatographic paper (JP40) used to fabricate the paper-based device was purchased from JProlab (Paraná, Brazil).

Electrochemical procedure

All the voltammograms were performed using a common system of three electrodes boron-dopped diamond (BDD), Ag/AgCl (Sat)/KCl (Sat.), and platinum as work, reference, and counter electrode, respectively. The BDD surface was electrochemically pre-treated applying amperometry procedure in sulfuric acid 0.5 mol L^{-1} solution: 3.0 V for the 30 s followed by -3.0 V for 120 s to surface activation and 3.0 V for 10 s followed by -3.0 V for 30 s to ensure a consistent condition of the electrode surface between experiments. Square Wave Voltammetry (SWV) experiments were carried out from 0 to 1.7 V with the parameters: step (ΔE_S) = 5 mV, amplitude (ΔE_A) = 25 mV and frequency (f) = 30 Hz, to extract the chemical information from the analytes.

Colorimetric device

The spot-test colorimetric experiments were performed using a paper-based system produced in the lab, procedure schematized in Figure 1. A previous pattern of the black background with white circles (diameter = 3 mm) was designed using CorelDRAW® for Windows and printed in chromatographic paper using a commercial wax print to produce the reactional zones. The printed paper was thermally pre-treated applying 120 °C for 3 minutes to fuse the wax printed and making a hydrophobic barrier in all the paper layers. Thus, eight reagent mixtures were immobilized on the constructed paper substrate to finish the device development. The composition of each reagent is found in Table I. Aim to perform the analysis; the device was exposed to the cutting agents' solutions. After 5 min of reaction, the photographic images were taken employing a chamber coupled to iPhone 4S to provide a fixed focus distance and homogeneous lighting during all the registrations. The chemical information was extracted as an RGB pattern for each cutting agent using GNU Image Manipulation Program (GIMP2).

In this procedure, we evaluated the slopes from the analytical calibration curves for each RGB value to remove the concentration effect in the measurement. This mathematical procedure was necessary once both intensity and color values change with the concentration. Then, the chemometric treatment could detect this information as different samples for a different concentration of the same analyzed compound. Hence, we added the investigated compound in the sample in various concentrations and mathematically calculated the linear regression for each color channel (R, G, and B). Based on this information, the regression slope in each channel was used as input to remove the concentration problem since each cutting agent will have a unique slope pattern in the linear regression.

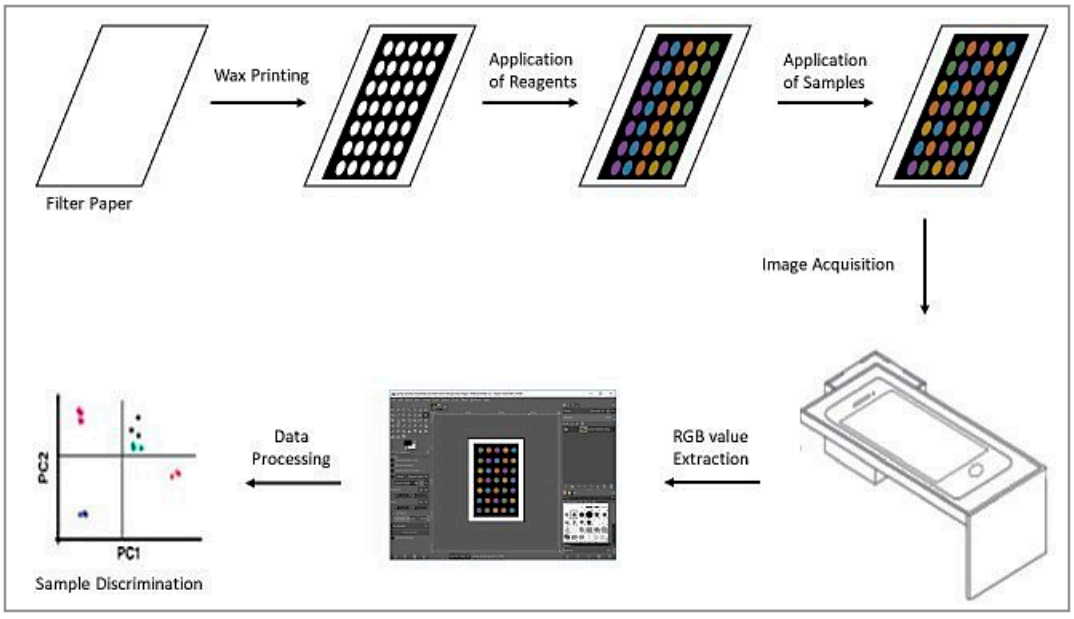


Figure 1. Schematic representation of the measurement process for extracting and analyzing RGB values to discriminate cutting agents in cocaine seized samples.

Table I. Composition of each reagent used in the paper-based-colorimetric device

Reagent Mixture	Components*	Medium	Oxidant**	nº
Bouchardat (Wagner)	I_2: 8 mmol L^{-1} KI: 24 mmol L^{-1}	NaOH	No	1
			Yes	2
		H_2O	No	3
			Yes	4
		HCl	No	5
			Yes	6
Methyl orange	Methyl orange: 20 mmol L^{-1}	NaOH	No	7
		H_2O	No	8
		HCl	No	9
Potassium Ferricyanide	$K_3[Fe(CN_6)]$: 20 mmol L^{-1}	NaOH	No	10
			Yes	11
Iron III Chloride	$FeCl_3$: 20 mmol L^{-1}	H_2O	No	12
			Yes	13
		HCl	No	14
			Yes	15
Cobalt II Sulphate	$CoSO_4 \cdot 7\ H_2O$: 20 mmol L^{-1}	NaOH	No	16
			Yes	17
		H_2O	No	18
			Yes	19

Table I. Composition of each reagent used in the paper-based-colorimetric device (Continuation)

Reagent Mixture	Components*	Medium	Oxidant**	nº
Silver Nitrate	$AgNO_3$: 20 mmol L^{-1}	NaOH	No	20
			Yes	21
		H_2O	No	22
			Yes	23
Ammonium Metavanadate	NH_4VO_3: 20 mmol L^{-1}	NaOH	No	24
			Yes	25
		H_2O	No	26
			Yes	27
		HCl	No	28
			Yes	29
Sims-Horn	NED: 5 mmol L^{-1} $NaNO_2$: 5 mmol L^{-1} Sodium Benzoate: 5 mmol L^{-1} Sulfanilic Acid: 5 mmol L^{-1}	NaOH	No	30
			Yes	31
		H_2O	No	32
			Yes	33
		HCl	No	34
			Yes	35

*Final concentration at the spot. **Oxidant: Potassium meta periodate.

Data treatment and analysis

The extracted values of current from SWV were mathematically treated using baseline correction and normalization [19]. The chemometric analysis was performed applying the unsupervised pattern recognition technique, principal component analysis (PCA), using the RGB pattern and the current values extracted from the cutting agents as input data. The chemometrics experiments were performed using Statistica 13.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSIONS

Electrochemical experiments

Cyclic voltammetry (CV) is commonly the first technique used in electroanalytic. It is possible to investigate electronic transfer processes and redox mechanisms due to their quick response to thermodynamic and kinetic parameters from the redox reactions. This work applied CV as an exploratory technique to choose the optimal conditions to extract the electrochemical information from the targets. Work electrode surface and hydrogen ion concentrations are among the varied parameters. Considering the purpose of qualitative study, to discriminate and classify cutting agents, the best signals separation was achieved with the boron-doped diamond as work electrode, besides its large potential window that promotes more negligible surface adsorption, and phosphate buffer 0.1 mmol L^{-1} (pH 6.8) as electrolyte.

We applied Square Wave (SW) voltammetry to obtain the discrimination model, combining all pulse techniques' best aspects. It shows a lesser background current that results in a higher sensibility between all voltammetry techniques. Figure 2 shows the behavior of all cutting agents studied when the BDD electrode is applied. Note that some of them manifested specifics responses: aminopyrine has three electronic transfer processes, with E_{ox} of 0.4, 0.6, and 1.2 V; acetaminophen shows a low value to the redox process, being close to 0.5 V; and three compounds, lidocaine, levamisole, and caffeine, present high E_{ox} values of 1.1, 1.3 and 1.4 V, respectively. However, three compounds, procaine, benzocaine, and phenacetin, showed a slight difference in the redox process's values, all-around 0.9 V.

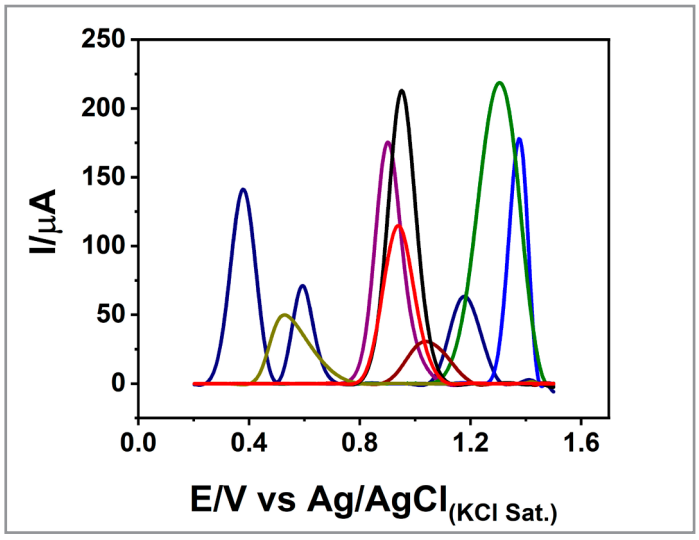


Figure 2. SW voltammograms in presence of phenacetin (black), procaine (red), caffeine (blue), levamisole (green), acetaminophen (yellow), aminopyrine (dark blue), lidocaine (wine) and benzocaine (purple) at 1 mmol L^{-1}. SWV Parameters: t_e = 10 s, E_i = 0.0 V, E_f = 1.7 V, ΔE_S = 5 mV, ΔE_A = 25 mV, f = 30 Hz.

The current values for potential were used as input data to the chemometric technique, PCA. In the beginning, the statistical model was constructed using the absolute current value without any pre-treatment. However, the model can classify or discriminate none of the groups due to the reflection of the concentration-effect in the current values when the cutting agents were studied in different concentrations (data not shown). Or rather, once an increase of the compound concentration promotes a current value increment, the model could not identify any pattern between the cutting agent groups and overlapped all the samples. Aiming to eliminate this problem, the current values were treated mathematically, using baseline correction and normalization between 0 and 1 as reported in the literature [19], before being inputted to the chemometric tool. Figure 3 shows the study applying the sensor to discriminate the cutting agents over different concentrations, varying between 0.1 and 1 mmol L^{-1}. The PCA model could satisfactorily discriminate six groups of cutting agents through the considerable dispersion of levamisole samples. This result shows significant differences between voltammetric behaviors from five groups over the similarities between redox processes of procaine, benzocaine, and phenacetin. Then, those three groups' characteristics promote a model comprehension as only one big group, with a dense overlap of procaine's and benzocaine's groups. Additionally, the mixture of these six groups well discriminated here was evaluated two in a composition 1:1, and good discrimination was observed in the PCA score plots, data not shown.

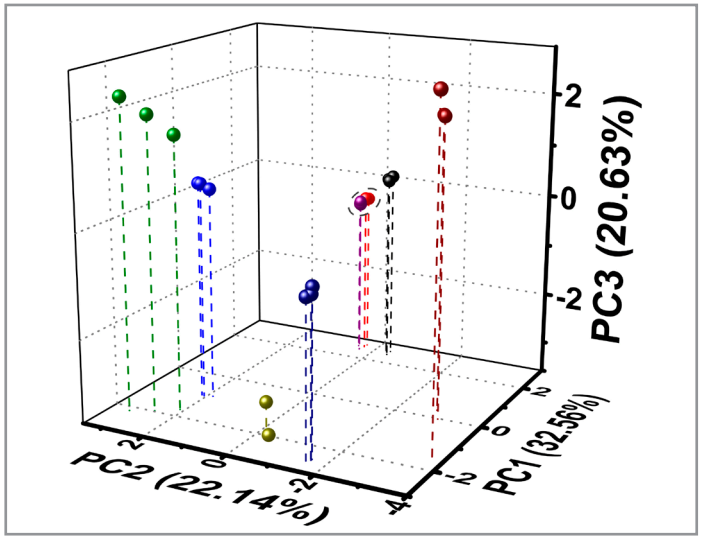


Figure 3. PCA scores plot using pre-processed current values recorded by SW voltammetry with BDD electrode in the presence of phenacetin (black), procaine (red), caffeine (blue), levamisole (green), acetaminophen (yellow), aminopyrine (dark blue), lidocaine (wine) and benzocaine (purple). Concentration range = 0.1 to 1.0 mmol L^{-1}.

Colorimetric experiments

In analytical chemistry, colorimetric uses color intensity and variation to identify and measure the presence and/or concentration. Commonly, spectrophotometry techniques based in Lambert-Beer register the chemical information through colorimetric signals. However, these classic instrumentations require complex and equipped lab. The colorimetric spot test method of analysis is quick, simple, and, due to its instant answer and easy-to-use procedure, this type of sensing has been used in several areas of study [15,18-24] in the last years. The molecular recognition applying an array of colorimetric reactions was started by Dr. Suslick [25,26]. The employed reactions are generally based on acid-base, Van der Waals interaction, adsorption; precipitation; and others. This report presents the development of a colorimetric method based on a paper spot test to classify and discriminate eight cutting agents commonly added to cocaine seized samples.

Initially, several reactions were tested applying the cutting agents simultaneously, and some were taken from the literature [27-30]. Thus, 35 reactions using eight different reactant mixtures were chosen to discriminate the eight targets satisfactorily. Figure 4 shows the RGB extracted color representation using the paper-based colorimetric device when the reactions are applied to each cutting agent, and analytical blank, at 10 mmol L^{-1}. Only employing eye visualization is impossible to identify the specific pattern of each cutting agent to classify them. Thereby, chemometric techniques are powerful tools when extensive data analysis is performed. In this case, 105 variables (35 reactions over 3 channels) were used as input data to the PCA model shown in Figure 5. The PCA model could satisfactorily discriminate all the nine groups, eight cutting agents, and analytical blank without any misclassified sample or group superposition. Although the manual technique to apply the reagents and analytes, the unsupervised pattern rightly recognized all the replicate samples, indicating the robustness of the developed method.

Figure 4. RBG in color representation in the cutting agents' presence at 10.0 mmol L^{-1} after 5 min of reaction. *Reaction 1 to 35 from left to right.*

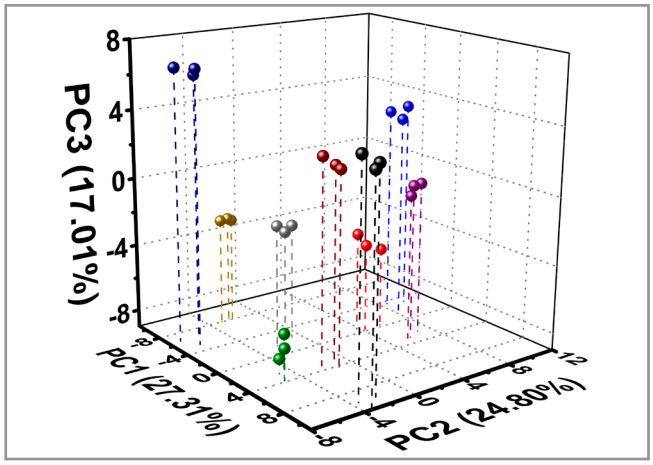


Figure 5. PCA scores plot using RGB values extracted in absence, blank (gray), and presence of phenacetin (black), procaine (red), caffeine (blue), levamisole (green), acetaminophen (yellow), aminopyrine (dark blue), lidocaine (wine) and benzocaine (purple) at 10.0 mmol L^{-1}.

They succeeded in detecting and discriminating the cutting agents in one concentration, an experiment to test the method potential when the compounds are present in a range of concentrations (1, 5, and 10 mmol L^{-1}) were performed to reconstruct the reality with higher quality. Unfortunately, the same problem previously faced applying different cutting agent concentrations using SWV was obtained using RGB values as input data. Once varying the concentration value changes the intensity of color and the color itself, the model misclassified all the samples making a chaotic dispersion of the groups (data not shown). Due to the impossibility of creating an RGB pattern using untreated values when the compound is applied in different concentrations, mathematical techniques of data treatment could be employed to deal with this problem. Therefore, the RGB values extracted were linearly regressed, as reported in the experimental section, and their slope values were used as input data to construct the discriminative model. Worth mentioning, the input data was 105 variables of 35 slope values (one by reaction) to each channel (R, G, and B). Figure 6 shows the PCA plot obtained with the eight groups adequately discriminated against, without any misclassified sample or group superposition. This good result shows the great potentiality of the developed method for classifying unknown cocaine samples based on the cutting agent presence.

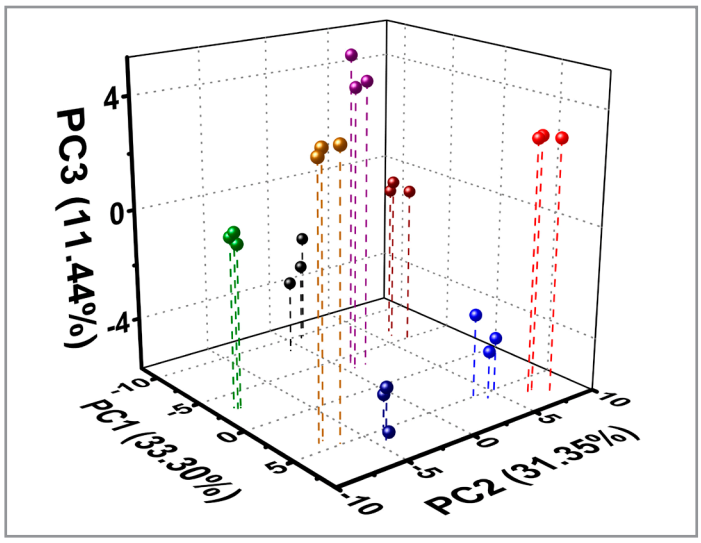


Figure 6. PCA scores plot using slopes values of RGB analytical curves in the presence of phenacetin (black), procaine (red), caffeine (blue), levamisole (green), acetaminophen (yellow), aminopyrine (dark blue), lidocaine (wine), and benzocaine (purple). Concentration range = 1.0 to 10.0 mmol L^{-1}.

CONCLUSIONS

This study shows successful electronic tongue applications to identify and classify the cutting agents individually in seized cocaine samples, aiming to determine the drug's chemical fingerprinting, demonstrating two good candidates for forensic analysis. The first device combines voltammetric technique, while the other applied colorimetric detection to chemometric data analysis. Both developed e-tongues set the main idea of first screening at the seizure moment, without the necessity of any complex equipment or sample pre-treatment. The proposed devices gather important characteristics to this field, such as fast, simple, easy-to-use, and low-cost compared to the classic analysis. In terms of fabrications, the sensor to the voltammetric device is commonly commercialized and accessible.

On the other hand, the colorimetric paper device is manually constructed by applying all reagents and targets using automatic pipettes, which may cause a robustness problem. However, nowadays, molecular printing is a growing field of study and might transform the manufacture of paper-based sensors. About the detection, the voltammetric e-tongue is portable to the remote analysis, though the necessity of a specific instrument. Simultaneously, the colorimetric approach includes one of the most used technology globally, a smartphone, which could be handled by any person and in any place. Both extracted data were analyzed using a chemometric method of pattern recognition, PCA. Each studied cutting agent's unique pattern was successfully obtained to the colorimetric device without any misclassified sample or group overlapped. On

the other hand, the voltammetric detection could discriminate only six of eight groups due to three studied compounds (procaine, benzocaine, and phenacetin). Still, it seems to be more promising in initial studies for the discrimination of cutting agents mixture.

Conflict of Interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Acknowledgements

This research was supported by São Paulo Research Foundation (FAPESP) [Grant numbers: 2017/10522-5, 2018/08782-1, 2019/16491-0], "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) [Pró-Forenses Edital 25/2014] and "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) [Grant Number: 305605/2017-8 and 302839/2020-8].

REFERENCES

1. https://wdr.unodc.org/wdr2020/index.html [Accessed 07 April 2021].
2. https://www.unodc.org/pdf/publications/report_st-nar-35.pdf 2005 [Accessed 07 April 2021].
3. https://www.unodc.org/unodc/en/scientists/drug-characterization-and-impurity-profiling---background-and-concepts.html [Accessed 07 April 2021].
4. Caligiorne, S. M.; Marinho, P. A. *Revista Criminalística e Medicina Legal*, **2016**, *1* (1), pp 34–45 (http://revistacml.com.br/wp-content/uploads/2017/01/RCML01-06.pdf).
5. Cole, C.; Jones, L.; McVeigh, J.; Kicman, A.; Syed, Q.; Bellis, M. *Drug Test. Anal.*, **2011**, *3* (2), pp 89–96 (https://doi.org/10.1002/dta.220).
6. Vlasov, Y.; Legin, A.; Rudnitskaya, A.; Di Natale, C.; D'Amico, A. *Pure Appl. Chem.*, **2005**, *77* (11), pp 1965–1983 (https://doi.org/10.1351/pac200577111965).
7. Wei, Z.; Yang, Y.; Wang, J.; Zhang, W.; Ren, Q. *J. Food Eng.*, **2018**, *217*, pp 75–92 (https://doi.org/10.1016/j.jfoodeng.2017.08.005).
8. de Morais, T. C. B.; Rodrigues, D. R.; Souto, U. T. C. P.; Lemos, S. G. *Food Chem.*, **2019**, *273*, pp 31–38 (https://doi.org/10.1016/j.foodchem.2018.04.136).
9. Wang, J.; Zhu, L.; Zhang, W.; Wei, Z. *Anal. Chim. Acta*, **2019**, *1050*, pp 60–70 (https://doi.org/10.1016/j.aca.2018.11.016).
10. Chapman, J.; Truong, V. K.; Elbourne, A.; Gangadoo, S.; Cheeseman, S.; Rajapaksha, P.; Latham, K.; Crawford, R. J.; Cozzolino, D. *Chemical Reviews*, **2020**, *120* (13), pp 6048–6069 (https://doi.org/10.1021/acs.chemrev.9b00616).
11. Zabadaj, M.; Szuplewska, A.; Kalinowska, D.; Chudy, M.; Ciosek-Skibińska, P. *Sens. Actuators B Chem.*, **2018**, *272*, pp 264–273 (https://doi.org/10.1016/j.snb.2018.05.137).
12. Saidi, T.; Moufid, M.; Zaim, O.; El Bari, N.; Bouchikhi, B. *Meas. J. Int. Meas. Confed.*, **2018**, *115*, pp 178–184 (https://doi.org/10.1016/j.measurement.2017.10.044).
13. Wesoły, M.; Ciosek-Skibińska, P. *Sens. Actuators B Chem.*, **2018**, *267*, pp 570–580 (https://doi.org/10.1016/j.snb.2018.04.050).
14. Guo, T.; Yin, T.; Ma, Z.; Wang, Z.; Sun, X.; Yuan, W. *IFAC-PapersOnLine*, **2018**, *51* (17), pp 683–688 (https://doi.org/10.1016/j.ifacol.2018.08.117).
15. Salles, M. O.; Meloni, G. N.; de Araujo, W. R.; Paixão, T. R. L. C. *Anal. Methods*, **2014**, *6* (7), pp 2047–2052 (https://doi.org/10.1039/C3AY41727A).
16. Sant'Ana, L. D.; de Sousa, V. C.; dos Santos, F. R.; Sabino, B. D.; Cardoso, A.; de Lima, M. E. F.; Castro, R. N. *Quim. Nova*, **2019**, *42* (4), pp 379–386 (https://doi.org/10.21577/0100-4042.20170346).

17. Villesen, P.; Nielsen, L. S. *Sci. Rep.*, **2017**, *7* (1), pp 1–8 (https://doi.org/10.1038/s41598-017-12042-x).
18. Botelho, É. D.; Cunha, R. B.; Campos, A. F. C.; Maldaner, A. O. *J. Braz. Chem. Soc*., **2014**, *25* (4), pp 611-618 (https://dx.doi.org/10.5935/0103-5053.20140008).
19. Selva, T. M. G.; Paixão, T. R. L. C. *New J. Chem.*, **2016**, *40* (3), pp 2514–2520 (https://doi.org/10.1039/C5NJ03524D).
20. Martinez, A. W.; Phillips, S. T.; Butte, M. J.; Whitesides, G. M. *Angew. Chemie - Int. Ed.*, **2007**, *46* (8), pp 1318-1320 (https://doi.org/10.1002/anie.200603817).
21. Dini, F.; Paolesse, R.; Filippini, D.; D'Amico, A.; Lundström, I.; Di Natale, C. *Procedia Engineering*, **2010**, *5*, pp 1228-1231 (https://doi.org/10.1016/j.proeng.2010.09.334).
22. Eaidkong, T.; Mungkarndee, R.; Phollookin, C.; Tumcharern, G.; Sukwattanasinitt, M.; Wacharasindhu, S. *J. Mater. Chem.*, **2012**, *22*, pp 5970-5977.
23. Jokerst, J. C.; Adkins, J. A.; Bisha, B.; Mentele, M. M.; Goodridge, L. D.; Henry, C. S. *Anal. Chem.*, **2012**, *84*, 6, pp 2900–2907 (https://doi.org/10.1021/ac203466y).
24. Rattanarat, P.; Dungchai, W.; Cate, D.; Volckens, J.; Chailapakul, O.; Henry, C.S. *Anal. Chem.,* **2014**, *86* (7), pp 3555–3562 (https://doi.org/10.1021/ac5000224).
25. Suslick, K. S.; Rakow, N. A.; Sen, A. *Tetrahedron*, **2004**, *60* (49), pp 11133-11138 (https://doi.org/10.1016/j.tet.2004.09.007).
26. Janzen, M. C.; Ponder, J. B.; Bailey, D. P.; Ingison, C. K.; Suslick, K. S. *Anal. Chem.*, **2006**, *78* (11), pp 3591–3600 (https://doi.org/10.1021/ac052111s).
27. Wisniak, J. *Revista CENIC Ciencias Biológicas*, **2018**, *48* (1), pp 40-48 (https://revista.cnic.cu/index.php/RevBiol/article/view/24).
28. Sims, F. H.; Horn, C. *Am. J. Clin. Pathol*., **1958**, *29* (5), pp 412-417 (https://doi.org/10.1093/ajcp/29.5.412).
29. Raal, A.; Meos, A.; Hinrikus, T.; Heinämäki, J.; Romāne, E.; Gudienė, V.; Jak Tas, V.; Koshovyi, O.; Kovaleva, A.; Fursenco, C.; et al. *Pharmazie*, **2020**, *75* (7), pp 299-306 (https://pubmed.ncbi.nlm.nih.gov/32635970/).
30. Arnab, A.; Goyal, A.; Middha, S. *J. Nat. Pharm.*, **2010**, *1* (1), pp 40-45 (https://www.semanticscholar.org/paper/Evaluation-of-the-DPPH-radical-scavenging-activity%2C-Arnab-Goyal/cb6ea2c9f7220c0f4dded5ee9c59cfeea67bc90c).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 198-209
doi: 10.30744/brjac.2179-3425.AR-62-2021

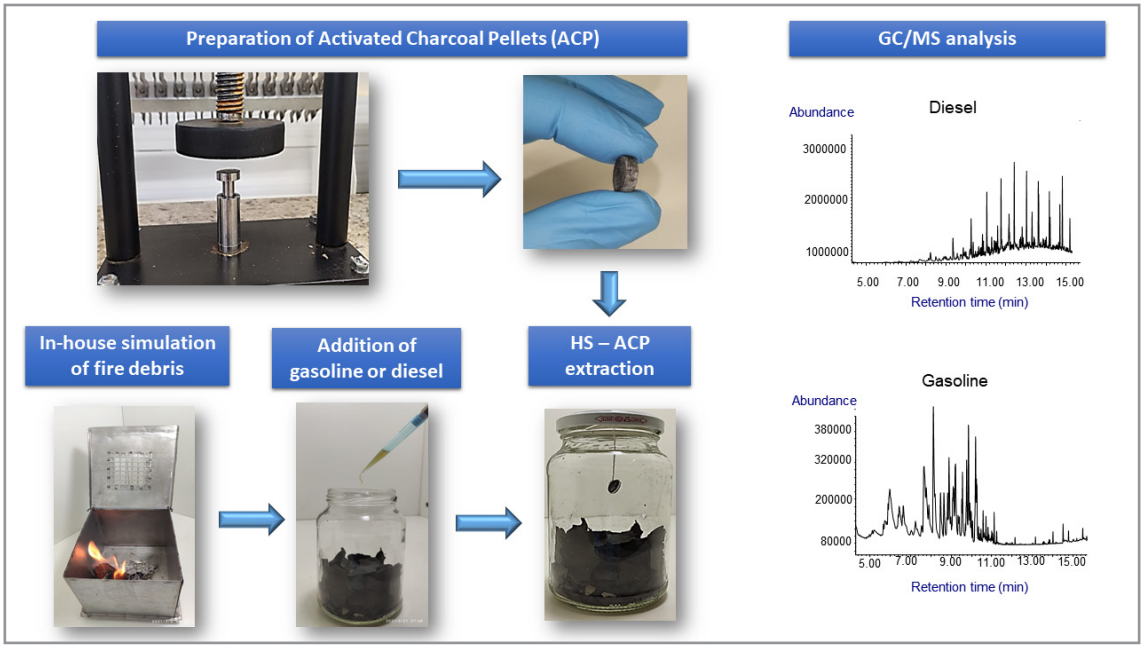
BrJAC

ARTICLE

Activated Charcoal Pellets as an Innovative Method for Forensic Analysis of Ignitable Liquid Residues from Fire Debris by GC-MS

Lais Helena Picolo Bueno Carmona[1]*, Eduardo Geraldo Campos[2], Vitor Luiz Caleffo Piva Bigão[1], Ana Luiza Cubas Pereira[1], Flávia Duarte Maia[1], Laíza Silva Telles[1], Bruno Spinosa De Martinis[1]

[1]Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, Ribeirão Preto, 14040-901, São Paulo, Brazil
[2]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, Ribeirão Preto, 14040-903, São Paulo, Brazil



In Forensic Chemistry, evidence collected at a crime scene is of paramount importance for any case to be properly elucidated. Ignitable liquid residues are important chemical evidence in investigations into cases of fire because these substances can be correlated to arson. Here, we describe an innovative technique for sampling and extracting gasoline and diesel from fire debris by using activated charcoal pellets (ACP). ACP can be an alternative to activated charcoal strips and can be easily produced on the laboratory scale.

Cite: Carmona, L. H. P. B.; Campos, E. G.; Bigão, V. L. C. P.; Pereira, A. L. C.; Maia, F. D.; Telles, L. S.; De Martinis, B. S. Activated Charcoal Pellets as an Innovative Method for Forensic Analysis of Ignitable Liquid Residues from Fire Debris by GC-MS. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 198-209. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-62-2021

Submitted 23 April 2021, Resubmitted 25 June 2021, 2nd time Resubmitted 11 August 2021, Accepted 23 August 2021, Available online 24 September 2021.

The ACP approach allowed all the target compounds selected for gasoline and diesel fuels to be extracted. Among the six tested extraction conditions, optimal extraction occurred at 100 °C, after 240 min. These preliminary results showed the potential of ACP for detecting gasoline and diesel in fire debris. However, the ACP approach still requires analytical validation, so that its applicability in an authentic forensic setting can be explored.

Keywords: activated charcoal pellets, fire debris, ignitable liquid residues, accelerants, forensic chemistry

INTRODUCTION

In Forensic Chemistry, evidence collected at a crime scene is of paramount importance for any case to be properly elucidated. During investigations into fires, the presence of ignitable liquid residues (ILR) in fire debris is an important finding that can discriminate between accidental fires and arsons [1–3]. ILR can be intentionally used to induce, to accelerate, and to spread fire [4,5]. These accelerants are usually volatile and easily flammable substances [6], such as products of petroleum distillation [2,6,7]. Some common substances include gasoline, diesel, kerosene, turpentine, paint thinners, and alcohols, among others [2,8,9].

Many factors make investigation of arson cases challenging. First, evidence collected in a fire scene may be damaged by the fire itself or by the efforts and measures used to control the fire [5,8,10]. For example, powders and foams employed in fire control may lead to interferences [5]. Moreover, given that large amounts of water are frequently used to control fires, ILR might be diluted or even washed [7], which requires sensitive analyses. It is important that any ILR analysis be carefully performed to avoid interpreting that the presence of low levels of ignitable liquids, which may be present in the scene for non-arson-related reasons, is due to arson [11]. The presence of ILR in fire debris does not necessarily confirm an arson because these substances may be present at the scene in normal conditions, especially if we consider that most products derive from petroleum [11,12]. Several matrixes including flooring, plastic materials, tires, and shoes may be composed of ignitable liquids [13]. In addition, the composition of IRL may vary, thereby hampering their classification and identification. One example is gasoline, which is commonly reported and easily detected in fire debris but may have variable compositions [13]. Another challenge is the potential degradation of ILR by the soil microbiome [5,13,14]. Microorganisms might interact with petroleum-derived products and degrade important target analytes (e.g., *n*-alkanes) [13], which could lead to a “false-negative” result if an ILR is completely degraded by microorganisms in soil.

During the scene investigation, samples of fire debris are collected and placed in clean containers, protected from leaks (to avoid loss of analyte), and further submitted to forensic laboratories for analysis, especially to search for ILR [4,6,9,15]. Information related to the type of ILR present in the sample can support the investigation in identifying the person that was responsible for the criminal fire [8]. Considering all the challenges inherent in this type of analysis, correctly selecting the analytical method and approach is fundamental. Many protocols have been developed and implemented for analysis of ILR in fire debris, especially by the American Society for Testing and Materials (ASTM) [16]. ILR in samples of fire debris are analyzed mainly by gas chromatography coupled to mass spectrometry (GC-MS) [1,2,5,15]. GC-MS has been replacing gas chromatography with flame ionization detector (GC-FID) because mass spectrometry (MS) can identify the structure of the compounds present in a sample [6,8]. A sample preparation step is necessary to isolate the ILR and to remove interferents before gas chromatography analysis is performed [1]. Such separation can be achieved via numerous techniques [8,17].

Examples of sample preparation techniques include solvent extraction, direct sampling, passive or dynamic headspace techniques, and solid phase microextraction (SPME) [4,10,18]. Regarding solvent extraction, this technique provides fast and simple sample preparation [18] and efficiently extracts heavier substances, with lower vapor pressure [5]. However, this technique requires a large volume for extraction, the amount of solvent must be enough solvent to cover the debris sample completely. Furthermore, solvent extraction is destructive and may be subject to potential interferences from the solvent employed during the extraction [5,18], which can be the same solvent used by the arsonist [18].

In turn, headspace techniques offer analytical gains such as easy manipulation, reduced interferences (compared to extractions involving solvents) [1], and fast and non-destructive analyses [18]. Besides that, headspace techniques are good for screening ignitable liquids that may be present in the fire debris, to support technique selection [19]. Passive headspace with a concentration step involves extracting ILR from fire debris samples to an adsorbent material inside a vessel, under heating [1]. Activated charcoal strips (ACS) and SPME are the traditional sorbents in passive headspace approaches [1,9]. Sampling/extraction based on SPME allows for fast, highly sensitive, and solvent-free analyses [5,6,9]. The fiber in the headspace is exposed for adsorption and the desorption, which occur directly in the GC injector [5,18]. Nevertheless, SPME fibers are expensive and frail, have a short lifetime [1], and may generate interferences after reuse [5,18]. In the case of passive headspace with ACS, a second step encompassing ILR extraction from the charcoal strips is performed with solvents (e.g., carbon disulfide) [9,10]. The ACS-based technique offers advantages like high adsorption capacity for a broad range of substances [5], high sensitivity, easy sample preparation, and possibility of restoring and reanalyzing the remaining materials [1], and ACS are commercially available. However, the technique requires the use of organic solvents and may be time-consuming [1,5].

In this context, we propose an innovative method for extracting ILR from fire debris. The method consists of adsorption/extraction by activated charcoal pellets (ACP) followed by hexane extraction and GC-MS analysis. ACP are an alternative to ACS and have the advantages of reduced cost and rapid preparation in the laboratory, dismissing the need to acquire commercially available strips.

MATERIALS AND METHODS

Materials and apparatus

Reference materials for unleaded gasoline and diesel fuel (5000 μg mL^{-1} in methanol) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used during method development. HPLC grade hexane was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gasoline and diesel fuels were acquired from a local store for extraction optimization assays. Analytical grade activated charcoal powder and anhydrous D-glucose were purchased from Synth (Diadema, SP, Brazil) and used to prepare the ACP materials by using a manual press.

ACP preparation and extraction

The use of charcoal powder as single component and the combination of charcoal powder with other substances (like pellet binder) were tested for ACP preparation. The tested substances included calcium dichloride, silica, and D-glucose as potential pellet binders. Several proportions of activated charcoal powder and binders were tested, including 1:1, 1:5, 1:10, 1:15, and 1:16 (m/m). After the best conditions to prepare the pellets were established, the powder materials were inserted into a cylindrical mold, manually pressed, and held in the supporting mold for five minutes. The pellets were further removed and heated at 60 °C for 12 h to remove potential contaminants.

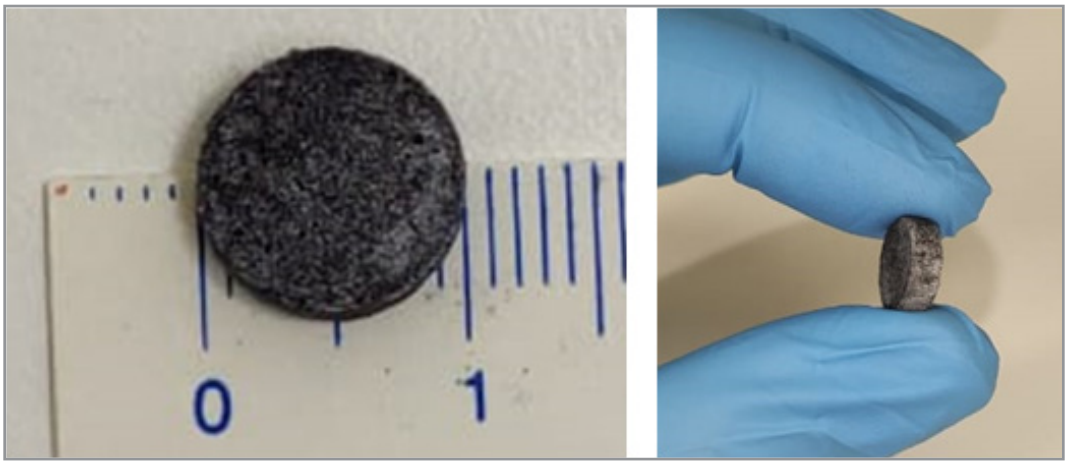


Figure 1. General aspects of the ACP.

Fire debris was simulated on the laboratory scale by burning cotton fabric and paper through direct flame contact. A portion of the simulated samples was placed in the bottom of the glass recipients, and 400 µL of pure gasoline and diesel were added to the top of the debris (except for blank extraction, to which no fuels were added). The ACP were attached to the glass container lid through a 1-mm-thick cotton string. The entire apparatus was placed in the laboratory oven at 100 ºC for 240 min (Figure 2).

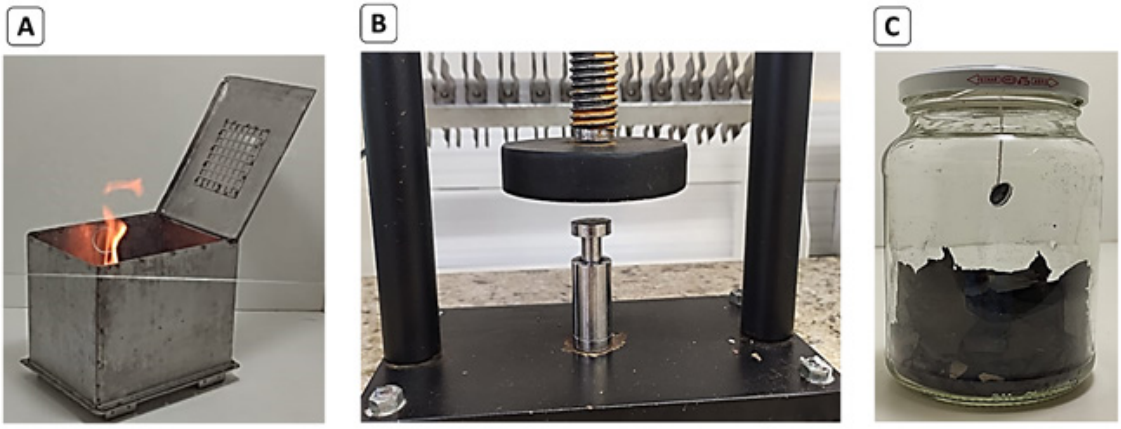


Figure 2. A: Process for burning materials to simulate fire debris. B: The manual press where the pellets were fabricated and the cylindrical mold and C: Glass vial containing the simulated fire debris and the pellets suspended in the headspace.

After the samples were heated, the pellets were removed from the glass containers and transferred to glass tubes, and 1 mL of hexane was added to the tubes for extraction. Solvent extraction was performed by horizontal agitation at 120 rpm for 5 min. Then, 10 µL of the extract was collected, added to a 1.5 mL clean glass vial, diluted with 50 µL of hexane, and injected into the GC-MS. Six different extraction assays were accomplished, in duplicate, according to Table I. The experiments involved two glass containers with debris, containing the pellet suspended with gasoline and diesel, as described.

Table I. Different temperatures and times tested for gasoline and diesel extraction from debris by using ACP

Condition	Temperature (°C)	Time (min)
1	50	240
2	60	155
3	60	325
4	75	120
5	75	240
6	100	240
Blank	100	240

Instrumentation

Analyses were carried out on an Agilent 7890A gas chromatograph equipped with an Agilent 7683 automatic sampler and coupled to an Agilent 5975C mass spectrometer (Agilent Technologies®, Santa Clara, CA, USA). Chromatographic separation was performed on a DB-5MS fused silica capillary column (5%-phenyl)-methylpolysiloxane phase (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies®, Santa Clara, CA, USA). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The injector temperature was maintained at 260 °C. The sample was introduced in the split injection mode. The following column temperature program was applied: initial temperature of 50 °C with holding time of 5 min, followed by an increase to 260 °C at 20 °C min^{-1}, with final holding time of 0.2 min. The total analysis time was 15.7 min. The temperatures of the mass spectrometer interface, source, and quadrupole were 230 and 150 °C, respectively. The mass spectrometer was operated in the full scan mode.

Selection of target compounds in gasoline and diesel

To select the target compounds in gasoline and diesel for further monitoring during chemical analysis, the gasoline and diesel fuels were initially analyzed. The fuels were diluted in hexane (5000 µg mL^{-1}) and analyzed by GC-MS in the full scan mode. The chromatographic peaks with the smallest width and highest abundance were selected, and their mass spectral profile was manually compared against the NIST library (NIST MS Search 2.0, 2009).

Qualitative ACP extraction evaluation

The ACP extraction efficiency was qualitatively assessed by verifying the volume of gasoline and diesel that was recovered and by identifying the target compounds. For this test, the ACP were attached to a polyamide string (0.25 mm diameter) in a 20 mL headspace vial, in the absence of debris. Headspace vials were used to standardize the extraction given that they have better seal capability, thereby avoiding losses by evaporation. The polyamide string was preferred to a cotton string because the former has no pores, and its diameter is smaller. Twelve samples (six with gasoline and six with diesel) were prepared and added at the bottom of the vials, in different amounts (1, 5, 10, 20, 30, and 40 µL). Blank samples consisting of the ACP suspended in the vial without, gasoline, or diesel were prepared and extracted, to evaluate any possible potential interferents from the vial or the polyamide string. The vials were incubated in a laboratory oven at 100 °C for 240 min. Gasoline and diesel were extracted from the ACP as previously described.

RESULTS AND DISCUSSION

ACP preparation

Using only charcoal powder to prepare the ACP gave poor results. When we pressed the charcoal powder, it did not agglutinate into pellets satisfactorily, and the pellet quickly disintegrated into the powder form. To address this problem, we tested three substances as pellet binder: calcium dichloride, silica, and D-glucose. The condition that provided more physically resistant pellets (i.e., pellets that did not disintegrate when they were removed from the mold or attached to the cotton string) was achieved by combining activated charcoal powder and D-glucose, as binder, at a 1:16 (m/m) ratio. Therefore, to prepare the ACP, we adopted a total mass of 0.3 mg of activated charcoal powder and D-glucose (1:16 m/m) for the study.

GC-MS analysis of fire debris by using ACP

Figures 3 and 4 show the total ion chromatogram of the analysis of gasoline and diesel fuels, in hexane. The identified peaks are numbered from 1 to 6 for gasoline and from 7 to 14 for diesel. Tables II and III list the identities of the compounds selected for the targeted analyses.

Table II. Selected target compounds for gasoline

Number	*m/z*	Retention Time (min)	Target Compound
1	105,120, 91	8.117	1-ethyl-3-methylbenzene
2	119,105,134	8.884	1,4-diethylbenzene
3	119,134, 91	9.548	1,2,3,5-tetramethylbenzene
4	117,132,115	9.754	1-methyl-2-(2-propenyl)benzene
5	117,132,115	9.84	1-methyl-4-(2-propenyl)benzene
6	131,146,115	10.191	2,3-dihydro-4,7-dimethyl-1H-indane

Table III. Selected target compounds for diesel

Number	*m/z*	Retention Time (min)	Target Compound
7	57, 43, 41	9.332	Undecane
8	57, 43, 71	10.24	Dodecane
9	57, 43, 41	11.023	Tridecane
10	57, 43 ,41	11.731	Tetradecane
11	57, 43, 41	12.39	Pentadecane
12	57, 43, 41	13.011	Hexadecane
13	57, 43, 41	13.594	Heptadecane
14	57, 43, 41	14.145	Octadecane

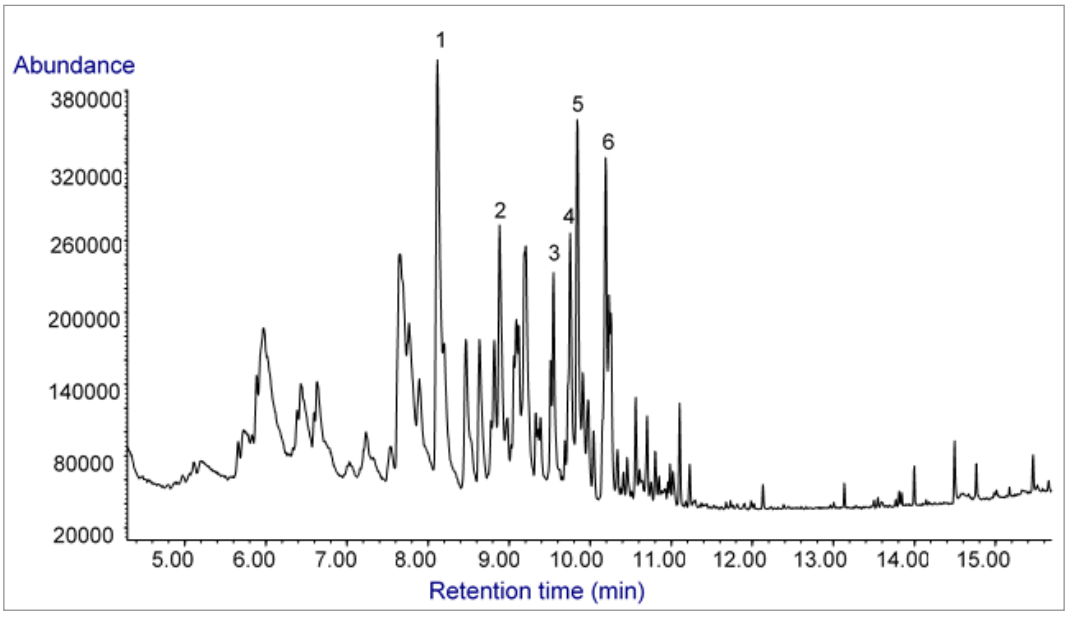


Figure 3. Chromatogram of 5000 µg mL^{-1} gasoline, in hexane. The identified compounds are numbered and listed in Table II.

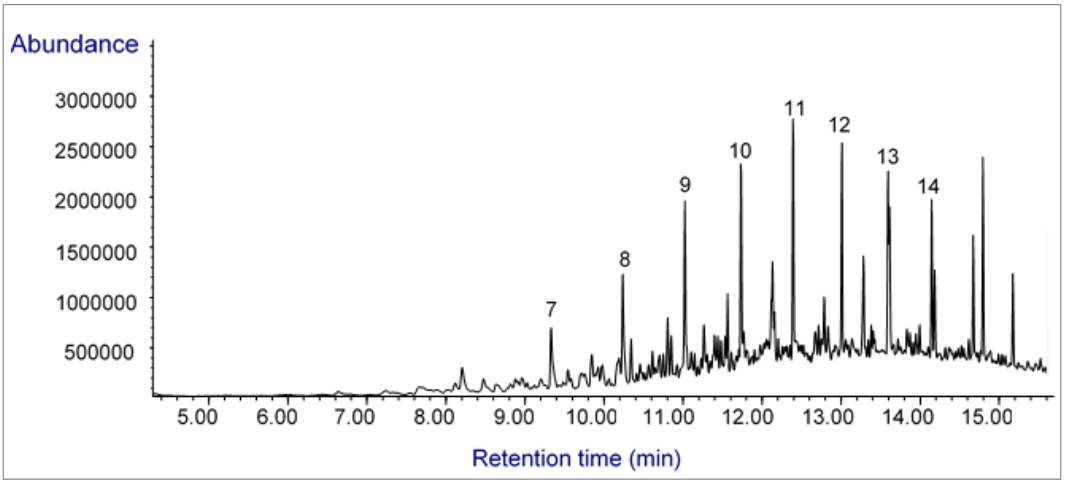
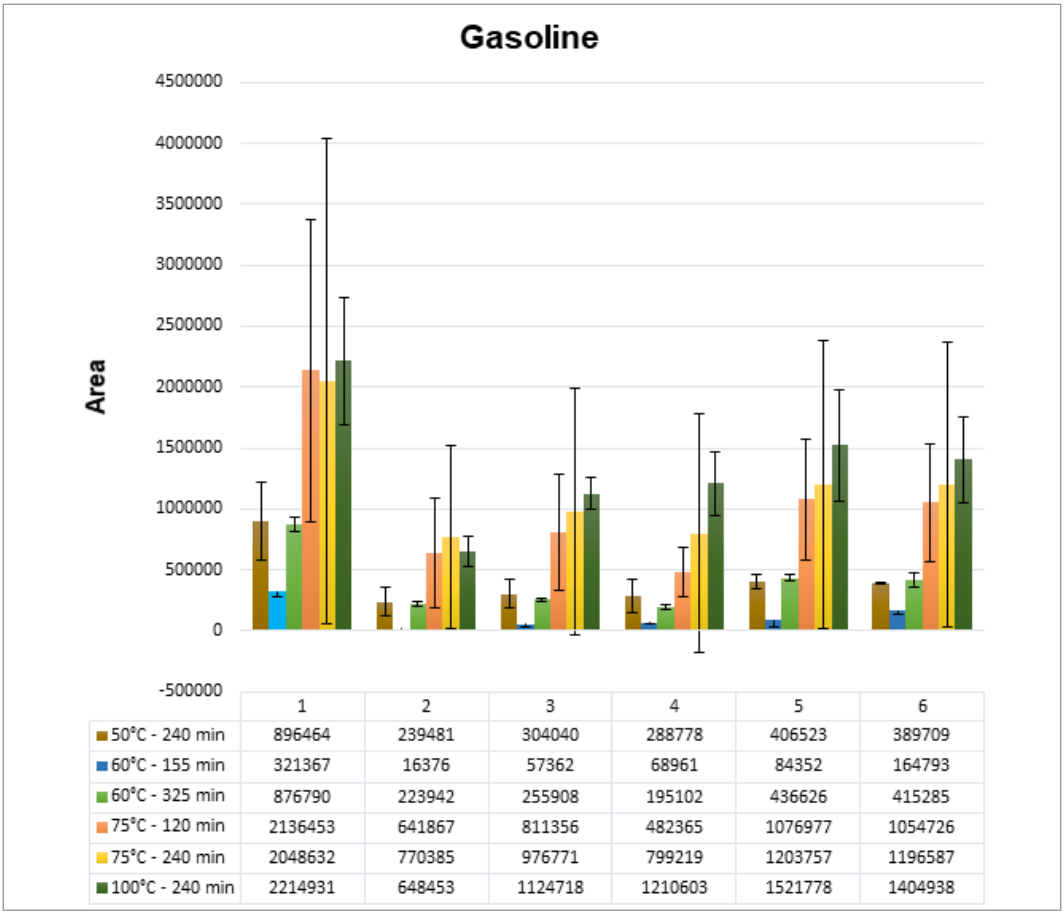


Figure 4. Chromatogram of 5000 µg mL^{-1} diesel, in hexane. The identified compounds are numbered and listed in Table III.

Evaluation of the optimal extraction conditions

After the target compounds were properly selected, we tested the extraction of gasoline and diesel fuels by the ACP to establish the conditions that would provide the best signal for each compound. We used a mixture of gasoline and diesel to optimize the extraction conditions for both fuels by considering the situation in which these two liquids would be found in a sample of fire debris. We randomly selected the extraction times to be tested in the experiments by considering extractions no longer than 6 h, to enable time-effective analyses. We tested each condition in duplicate and, to avoid carryover, we injected 1µL of hexane into the GC-MS between each chromatographic run. Initially, we tested temperatures higher than 100 °C for extraction (e.g., 105, 135, and 150 °C). However, at 135 and 150 °C, the pellets melted down. The results at 105 °C resembled the results obtained at 100 °C, so we conducted the tests at 100 °C to ensure that the pellets would not deform or melt, thereby avoiding poor or no gasoline and diesel extraction.

Figures 5 and 6 illustrate the chromatogram peak areas obtained for each analyte, in each of the tested conditions. In the graph, the area values are the average area of the duplicate experiments in the same conditions for each compound; the standard deviation of these measures is shown. The numbers refer to the target compound monitored for gasoline and diesel according to Tables II and III, respectively.

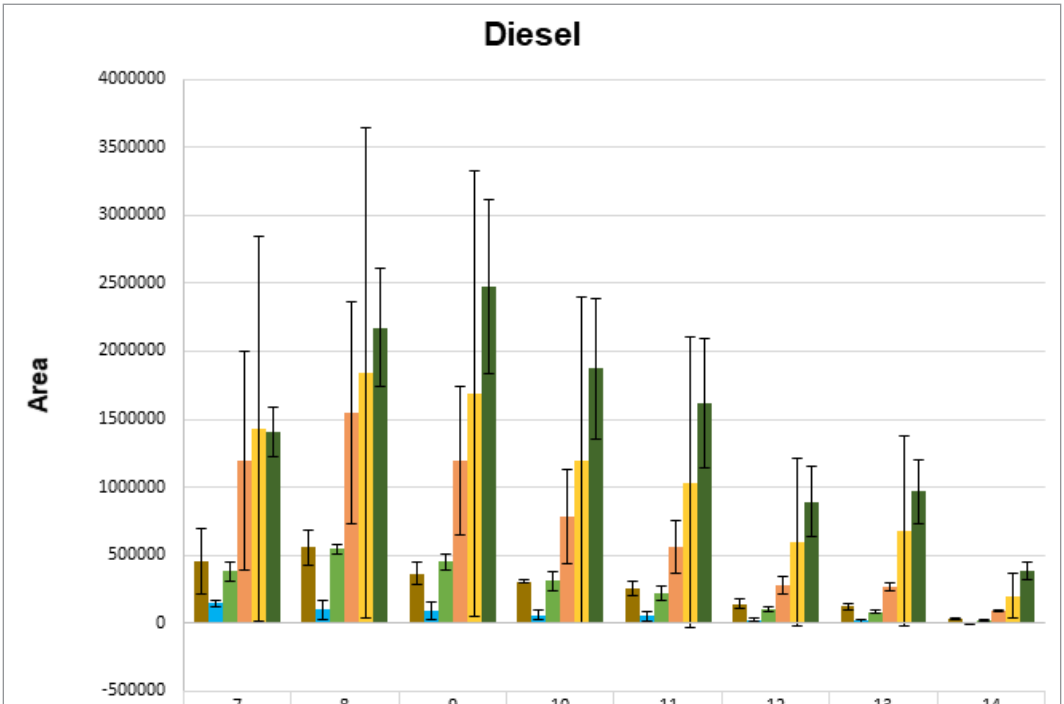


	1	2	3	4	5	6
50°C - 240 min	896464	239481	304040	288778	406523	389709
60°C - 155 min	321367	16376	57362	68961	84352	164793
60°C - 325 min	876790	223942	255908	195102	436626	415285
75°C - 120 min	2136453	641867	811356	482365	1076977	1054726
75°C - 240 min	2048632	770385	976771	799219	1203757	1196587
100°C - 240 min	2214931	648453	1124718	1210603	1521778	1404938

Figure 5. Response (peak areas) of each compound in gasoline (listed in Table II) obtained at different temperatures and extraction times.

The optimal extraction conditions for both gasoline and diesel (based on the best signal areas of each target compound) were 100 °C and 240 min (dark green bars). This temperature was the highest tested temperature at which the pellet maintained its physical stability, but heat could lead to interferences [20]. Therefore, we submitted a blank sample consisting of the glass container with debris and the pellet suspended without gasoline or diesel fuels to extraction at 100 °C for 240 min to investigate possible whether heat generated interferents from the pellets, glass container, or debris. We found no interferents in the blank chromatogram. Condition number 5 (yellow bars) resembled condition number 6 (dark green bars) for both gasoline and diesel, with the target compounds showing larger peak areas in condition 6. However, the standard deviation observed for condition 5 was higher if compared to condition 6, suggesting better (considering the higher areas) and more reliable/robust results under condition 6. We achieved the worst results for condition number 2 (60 °C for 155 min – blue bars), which gave reduced peak areas for all the compounds. In general, longer extraction time increased the peak area of each target analyte, except for compound 1 (1-ethyl-3-methylbenzene) in condition number 5 (75 °C for 240 min orange bars). For example, extractions performed at 50 °C for 240 min (brown bars) produced large peak areas compared to extractions conducted at 60 °C for 155 min (blue bars) even though higher temperatures provided

better responses (in terms of peak areas). In this sense, longer extraction times were positive factors for the overall quality of extraction with the ACP. However, further studies are necessary to understand the mechanism of analyte adsorption onto the ACP: some compounds were poorly adsorbed on the pellets, which could also explain the low recoveries in these tested conditions.



	7	8	9	10	11	12	13	14
50°C - 240 min	454790	555648	363758	304229	257819	141692	120577	34428
60°C - 155 min	145333	98087	93151	58393	50884	22856	17529	3696
60°C - 325 min	381455	544517	451085	312745	219301	106025	84193	20830
75°C - 120 min	1197049	1546468	1198325	780697	561802	277547	268738	85589
75°C - 240 min	1429689	1837712	1687161	1197407	1036142	594516	672531	201194
100°C - 240 min	1405345	2173293	2473557	1873977	1615559	892776	969660	384578

Figure 6. Response (peak areas) of each diesel compound (listed in Table III) obtained at different temperatures and extraction times.

We assessed extraction efficiency at the optimal condition (100 °C, 240 min) by extracting a set of samples using headspace vials without debris and separately adding different volumes of gasoline and diesel at the bottom of the vial (1, 5, 10, 20, 30, and 40 µL). We analyzed the chromatograms and searched for the target compound. According to the standard ASTM E1618-11 (2011), “an adequate chromatogram with sufficient data for comparison work is one in which the peaks of interest are 50 to 100% of full scale” [21]. On the basis of this information and by considering only the chromatograms that had all the target compounds, we found that the limit volume of extraction was 20 and 40 µL for diesel and gasoline, respectively. The volumes of 20 and 40µL represent the quantities present in the vial, recovered by the pellet, which resulted in the positive identification of diesel and gasoline, respectively, after extraction process.

Comparison between ACP and other sampling/extraction methods

Table IV compares the extraction efficiency/sensitivity findings of this study and of other studies is provided, encompassing traditional extraction methods standardized by ASTM [18,19,22–24] and new materials [7,15,25], such as ACP. The headspace technique is the least sensitive among all the traditional

methods [18], and volumes below 10 µL may not be sufficient for recovery and extraction [19]. However, the efficiency/sensitivity seems to be controversial [18], with limit volumes of extraction ranging from 1 to 10 µL [18], but also being as low as 0.1 µL [22]. Being vapor concentration-based techniques, ACS and SPME are more sensitive than the headspace and solvent extraction techniques, [18]. Moreover, ACS and SPME are highly sensitive, reaching volumes lower than 0.1 µL [18]. As for new adsorbent materials, some exhibit limit volumes of extraction in line with the results reached with the ACP technique developed in this study. Hydrophobic pads and ACP produce closer results, but the former is more efficient in the recovery of lighter compounds [7], whereas ACP is more efficient for heavier compounds. A major advantage of the ACP approach proposed in this study is the possibility of rapidly producing adsorbents in the laboratory, at low cost.

Table IV. Comparison between the efficiency of the ACP technique and some traditional techniques and new adsorbent materials

	Reference	Methods	Limit volume of extraction	Compounds
1	[19]	Headspace	Above 10 µL	Better for low molecular weight compounds
2	[18,22]	Solvent extraction	1 µL [22] / 10 µL [18]	Better for high molecular weight compounds
3	[23]	ACS	0.1 µL	All range of compounds
4	[24]	SPME	0.1 µL	All range of compounds
5	[15]	Limestone and British Fuller's earth; 10:1 w/w	Not specified	All range of compounds
6	[7]	Hydrophobic Pads	10 µL (A) and 25 µL (B)	A: heavier and B: lighter (six atoms of carbon or less) compounds
7	[25]	Activated Charcoal Cloth (ACC)	10 µL	Lighter fluid, camp fuel, thinners, lamp oil, and kerosene
8	This study	ACP	20 µL (C) and 40 µL (D)	C: diesel and D: gasoline

CONCLUSIONS

ACP are a promising alternative for extracting gasoline and diesel from fire debris. This technique allowed all the target compounds selected from gasoline and diesel and added to the debris to be detected. ACP have two main advantages: reduced costs and possibility of preparing the pellets in a laboratory setting, dismissing the need for purchasing an adsorbent. The goal of this study was to report the preliminary development of this new approach, and we have shown its promising applicability in fire debris analyses. However, further studies are still needed to validate this method for routine applications, to understand the adsorption mechanisms occurring on the ACP and to evaluate analytical parameters such as recovery, limit of detection, and selectivity. These further studies will be essential to explore and to discuss other advantages and limitations of the method.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

This study was financed in part by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil* (CAPES) – Finance Code 001 (doctoral fellowship and Pro-Forenses Grant – N. 25/2014).

REFERENCES

1. Fabritius, M. M.; Broillet, A.; König, S.; Weinmann, W. *Forensic Sci. Int.*, **2018,** *289*, pp 232–237 (https://doi.org/10.1016/j.forsciint.2018.05.048).
2. Ferreiro-González, M.; Barbero, G.; Palma, M.; Ayuso, J.; Álvarez, J.; Barroso, C. *Sensors*, **2016,** *16* (5), 695 (https://doi.org/10.3390/s16050695).
3. Choi, S.; Yoh, J. J. *Spectrochim. Acta – Part B At. Spectrosc.*, **2017,** *134*, pp 75–80 (https://doi.org/10.1016/j.sab.2017.06.010).
4. Smith, R. W.; Brehe, R. J.; McIlroy, J. W.; McGuffin, V. L. *Forensic Chem.*, **2016,** *2*, pp 37–45 (https://doi.org/10.1016/j.forc.2016.08.005).
5. Martín-Alberca, C.; Ortega-Ojeda, F. E.; García-Ruiz, C. *Anal. Chim. Acta*, **2016,** *928*, pp 1–19 (https://doi.org/10.1016/j.aca.2016.04.056).
6. Dolan, J. *Anal. Bioanal. Chem.*, **2003,** *376* (8), pp 1168–1171 (https://doi.org/10.1007/s00216-003-1890-5).
7. Totten, V.; Willis, J. *Forensic Sci. Int.*, **2020,** *312*, 110309 (https://doi.org/10.1016/j.forsciint.2020.110309).
8. Cacho, J. I.; Campillo, N.; Aliste, M.; Viñas, P.; Hernández-Córdoba, M. *Forensic Sci. Int.*, **2014,** *238*, pp 26–32 (https://doi.org/10.1016/j.forsciint.2014.02.006).
9. Kabir, A.; Holness, H.; Furton, K. G.; Almirall, J. R. *TrAC - Trends Anal. Chem.*, **2013,** *45*, pp 264–279 (https://doi.org/10.1016/j.trac.2012.11.013).
10. Sinkov, N. A.; Sandercock, P. M. L.; Harynuk, J. J. *Forensic Sci. Int.*, **2014,** *235*, pp 24–31 (https://doi.org/10.1016/j.forsciint.2013.11.014).
11. Abel, R. J.; Zadora, G.; Sandercock, P. M. L.; Harynuk, J. J. *Separations*, **2018,** *5* (4), pp 1–18 (https://doi.org/10.3390/separations5040058).
12. St. Pierre, K. A.; Desiderio, V. J.; Hall, A. B. *Forensic Sci. Int.*, **2014,** *240*, pp 137–143 (https://doi.org/10.1016/j.forsciint.2014.02.017).
13. Baerncopf, J.; Hutches, K. *Forensic Sci. Int.*, **2014,** *244*, pp e12–e20 (https://doi.org/10.1016/j.forsciint.2014.08.006).
14. Turner, D. A.; Pichtel, J.; Rodenas, Y.; McKillip, J.; Goodpaster, J. V. *Forensic Sci. Int.*, **2015,** *251*, pp 69–76 (https://doi.org/10.1016/j.forsciint.2015.03.013).
15. Hall, S.; White, G.; Gautam, L. *J. Anal. Appl. Pyrolysis*, **2016,** *122*, pp 304–314 (https://doi.org/10.1016/j.jaap.2016.09.012).
16. Stauffer, É.; Lentini, J. J. *Forensic Sci. Int.*, **2003,** *132* (1), pp 63–67 (https://doi.org/10.1016/S0379-0738(02)00459-0).
17. Grafit, A.; Muller, D.; Kimchi, S.; Avissar, Y. Y. *Forensic Sci. Int.*, **2018,** *292*, pp 138–147 (https://doi.org/10.1016/j.forsciint.2018.09.004).
18. Stauffer, E.; Dolan, J. A.; Newman, R. (Eds.) *Fire Debris Analysis.* Elsevier, Burlington, MA, **2008**, Chapter 11: Extraction of Ignitable Liquid Residues from Fire Debris, pp 377–439 (https://doi.org/10.1016/B978-012663971-1.50015-4).
19. ASTM International. ASTM E1388-12. *Standard Practice for Sampling of Headspace Vapors from Fire Debris Samples*. West Conshohocken, USA: ASTM, **2012**.
20. Stauffer, E. Sources of interference in fire debris analysis. In: Daeid, N. N. (Ed.). *Fire Investigation*. CRC Press, Boca Raton, **2004**, pp 191-226.
21. ASTM International. ASTM E1618-11. *Standard Test Method for Ignitable Liquid Residues in Extracts from Fire Debris Samples by Gas Chromatography-Mass Spectrometry.* West Conshohocken, USA: ASTM, **2011**.
22. ASTM International. ASTM E1386-15. *Standard Practice for Separation of Ignitable Liquid Residues from Fire Debris Samples by Solvent Extraction*. West Conshohocken, USA: ASTM, **2015**.
23. ASTM International. ASTM E1412-07. *Standard Practice for Separation of Ignitable Liquid Residues from Fire Debris Samples by Passive Headspace Concentration With Activated Charcoal*. West Conshohocken, USA: ASTM, **2007**.

24. ASTM International. ASTM E2154-15a. *Standard Practice for Separation and Concentration of Ignitable Liquid Residues from Fire Debris Samples by Passive Headspace Concentration with Solid Phase Microextraction (SPME)*. West Conshohocken, USA: ASTM, **2015**.
25. Sandercock, P. M. L. *Can. Soc. Forensic Sci. J.*, **2016,** *49* (4), pp 176–188 (https://doi.org/10.1080/00085030.2016.1189226).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 210-228
doi: 10.30744/brjac.2179-3425.AR-65-2021

BrJAC

ARTICLE

Disposable Stencil-Printed Carbon Electrodes for Electrochemical Analysis of Sildenafil Citrate in Commercial and Adulterated Tablets

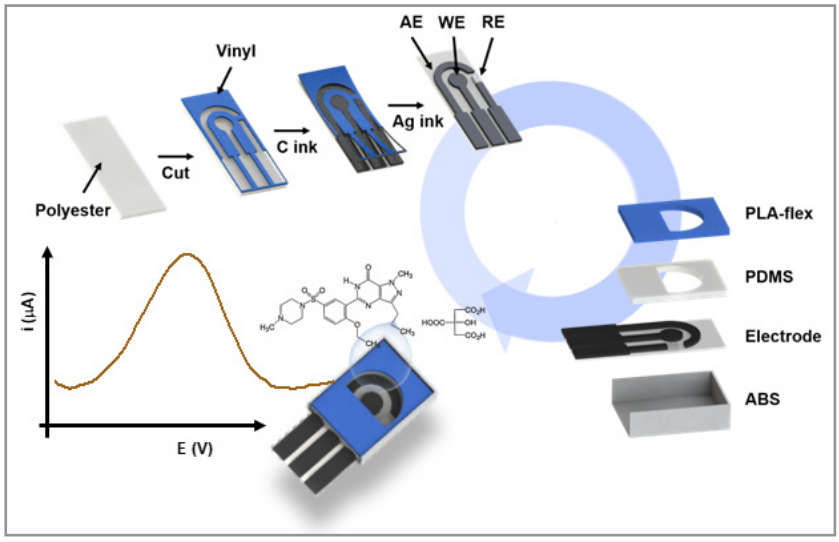
Danielly Santos Rocha[1,a], Habdias Araujo Silva-Neto[1,a], Laísa Cristina de Oliveira[1], Shellyda Gonçalves Guimarães Souza[1], Mário Henrique Palis Santana[2], Wendell Karlos Tomazelli Coltro[1,3]*

[1]*Instituto de Química, Universidade Federal de Goiás, Goiânia, GO, 74690-900, Brazil*

[2]*Unidade Técnico-Científica, Superintendência Regional da Polícia Federal, Uberlândia, MG, 38408-6630, Brazil*

[3]*Instituto Nacional de Ciência e Tecnologia de Bioanalítica, Campinas, SP, 13083-861, Brazil*

[a]*These authors contributed equally to this work.*



Forensic studies are extremally important to investigate suspected adulterations of consumable products, such as Viagra®. This report describes the determination of sildenafil citrate (SC) in commercial and adulterated tablets based on square-wave voltammetry (SWV) measurements using disposable stencil-printed carbon electrodes. The conductive ink used for the manufacture of integrated electrodes was produced by combining graphite powder and glass varnish. To promote a reusable strategy for limiting the geometric area of the electrodes, a 3D-printed holder was constructed. Detailed morphological and electrochemical characterization studies revealed well-defined graphite flakes incorporated on the polymeric substrate and a fast heterogeneous electron-transfer rate constant (Ks = 1.3 × 10^{-3} cm s^{-1}). Based on the analytical performance, a linear behavior was observed in a SC concentration range from 1 to 20 μmol L^{-1} with limit of detection equal to 0.2 μmol L^{-1}. The selectivity of the proposed method was evaluated and the presence of potentially interfering compounds like phosphate, lactose, paracetamol and tadalafil and no difference higher than 15% was observed. The analysis of SC was performed in commercial and seized tablets and the achieved values were 50 ± 1 mg

Cite: Rocha, D. S.; Silva-Neto, H. A.; Oliveira, L. C.; Souza, S. G. G.; Santana, M. H. P.; Coltro, W. K. T. Disposable Stencil-Printed Carbon Electrodes for Electrochemical Analysis of Sildenafil Citrate in Commercial and Adulterated Tablets. *Braz. J. Anal. Chem.*, 2022, *9* (34), pp 210-228. doi: http://dx.doi.org/10.30744/brjac.2179-3425.AR-65-2021

Submitted 27 April 2021, Resubmitted 30 June 2021, Accepted 30 July 2021, Available online 25 August 2021.

for Viagra® tablet, 54 ± 1 mg for generic formulations 38 ± 1 mg for seized tablet. In addition, the proposed method offered satisfactory accuracy (98.2 – 102.0%) no noticeable matrix effect. Lastly, considering the achieved results, the use of stencil-printed carbon electrodes and SWV has demonstrated to be a powerful and robust analytical tool for forensic investigations.

Keywords: carbon-based sensors, electrochemical analysis, erectile dysfunction, forensic analysis, Viagra.

INTRODUCTION

In recent years, the development of compact electrochemical cells combining alternative and/or commercial conductive carbon-based inks and affordable substrates through “do it yourself” protocols has become increasingly popular. In general, electrochemical devices have been fabricated using low-cost and emerging platforms, including paper and plastic materials, and assembled into portable apparatus [1–3]. Besides the low-cost and affordability, instrumental simplicity, ease of operation, reproducibility, portability, and reduced sample consumption are also attractive features which boost the development of alternative electroanalytical devices [4-8]. As well-known, electrochemical sensors are often fabricated by printing-based methods such as inkjet printing [9], screen-printing [10] and stencil-printing [11], which enable the large-scale production with satisfactory fidelity [2]. Cellulose fibers, ceramics and plastics are the most commonly employed substrates to produce printing-based electrodes [2,12,13]. Plastic is particularly advantageous over cellulose and ceramics because it presents interesting physical-chemical and mechanical proprieties, such as robustness, flexibility, durability, hydrophobicity, and optical transparency [4,8,14]. Furthermore, different polymeric materials are commercially available including polyester-based substrates.

As mentioned above, polymers are important substrates in the construction of robust printing-based electrodes. Electrochemical sensors have been explored for applications in different fields, including environmental [15], clinical, pharmaceutical, food and forensics sciences and even point-of-care or on-site studies [2,12,16]. Considering forensic applications, impact areas such as authenticity screening or detection of adulteration in pharmaceutical compounds are extremely important to society because counterfeit or adulterated formulations are a threat to health and the purchase of these drugs through internet is quite easy [17,18].

According to the World Health Organization (WHO) and the U.S. Food Drug Administration, falsified, adulterated, smuggled or illegally marketed drugs do not comply with specifications and quality standards [19,20]. In this sense, the drug may contain the active principal in quantities lower than necessary or the consumer may even be defrauded by the addition of substances that do not have an active effect. It is important to mention that the absence of quality drugs is a recurring problem in the world and exposes people to serious risks, once the inadequate intake of falsified products may cause intoxication or even death [20–22].

Pharmaceutical products associated with erectile dysfunction treatment are one of the classes of drugs most often adulterated in Brazil [17]. Active principles based on tadalafil and sildenafil citrate (SC) are the most commercially popular drugs, in which SC is well-known as Viagra®. Their activity principles are based on selective inhibition of the enzyme phosphodiesterase type 5 (PDE-5), which is specific to cyclic guanosine (cGMP) and it is directly related to the regulation of erectile function. This drug has also been reported as an adulterant in food supplements and herbal products aiming to promote improvements in the sexual performance [23–25]. In this way, it is important to investigate possible irregularities in pharmaceutical products based on SC. Selective and robust analytical techniques, such as high-performance liquid chromatography associated to UV detection [26–27], Raman spectroscopy [28] and electrochemistry [29–31] have been explored to aid the forensic authorities [31,36, 37]. In addition, flow injection analysis and batch injection analysis systems coupled with amperometric detection have been also explored as simple as powerful analytical tools for determining SC in erectile dysfunction drugs [38,39].

As demonstrated by Backes and colleagues [38], the use of carbon-based printed electrodes has offered great analytical performance for accurate determination of SC in commercial drugs including Viagra® and Generics. In this report, we propose a simple combination of disposable stencil-printed carbon electrodes (SPCE) and square-wave voltammetry (SWV) measurements for the determination of SC in commercially available tablets. A compact electrochemical device containing working, reference and auxiliary electrodes was constructed in a single plastic platform using readily affordable materials like glass varnish [8,40] and graphite powder [14]. The electrode surface was characterized using scanning electron microscopy, Raman spectroscopy, contact angle and electrochemical measurements. The forensic feasibility of the proposed approach was demonstrated through the analysis of SC in six commercial tablets and one adulterated sample.

MATERIALS AND METHODS

Reagents and materials

Sildenafil citrate, tadalafil, acetonitrile, potassium ferrocyanide, potassium ferricyanide, potassium chloride, chloride acid, boric acid, phosphoric acid, acetone, paracetamol and lactose were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Apprehended tablets of SC were provided by the Brazilian Federal Police. Commercial tablets of SC were purchased from a local pharmacy. Stock and standard solutions were prepared using ultrapure water processed through a water purification system (Direct-Q®3, Millipore, Darmstadt, Germany) with resistivity of 18.2 MΩ cm.

Graphite powder, glass varnish and silver ink were acquired from Fisher Chemical™ (New Jersey, USA), ACRILEX® (São Paulo, SP, Brazil) and Method Development Co. (Chicago, IL, USA), respectively. Thermoplastic filaments (ø = 1.75 mm), acrylonitrile butadiene styrene (ABS) and flexible poly(lactic) acid (Flex-PLA) were provided by 3D Fila (Belo Horizonte, MG, Brazil). Poly(dimethylsiloxane) (PDMS) structures were prepared using a Sylgard 184 elastomer kit (monomer and curing agent) from Dow Corning (Midland, MI, USA). Polyester thermal lamination pouches (250 µm thick) and vinyl adhesive (125 µm thick) were acquired from Yidu Group Co., Ltd (Hsi-Chih, Taipei, Taiwan) and Imprimax® (São Paulo, SP, Brazil), respectively.

Fabrication of the disposable carbon-based electrodes

The integrated electrochemical cell was manufactured using the stencil-printing technique [15,41]. Firstly, two layers vinyl adhesive (8 x 11 cm) were combined on the polyester substrate using a spatula tool. The layout of the integrated electrodes was designed using Silhouette Studio software and contained a working electrode (WE; ø = 4 mm), reference electrode (RE) and auxiliary electrode (AE). This layout was printed reductively on the vinyl adhesive using a cutting plotter from Silhouette (Belo Horizonte, MG, Brazil). Then, the adhesive structures containing only the electrode designs were removed from the vinyl adhesive/polyester backing using metal tweezers. This step allowed a vinyl stencil to be incorporated with the proposed conductive ink.

The conductive ink was prepared using a procedure adapted from the protocol described by Silva-Neto and coworkers [40]. In brief, the graphite-based ink was based on a mixture composed of 1.5 g of graphite powder, 1.5 g of glass varnish (binder) and 4.0 mL of acetone. To ensure the homogeneity of the ink, these components were mixed under stirring at 1000 rpm for 30 min. Then, the conductive ink was incorporated onto the stencil and polyester substrate using a spatula. After 15 min, the electrode surface was exposed to mechanical polishing process through an alumina sandpaper (1200 mesh). The surface treatment was carried out based on circular mechanical movements. Next, the plastic stencil was manually removed for exposing the integrated electrochemical cell, as illustrated in Figure 1A. It is important to mention that this amount of conductive ink was enough to manufacture *ca.* 60 electrochemical cells. To forming a pseudo-reference electrode, the RE was painted with silver ink. The geometric area of the electrochemical devices was delimited using a 3D printed holder, as recently reported [42]. This 3D-printed holder was designed through the SolidWorks® software and printed through a 3D printer model

open-source from Prusa research (Prague, Czech Republic) via fused deposition modeling employing acrylonitrile butadiene styrene (ABS), flexible polylactic acid (PLA-flex) as the thermoplastic filaments (ø=1.75 mm). The geometric area delimitation was based on a sandwich assembling of the printed sensing electrodes, PLA-flex, PDMS and ABS films, as demonstrated in Figure 1B.

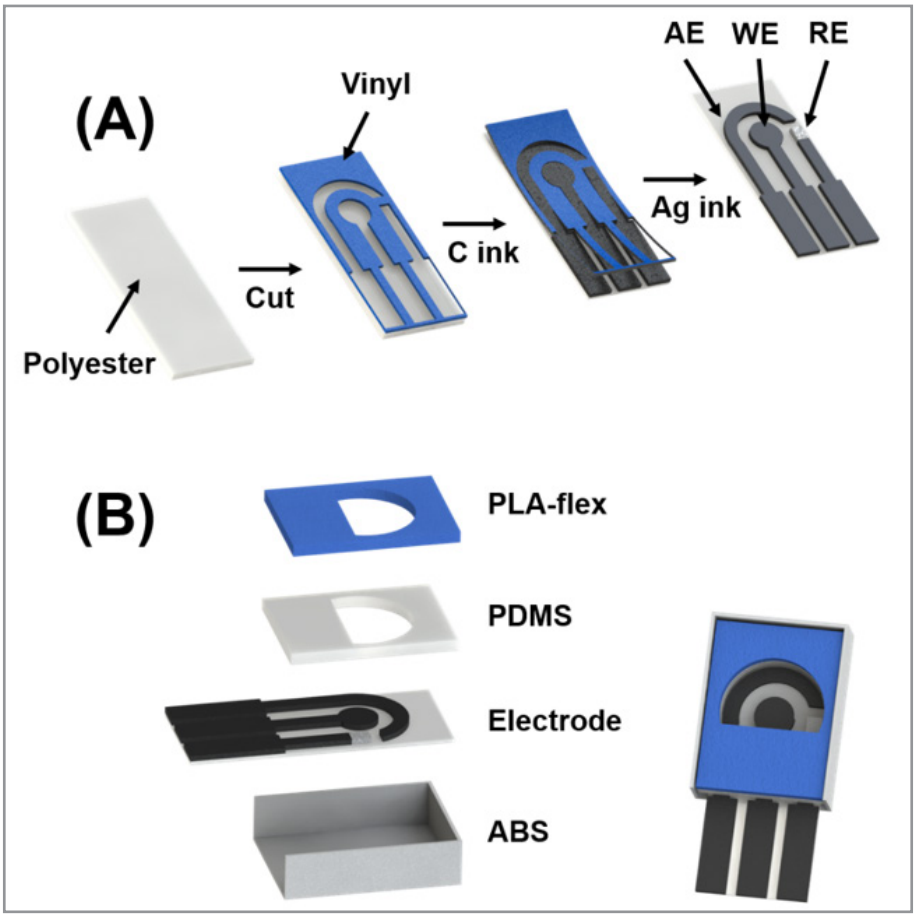


Figure 1. (A) Schematic representation of the stencil-printing method for producing disposable carbon-based electrodes and (B) their assembling in a 3D printed holder combining thermoplastic materials and PDMS film.

Instrumentation and SWV measurements

Electrochemical measurements were performed using a portable bipotentiostat/galvanostat, model µStat 400 from DropSens S.L. (Oviedo, Spain) equipped with DropView 2.9 software. The stock solution of SC (5 mmol L^{-1}) was prepared in a mixture of acetonitrile and ultrapure water at the ratio 30:70 (v/v).

For preparing the Viagra® tablet samples, six different products purchased at a local pharmacy (Goiânia, Goiás, Brazil) and one tablet supplied by the Brazilian Federal Police were used. Each tablet was individually ground and solubilized in a mixture of acetonitrile and water (30:70 v/v) and exposed to an ultrasound bath for 20 min. Finally, samples were filtered (pore size = 0.22 µm) prior to use. To realize the SWV measurements, standard solutions and tablet samples of SC were prepared in Britton-Robinson buffer (0.03 mol L^{-1}, pH = 8.0). The SWV parameters were optimized, and the best results were achieved applying 10 Hz frequency, 0.025 V amplitude and 0.004 V step ranging from 0.9 to 1.5 V *vs* Ag. All electrochemical experiments were performed at room temperature (25 ± 2 ºC).

Characterization

The surface morphology, the structural characteristics as well as the faradaic performance of the proposed SPCE were characterized by scanning electron microscopy (SEM), Raman spectroscopy, contact angle (CA) and cyclic voltammetry (CV) measurements. SEM analyses were performed with a JEOL microscope (model JSM, 6610, Waltham, MA, USA). Raman spectra were obtained with a confocal Raman spectrometer (model Horiba LabRAM HR Evolution, HORIBA France SAS) using a laser of wavelength 532 nm and a spot size of 2.6 μm. CA experiments were carried out by a Xiaomi Redmi note 10 smartphone camera fixed to a modular 3D-printed holder [43] and ImageJ software. CV measurements were recorded in 1.0 mmol L^{-1} $[Fe(CN)_6]^{4-/3-}$ prepared in 0.1 mol L^{-1} KCl at scanning rates ranging from 10 to 100 mV s^{-1}. These electrochemical experiments were realized with the goal of estimating the standard heterogeneous rate constant (K_S) based on the Nicholson method [44].

RESULTS AND DISCUSSION

Fabrication of the SPCEs

The stencil-printing technique is one of the most versatile techniques for manufacturing electrochemical sensors [45]. This fabrication strategy is particularly advantageous because it can use portable apparatus and low-cost resources, thus offering ability of creating microstructures in large scale with satisfactory fidelity [2,11]. The instrumentation required to prepare the masks by cutting plotter can be acquired for *ca.* USD 200. Also, this technology can enable the creation of alternative “do it yourself” conductive inks, which further reduces the cost of preparing components of the target analytical devices. In general, the electrochemical performance of SPCEs manufactured with alternative inks allows appreciable faradaic results with a huge potential for applications in different fields.

For the development of the alternative conductive ink, graphite powder was used as the conductive material and glass varnish as binder, as reported in a recent study [40]. The glass varnish is composed of an alkyd resin, which allows the formation of a solid composite with excellent conductive properties when incorporated with graphite flakes. Recently, Pradela et al. [8] manufactured carbon-based electrodes combining glass varnish and graphite powder on a PET substrate. The authors used permanent adhesive tape to delimit the geometric area of the electrochemical cell. As proof of concept, the proposed electrochemical cell was explored to detect estriol hormones in tap water and pharmaceutical formulation samples. In this current study, the SPCE was fabricated through a stencil-printing technique using these well-known low-cost materials. However, we used a reusable 3D-printed holder to delimitate the geometric area of the SPCE aiming drug analysis for forensic applications.

Morphological, structural, and electrochemical characterization

To evaluate the morphology and surface characteristics of the carbon-based material proposed herein, SEM images and CA measurements were recorded for WE surface. Figures 2A and 2B show SEM images recorded at different magnifications (30, 500 and 1000x) and captured images for CA measurements, respectively.

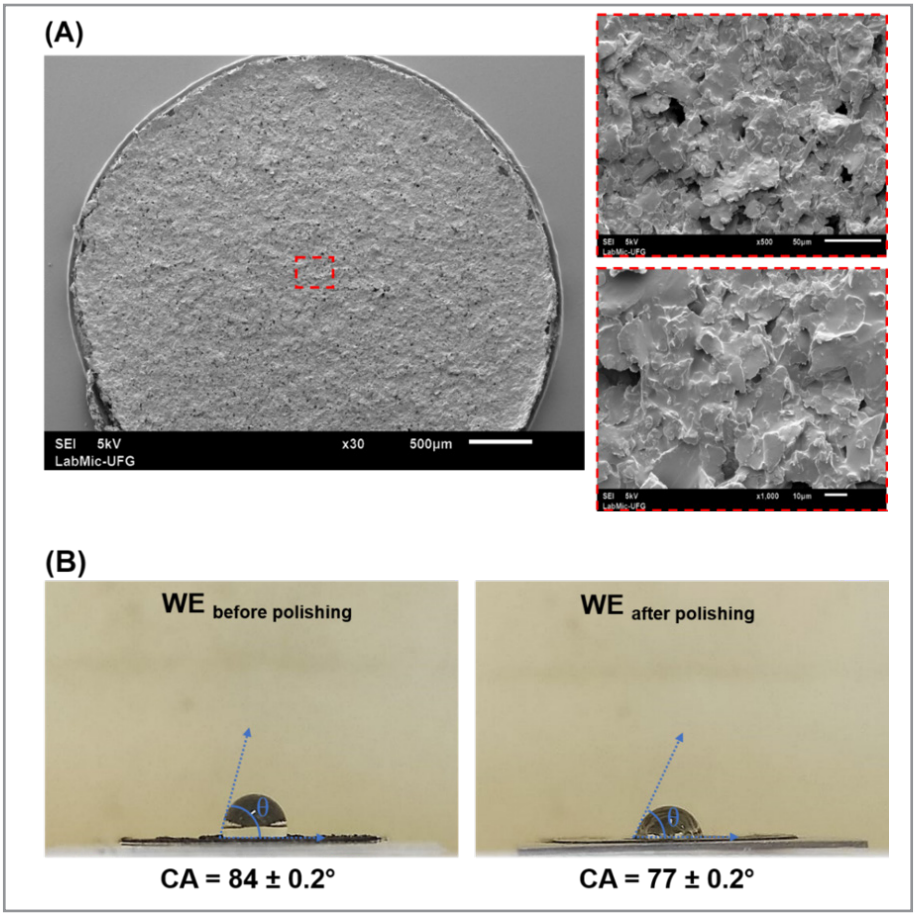


Figure 2. (A) SEM captured imagens of the WE and (B) CA measurements to WE surface before and after the polishing process.

As it can be seen, the conductive material presents a surface with graphite flakes homogenously and extensively incorporated on the polyester surface with an absence of microcracks on the carbon-based flakes. This characteristic suggests that the transfer of the conductive ink to the substrate surface was successfully achieved. Based on the recorded images, the size of the graphite flakes and the real diameter of the WE were estimated to be 9 ± 4 µm and 3.7 ± 0.2 mm, respectively.

The calculated value for the WE diameter through SEM images was compared to the theoretical dimension (Ø = 4 mm) and it revealed satisfactory fidelity (~91%). Regarding the physical characteristics of the conductive material adhered to the substrate, it was noticed that the binder appeared partially to cover the conductive graphite flakes. For this reason, a mechanical polishing step was added to the preparation method aiming to remove the excess binder and expose more graphite flakes on the surface. This strategy was adopted to enhance the electrochemical performance of the proposed SPCE. The electrochemical responses achieved before and after polishing pre-treatment are summarized in Figure S1, available in the electronic supplementary information (ESI).

Likewise SEM images, CA measurements were obtained for the proposed SPCE surface before and after the polishing process. As denoted in Figure 2, the captured images of the electrode surface before and after mechanical polishing revealed CA values equal to 84 ± 0.2º and 77 ± 0.2º, respectively. These results suggest that the excess hydrophobic material was satisfactorily removed by mechanical polishing. In addition, Cumba et al. [46] demonstrated that the mechanical polishing process on screen-printed carbon electrodes surface promotes the insertion of carbon-oxygen groups, which can improve the electron transfer rate kinetics. For this reason, the noticeable improvement observed for the electrochemical response

is probably associated to a lower repulsion to the target analyte upon the electrode solid surface. It is important to mention that carbon-based electrodes with hydrophilic properties may have greater potential in biofunctionalization, which can be interesting for applications involving sensors and biosensors.

Raman spectra provide fundamental information of the sp^2 and sp^3 carbon-based microstructures [47]. For this reason, the carbon-based ink incorporated on the plastic structure was evaluated using unpolished electrodes. Figure 3A shows the Raman spectrum with bands D, G and 2D at 1350, 1586 and 2702 cm^{-1}, respectively.

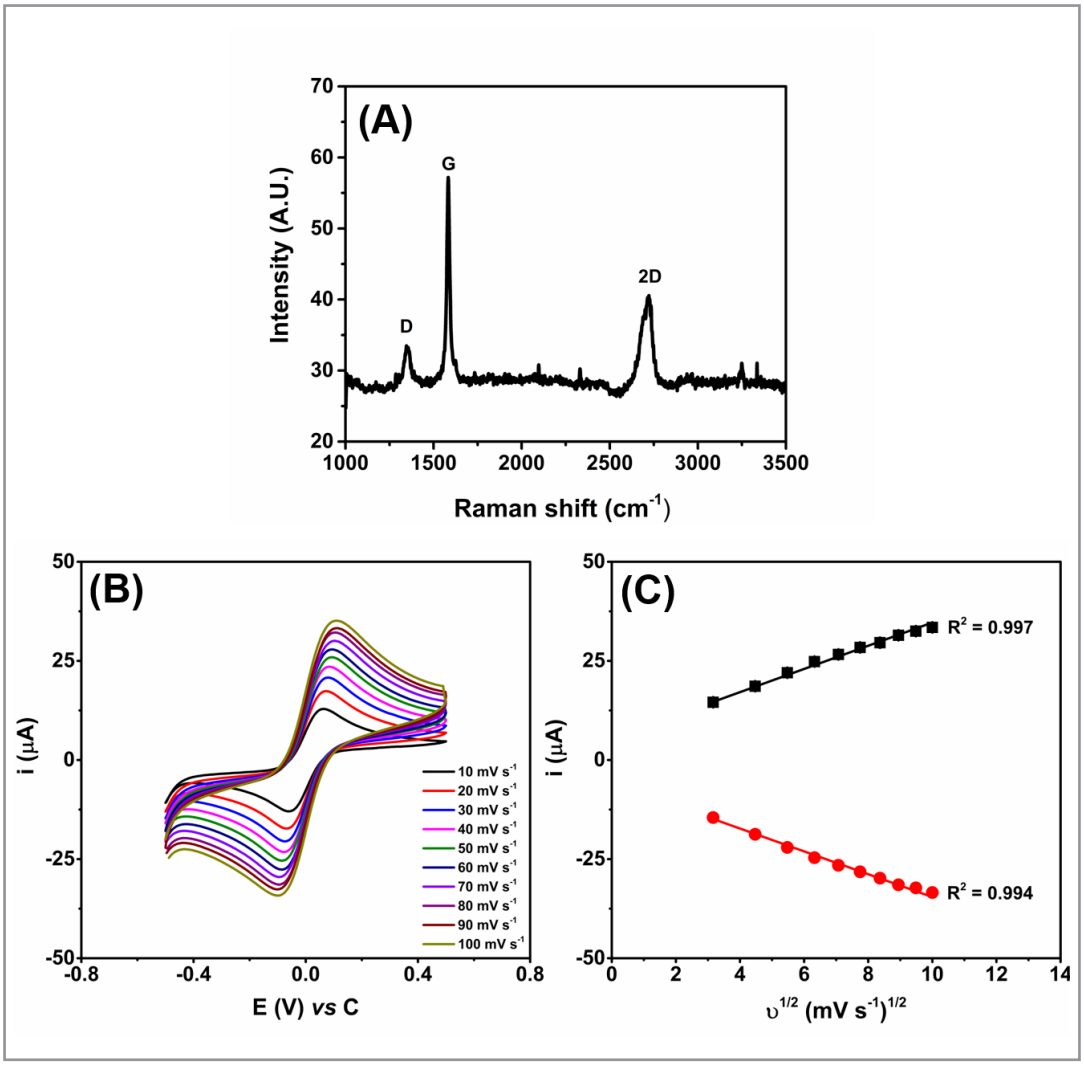


Figure 3. (A) Raman spectra ranging from 1000 to 3500 cm^{-1}, (B) cyclic voltammograms and (C) peak current *vs* scanning rate$^{1/2}$ plots involving electrochemical experiments in the presence of 1.0 mmol L^{-1} $[Fe(CN)_6]^{4-/3-}$ prepared in 0.1 mol L^{-1} KCl at scanning rates ranging from 10 to 100 mV s^{-1}.

Based on the recorded intensity of D and G bands (I_D and I_G, respectively), the I_D/I_G ratio was calculated and the resulting value was 0.19. The obtained response provides structural defects information involving the graphite flakes incorporated on polymeric substrate. When comparing this value with that reported in the literature for pure graphite (0.014) [47], it is possible to observe a drastic increase in the structural defects in the carbon microstructures distributed on the SPCE surface. This behavior may be associated with the incorporation of the graphite flakes in the amorphous polymeric material used as binder, as

recently reported [48]. It is important to highlight that carbon-based microstructures with high numbers of structural defects have been used as material to manufacture of electrochemical sensors. Examples that are gaining prominence are the carbon allotropes such as graphene, graphite and carbon black. These materials present considerable structural defects, with I_D/I_G ratio ranging from 0.16 to 1.0 [47,49,50].

The electrochemical performance of the proposed electrochemical cell was investigated using $[Fe(CN)_6]^{4-/3-}$ as redox probe aiming to evaluate the kinetic mass transfer (considering one electron) on the heterogenous electrolyte support/SPCE interface. Figure 3B depicts the recorded cyclic voltammograms at scanning rates ranging from 10 to 100 mV s^{-1}. The peak-to-peak separation (ΔEp) values calculated for low (10 mV s^{-1}) and high (100 mV s^{-1}) scanning rates were 114 ± 6 and 195 ± 10 mV, respectively (n = 3). A linear behavior (R^2 = 0.99) was observed for the peak current intensity versus square root of the scanning speed rate (Figure 3C), thus suggesting that the mass transfer is diffusion-controlled in a quasi-reversible process. For this reason, the standard heterogeneous rate constant (Ks) was estimated based on the Nicholson method (n = 3). The achieved value was 1.3 (±0.2) × 10^{-3} cm s^{-1} and it is good agreement with other reports found in the literature for carbon-based electrodes [44,51,52], thus highlighting the high electrochemical performance of the proposed SPCE. It is important to mention that the current study has successfully demonstrated for the first time the Ks value for an electrode manufactured using glass varnish-based carbon conductive ink.

3D-printed holder, repeatability and reproducibility

A reusable 3D-printed holder was fabricated and combined with the electrode aiming to simplify the manipulation of the hydrophobic barriers on the SPCE. This step is important to promote the isolation of the electrical contacts and to delimit the electrode geometric area. To demonstrate that the present strategy is similar to non-reusable and traditional methods, a comparative study was performed using the 3D-printed holder and other reported strategies [4,48]. The CV technique was used in the presence of $[Fe(CN)_6]^{4-/3-}$ using the SCPE combined with the present hydrophobic barrier, nail polish binder and laminated thermoplastic polyester. Figures 4A and 4B show the voltammograms and the resulting peak current intensities, respectively.

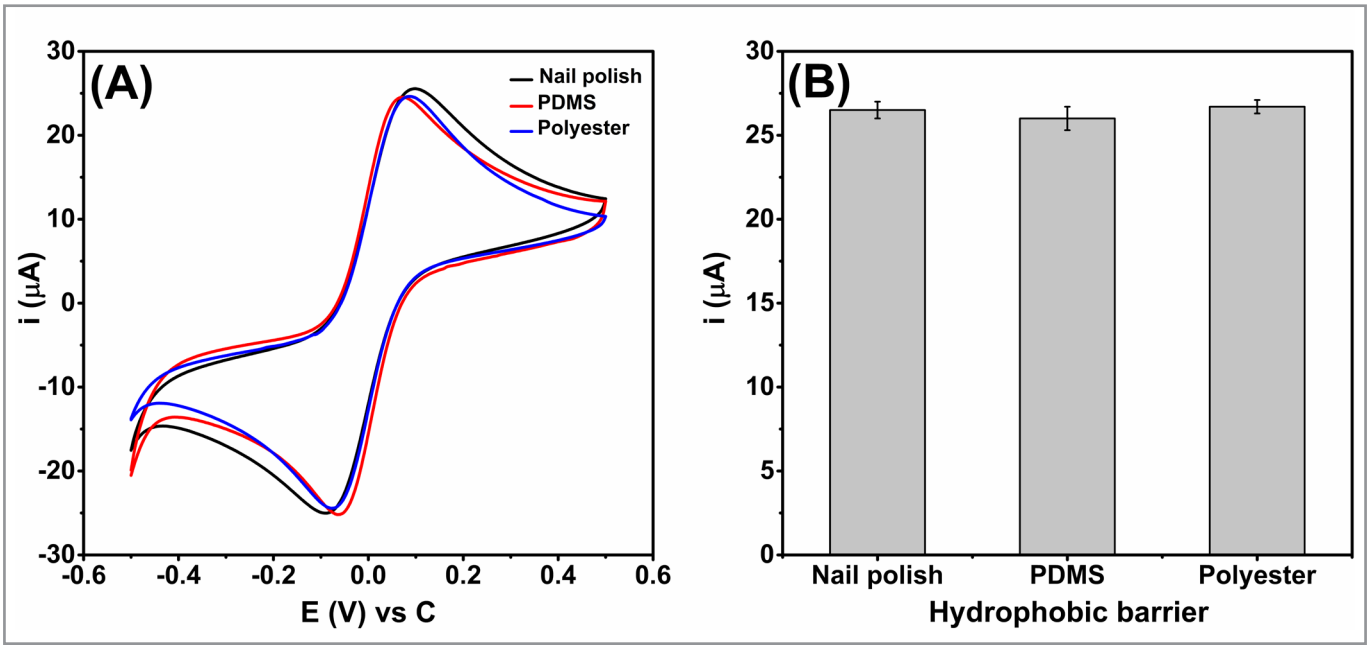


Figure 4. (A) Cyclic voltammograms and (B) current peak intensities recorded for a redox probe composed of 1.0 mmol L^{-1} $[Fe(CN)_6]^{4-/3-}$ prepared in 0.1 mol L^{-1} KCl using the proposed SPCE combined with hydrophobic barriers like nail polish, PDMS and polyester. CV conditions: scanning rate = 50 mV s^{-1} at potential window ranging from –0.5 to 0.5 V *vs* C.

As it can be seen in Figure 4, the obtained anodic peak current intensities did not change significantly between the PDMS, polyester and the 3D-printed support hydrophobic barrier. The electrochemical results in each case presented a relative standard deviation (RSD) lower than 1.5%. It is important to state that the 3D-holder associated with stencil-printing technology made use of a disposable SPCE and a reusable hydrophobic barrier to simplify the day-to-day use.

In addition, it is important to mention that the creation of hydrophobic barriers based on nail polish and thermal lamination requires manual painting and heating steps, respectively. Both manual protocols are instrumentally simple, however, they are dependent on visual perception and can be susceptible to low reproducibility during the fabrication step. When nail polish is used, for example, the binder invasion into working structure leads to the formation of different electroactive areas. In a similar way, the thermal stress used in the thermosensitive barrier process can create micro-fissures in the graphite surface, thus negatively affecting their electrical properties. On the other hand, the PDMS hydrophobic barrier is reusable as well as the 3D printed holder, i.e., once successfully printed, they can be used repeatedly [42]. This strategy is based on the formation of a sandwich arrangement (ABS support, electrode, PDMS film and PLA-Flex), which can be easily assembled or disassembled through compression and decompression of the 3D printed holder, respectively.

The repeatability and reproducibility of the SPCE fabrication protocol was also investigated through electrochemical measurements and the achieved data are summarized in Figure S2, available in the ESI. The obtained RSD values for repeatability and reproducibility were 1.4% (n = 10) and 7.8% (n = 5), respectively. The achieved results are comparable with those reported in studies on carbon-based electrodes constructed via screen-printing and pencil-drawn methods [5,53].

Analytical performance and forensic application

Forensic investigations by security and product control organizations are fundamental for human society [16]. Inspection of the quality of commercial products is one of the most important ability of forensic police, mainly in locations close to airports, docks and highways [16]. Products considered to be in high circulation in society and with considerable aggregate value, such as SC (Viagra®), are manufactured and sold illegally. These irregular formulations either have an absence or minimal presence of the expected active ingredient, or contain inactive substances or those harmful to human health [54]. For this reason, the manufactured SPCE was used in the analysis of SC in tablets of suspect origin.

To obtain a satisfactory analytical performance, important chemical conditions and electrochemical parameters were carefully evaluated, such as the pH and SWV parameters including frequency, amplitude and step. As can be seen in Figure S3, the potential (*vs* Ag) and peak current involved in the redox activity of SC are strongly influenced by the pH range of the supporting electrolyte solution. It is well-known that when buffer pH increases, the potential (*vs* Ag) changes to less positive values, as previously reported to the electrochemical cell based on graphite electrode *vs* Ag as pseudo reference electrode [38]. In addition, it was possible to observe a noticeable improvement of the electrochemical oxidation response to SC when the analyte solution is prepared in BR buffer at pH 8. In this way, the chemical conditions mentioned above were chosen for the subsequent electrochemical experiments. Likewise, it is well-known that the operating parameters such as frequency, step and amplitude influence on SWV responses. Then, these conditions were also investigated and the recorded results are displayed in Figures S3-S6 (available in the ESI). The optimized values revealed a noticeable gain in the peak current magnitude so that the chosen parameters were 10 Hz, 0.025 V e 0.004 V for frequency, amplitude and step, respectively. The best achieved conditions and parameters are summarized in Table I.

Table I. Evaluated and optimized conditions for the SWV detection of sildenafil citrate

Parameter	Evaluated range	Optimized value	Unit
pH	2; 4; 8	8	-
Frequency	5; 10; 15	10	Hz
Amplitude	0.025; 0.050; 0.075	0.025	V
Step	0.002; 0.003; 0.004	0.004	V

The analytical performance of the proposed SPCE was studied under the optimized conditions. As presented in Figure 5, a linear behavior (R^2 = 0.990) was observed in the SC concentration range from 1 to 20 µmol L^{-1}. Considering the data presented in Figure 5B, the limit of detection (LOD) was estimated based on the ratio between three times the standard deviation for the blank signal and the slope of the analytical curve (LOD = 3.0 × SD_{blank}/slope). The obtained value was 0.2 µmol L^{-1} (n = 3) and this value is in the same magnitude order than those LODs reported in other studies employing glassy carbon [36] and boron-doped diamond [33] electrodes. It is important to emphasize that the LOD achieved using SPCE is suitable for forensic studies aiming the detection of SC since its analytical performance was considered satisfactory when compared to other electrochemical methods based on carbon-based electrodes [31,33,35,36,38]. The analytical parameters found in this current study were compared to reported electrochemical methods and they are summarized in Table SI (available in the ESI).

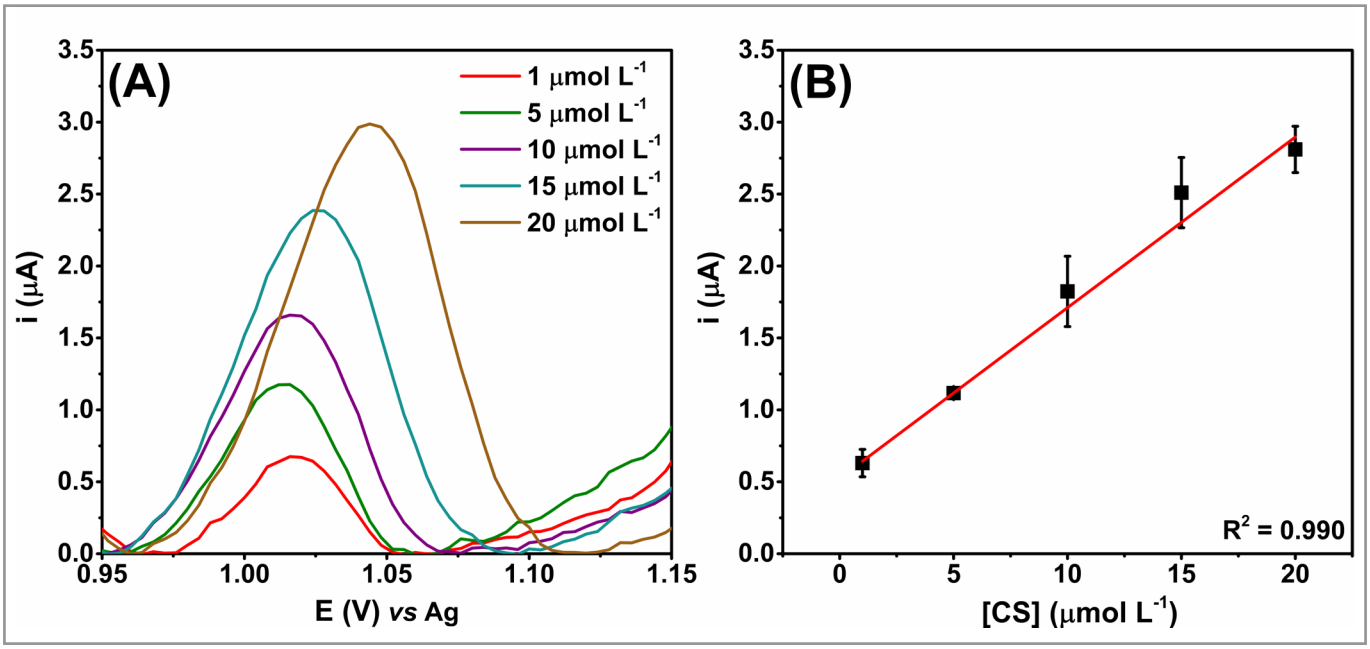


Figure 5. (A) SWV responses and (B) the resulting analytical curve obtained for standard solutions of SC prepared at different concentrations (1 to 20 µmol L^{-1}). Standard solutions were prepared in 0.03 mol L^{-1} Britton-Robinson buffer at pH 8. Linear regression equation: i(µA) = 0.52 + 0.118[SC].

Interference analyses

Pharmaceutical formulations are manufactured using the active principle and multiple other compounds to promote proper digestibility and functional performance. However, when considering adulterated tablets,

the interference possibilities are complex. For this reason, the selectively of the present method was investigated using compounds used as tablet excipients and other drugs, including those used for the treatment of erectile dysfunction (phosphate, lactose, paracetamol and tadalafil).

Electrochemical measurements were performed in the absence and presence of the possible interfering compounds considering a ratio of 10:1 (SC/interfering). The recorded CV measurements are presented in Figure S7. Based on achieved results, the percentage of interference causing an electrochemical signal increase in the presence of phosphate, lactose, tadalafil and paracetamol ranged from 6 to 14%. Electrochemical analysis of SC in the presence of tadalafil showed the greatest signal variation (14%). This level is statistically acceptable [55] and it can be associated with the similar oxidation potential (~1.0 V *vs* Ag). However, this oxidation potential can be strongly affected by the pH of the prepared samples [56].

Analysis of SC in commercial and seized tablets

The proposed SPCE devices were then explored to determine SC content in commercial samples including Viagra® (#1) and six different suppliers of generic products (#2, #3, #4, #5 and #6). In addition, the SC content was also determined in one sample seized and donated by the federal police (#7). The results are displayed in Table II.

Table II. Summary of the SC content in commercial and seized tables using SPCE and SWV measurements (n = 3)

Samples	Labeled (mg)	Found (mg)
#1	50	50 ± 1
#2	50	54 ± 6
#3	50	54 ± 4
#4	50	54 ± 6
#5	50	52 ± 1
#6	50	54 ± 1
#7	50	38 ± 1

As it can be seen in Table II, the achieved data using SPCE were compared to the labelled content by different suppliers. For samples #1-6, the differences observed ranged between 2 and 8%. On the other hand, the SC content obtained for sample #7 was ca. 22% lower than the labeled value. This was somehow expected since this sample was seized by the Federal Police due to suspected adulteration. The obtained data was statistically compared through t-test (95% significance) and the calculated t and t-critical values were –0.38 and 2.44, respectively. Considering the paired t-test, it can be inferred that the results obtained for samples #1-6 did not reveal statistical differences between the achieved and labeled values at confidence level of 95%. On the other hand, the paired t-test confirmed (calculated t value lower than t-critical) that the obtained SC values for seized tablet (sample #7) was lower than the expected content thus suggesting the adulteration of the active principle amount. Based on the achieved data, it can be inferred that the proposed SPCE emerges as huge potential as analytical tool for forensic investigations.

The accuracy of the proposed SWV experiments using SPCE was investigated through recovery experiments. For this purpose, a solution containing SC ((50 mg per tablet) sample #1) was diluted to 5 μmol L^{-1} and then spiked with three different levels of SC standard solution (5, 10 and 15 μmol L^{-1}). The achieved results of the recovery experiment are shown in Table III. The recovery studies demonstrated

values ranging from 98.2 to 102.0% (n = 3), which suggest that the proposed analytical procedure did not suffer significant interference from the tablet matrix. In addition, the obtained percentages were within the range allowed by the regulatory agencies [55] and they are similar to the values reported in other studies dedicated to the determination of SC in commercial tablets [37,57].

Table III. Standard addition method for quantification of SC and recovery values considering to SWV experiments (n=3)

Spiking level	Sildenafil citrate (μmol L^{-1})		Recovery (%)
	Added	Found	
#1	5	5.1 ± 0.1	102.0
#2	10	10.2 ± 0.4	101.3
#3	15	14.7 ± 0.3	98.2

CONCLUSIONS

In summary, simple and disposable stencil-printed electrodes have demonstrated great feasibility for forensic investigations based on voltametric determination of sildenafil citrate in commercial and seized tablets. The proposed device was fabricated using affordable materials and assembled in a sandwich arrangement composed of a 3D-printed piece, a PDMS film and a PLA-flex, offering robustness and reproducibility. The mechanical polishing pre-treatment of the electrode surface was essential to expose more conductive particles allowing to achieve noticeable improvements on the electrochemical performance. The proposed approach revealed to be a promising analytical tool for forensic screening since it has ensured accurate analysis of SC in commercial and seized tablets. Future studies will focus on a complete validation of the proposed approach involving a larger and more representative sampling and a comparison with a reference analytical technique.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank CAPES [grant 3363/2014], CNPq [grants 426496/2018-3, 308140/2016-8 and 307554/2020-1] and INCTBio [grant 465389/2014-7] for the financial support and granted scholarships. The authors acknowledge the Multi-user Laboratory of high-resolution microscopy (LabMic/UFG) for using their facilities during SEM analysis. Lastly, we thank Professor Dr. Janegitz, B. C. for providing the graphite powder.

REFERENCES

1. Dossi, N.; Terzi, F.; Piccin, E.; Toniolo, R.; Bontempelli, G. *Electroanalysis*, **2016**, *28* (2), pp 250-264 (https://doi.org/10.1002/elan.201500361).
2. Ataide, V. N.; Mendes, L. F.; Gama, L. I . L. M.; de Araujo, W. R.; Paixão; T. R. L. C. *Anal. Methods,* **2020**, *12*, pp 1030-1054 (https://doi.org/10.1039/c9ay02350j).
3. de Moraes, N. C.; da Silva, E. N. T.; Petroni, J. M.; Ferreira, V. S.; Lucca, B. G *Electrophoresis*, **2019**, *41* (5-6), pp 278-286 (https://doi.org/10.1002/elps.201900270).
4. Foster, C. W.; Metters, J. P.; Banks, C. E. *Electroanalysis*, **2013**, *25*, pp 2275-2282 (https://doi.org/10.1002/elan.201300274).

5. Camargo, J. R.; Andreotti, I. A. A.; Kalinke, C.; Henrique, J. M.; Bonacin, J. A.; Janegitz, B. C. *Talanta*, **2020**, *208*, 120458 (https://doi.org/10.1016/j.talanta.2019.120458).
6. Petroni, J. M.; Lucca, B. G.; Ferreira, V. S. *Electroanalysis*, **2017**, *29* (7), pp 1762-1771 (https://doi.org/10.1002/elan.201700117).
7. Sameenoi, Y.; Mensack, M. M.; Boonsong, K.; Ewing, R.; Dungchai, W.; Chailapakul, O.; Cropek, D. M.; Henry, C. S. *Analyst,* **2011**, *136* (15), pp 3177-3184 (https://doi.org/10.1039/c1an15335h).
8. Pradela-Filho, L. A.; Andreotti, I. A. A.; Carvalho, J. H. S.; Araújo, D. A. G.; Orzari, L. O.; Gatti, A.; Takeuchi, R. M.; Santos, A. L.; Janegitz, B. C. *Sens. Actuators B Chem*., **2020**, *305*, 127433 (https://doi.org/10.1016/j.snb.2019.127433).
9. Tortorich, R. P.; Shamkhalichenar, H.; Choi, J. W. *Appl. Sci*., **2018**, *8* (2), 288 (https://doi.org/10.3390/app8020288).
10. Lamas-Ardisana, P. J.; Casuso, P.; Fernandez-Gauna, I.; Martínez-Paredes, G.; Jubete, E.; Añorga, L.; Cabañero, G.; Grande, H. J. *Electrochem Commun*., **2017,** *75*, pp 25-28 (https://doi.org/10.1016/j.elecom.2016.11.015).
11. Kava, A. A.; Beardsley, C.; Hofstetter, J.; Henry, C. S. *Anal. Chim. Acta*, **2020**, *1103*, pp 58-66 (https://doi.org/10.1016/j.aca.2019.12.047).
12. Adkins, J.; Boehle, K.; Henry, C. *Electrophoresis*, **2015**, *36*, pp 1811-1824 (https://doi.org/10.1002/elps.201500084).
13. Pradela-Filho, L. A.; Araújo, D. A. G.; Takeuchi, R. M.; Santos, A. L. *Electrochim. Acta*, **2017,** *258*, pp 786-792 (https://doi.org/10.1016/j.electacta.2017.11.127).
14. Andreotti, I. A. A.; Orzari, L. O.; Camargo, J. R.; Faria, R. C.; Marcolino-Junior, L. H.; Bergamini, M. F.; Gatti, A.; Janegitz, B. C. *J. Electroanal. Chem*., **2019**, *840*, pp 109-116 (https://doi.org/10.1016/j.jelechem.2019.03.059).
15. Martín-Yerga, D.; Álvarez-Martos, I.; Blanco-López, M. C.; Henry, C. S.; Fernández-Abedul, M.T. *Anal. Chim. Acta*, **2017**, *981*, pp 24-33 (https://doi.org/10.1016/j.aca.2017.05.027).
16. de Araujo, W. R.; Cardoso, T. M. G.; da Rocha, R. G.; Santana, M. H. P.; Muñoz, R. A. A.; Richter, E. M.; Paixão, T. R. L. C.; Coltro, W. K. T. *Anal. Chim. Acta,* **2018***, 1034*, pp 1-21 (https://doi.org/10.1016/j.aca.2018.06.014).
17. Jung, C. R.; Ortiz, R. S.; Limberger, R.; Mayorga, P. *Forensic Sci. Int*., **2012**, *216* (1-3), pp 92-96 (https://doi.org/10.1016/j.forsciint.2011.09.002).
18. Park, M.; Ahn, S. *Forensic Sci. Int*., **2012**, *57* (6), pp 1637-1640 (https://doi.org/10.1111/j.1556-4029.2012.02164.x).
19. Shinde, S. R.; Bhavsar, K.; Kimbahune, S.; Khandelwal, S.; Ghose, A. *Annu. Int. Conf. IEEE Eng. Med. Biol. Soc.*, **2020**, pp 6155-6158 (https://doi.org/10.1109/EMBC44109.2020.9176419).
20. Orubu, E. S. F.; Ching, C.; Zaman, M. H.; Wirtz, V. J. *J. Pharm. Policy Pract*., **2020**, *13* (1), pp 1–10 (https://doi.org/10.1186/s40545-020-00208-4).
21. Sanada, T.; Yoshida, N.; Matsushita, R.; Kimura, K.; Tsuboi, H. *Forensic Sci. Int*., **2020**, *307*, 110143 (https://doi.org/10.1016/j.forsciint.2020.110143).
22. Sanada, T.; Yoshida, N.; Kimura, K.; Tsuboi, H. *Pharmacy*, **2020**, *9* (1) (https://doi.org/10.3390/pharmacy9010003).
23. Patel, D. N.; Li, L.; Kee, C. L.; Ge, X.; Low, M. Y.; Koh, H. L. *J. Pharm. Biomed. Anal*., **2014**, *87*, pp 176-190 (https://doi.org/10.1016/j.jpba.2013.04.037).
24. Kass, D. A.; Champion, H. C.; Beavo, J. A. *Circulation Research,* **2007**,*101* (11), pp 1084-1095 (https://doi.org/10.1161/CIRCRESAHA.107.162511).
25. da Silveira, G. D.; Bressan, L. P.; Schmidt, M. E. P.; Molin, T. R. D.; Teixeira, C. A.; Poppi, R. J.; da Silva, J. A. F. *J. Solid State Electrochem*., **2020**, *24* (8), pp 1999-2010 (https://doi.org/10.1007/s10008-020-04533-1).
26. Al-Hroub, H.; Alkhawaja, B.; Alkhawaja, E.; Arafat, T. *J. Chromatogr. B*., **2016**, *1009-1010*, pp 1-6 (https://doi.org/10.1016/j.jchromb.2015.11.059).

27. Salem, H.; Aziz, B. E. A. *Anal. Chem. Lett.,* **2020**, *10* (3), pp 321-335 (https://doi.org/10.1080/22297928.2020.1779813).
28. Keizers, P. H. J.; Wiegard, A.; Venhuis, B. J. *J. Pharm. Biomed. Anal.*, **2016**, *131*, pp 133-139 (https://doi.org/10.1016/j.jpba.2016.08.027).
29. Sopha, H.; Hocevar, S. B.; Pihlar, B.; Ogorevc, B. *Electrochim. Acta*, **2012**, *60*, pp 274-277 (https://doi.org/10.1016/j.electacta.2011.11.049).
30. Lović, J.; Trišović, N.; Antanasijević, J.; Ivić, M. A. *J. Electrochem. Sci. Eng.*, **2018**, *8* (2), pp 163-170 (https://doi.org/10.5599/jese.481).
31. Tyszczuk, K.; Korolczuk, M. *Bioelectrochemistry*, **2010**, *78* (2), pp 113-117 (https://doi.org/10.1016/j.bioelechem.2009.08.005).
32. Rouhani, M.; Soleymanpour, A. *Microchim. Acta*, **2020**, *187* (9), 512 (https://doi.org/10.1007/s00604-020-04482-6).
33. Batista, E. F.; Sartori, E. R.; Medeiros, R. A.; Rocha-Filho, R. C.; Fatibello-Filho, O. *Anal. Lett.*, **2010**, *43* (6), pp 1046–1054 (https://doi.org/10.1080/00032710903491153).
34. Berzas, J. J.; Rodriguez, J.; Castañeda, G.; Villaseñor, M. J. *Anal. Chim. Acta*, **2000**, *417* (2), pp 143-148 (https://doi.org/10.1016/S0003-2670(00)00932-6).
35. Staden, R. S.; Staden, J. F. V.; Aboul-Enein, H. Y. *J. Solid State Electrochem.*, **2010**, *14* (6), pp 997–1000 (https://doi.org/10.1007/s10008-009-0901-7).
36. Özkan, S. A.; Uslu, B.; Zuman, P. *Anal. Chim. Acta*, **2004**, *501* (2), pp 227-233 (https://doi.org/10.1016/j.aca.2003.09.033).
37. Farghali, R. A.; Ahmed, R. A. *Int. J. Electrochem. Sci.*, **2015**, *10* (2), pp 1494-1505.
38. Backes, R. S.; Guedes, T. J.; dos Santos, W. T. P.; da Silva, R. A. B. *Quim. Nova*, **2017**, *40* (7), pp 752-759 (http://dx.doi.org/10.21577/0100-4042.20170047).
39. Júnior, A. C. V. L.; Luz, R. C. S.; Damos, F. S.; dos Santos, A. S.; Franco, D. L.; dos Santos, W. T. P. *J. Braz. Chem. Soc.*, **2012**, *23* (10), pp 1800-1806 (https://doi.org/10.1590/S0103-50532012005000047).
40. Silva-Neto, H. A.; Cardoso, T. M. G.; McMahon, C. J.; Sgobbi, L. F.; Henry, C. S.; Coltro, W. K. T. *Analyst,* **2021**, *146* (11), pp 3463-3473 (https://doi.org/10.1039/d1an00176k).
41. Afonso, A. S.; Uliana, C. V.; Martucci, D. H.; Faria, R. C. *Talanta,* **2016***, 146*, pp 381-387 (https://doi.org/10.1016/j.talanta.2015.09.002).
42. Rocha, D. S.; Duarte, L. C.; Silva-Neto, H. A.; Chagas, C. L. S.; Santana, M. H. P.; Antoniosi Filho, N. R.; Coltro, W. K. T. *Talanta*, **2021**, *232*, 122408 (https://doi.org/10.1016/j.talanta.2021.122408).
43. da Silva, V. A. O. P.; Tartare, V. A. P.; Kalinke, C.; de Oliveira, P. R.; Bonacin, J. A.; Janegitz, B. C. *Quim. Nova*, **2020**, *43* (9), pp 1312-1319 (http://dx.doi.org/10.21577/0100-4042.20170624).
44. Nicholson, R. S. *Anal. Chem.*, **1965**, *37* (11), pp 1351-1355 (https://doi.org/10.1021/ac60230a016).
45. Noviana, E.; McCord, C. P.; Clark, K. M.; Jang, I.; Henry, C. S. *Lab Chip*, **2020**, *20* (1), pp 9-34 (https://doi.org/10.1039/c9lc00903e).
46. Cumba, L. R.; Foster, C. W.; Brownson, D. A. C.; Smith, J. P.; Iniesta, J.; Thakur, B.; do Carmo, D. R.; Banks, C. E. *Analyst*, **2016**, *141*, pp 2791-2799 (https://doi.org/10.1039/c6an00167j).
47. Pimenta, M. A.; Dresselhaus, G.; Dresselhaus, M. S.; Cançado, L. G.; Jorio, A.; Saito, R. *Phys. Chem. Chem. Phys.*, **2007**, *9* (11), pp 1276-1291 (https://doi.org/10.1039/b613962k).
48. Dias, A. A.; Chagas, C. L. S.; Silva-Neto, H. A.; Lobo-Junior, E. O.; Sgobbi, L. F.; de Araujo, W. R.; Paixão, T. R. L. C.; Coltro, W. K. T. *ACS Appl. Mater. Interfaces*, **2019**, *11* (43), pp 39484-39492 (https://doi.org/10.1021/acsami.9b12797).
49. Klunder, K. J.; Nilsson, Z.; Sambur, J. B.; Henry C. S. *J. Am. Chem. Soc.*, **2017**, *139* (36), pp 12623-12631 (https://doi.org/10.1021/jacs.7b06173).
50. de Araujo, W. R.; Frasson, C. M. R.; Ameku, W. A.; Silva, J. R.; Angnes, L.; Paixão, T. R. L. C. *Angew. Chem.*, **2017**, *129* (47), pp 15309-15313 (https://doi.org/10.1002/anie.201708527).
51. Arduini, F.; Cinti, S.; Mazzaracchio, V.; Scognamiglio, V.; Amine, A.; Moscone, D. *Biosens. Bioelectron.*, **2020**, *156*, 112033 (https://doi.org/10.1016/j.bios.2020.112033).

52. Santhiago, M.; Strauss, M.; Pereira, M. P.; Chagas, A. S.; Bufon, C. C. B. *ACS Appl. Mater. Interfaces*, **2017**, *9* (13), pp 11959-11966 (https://doi.org/10.1021/acsami.6b15646).
53. Dossi, N.; Petrazzi, S.; Terzi, F.; Toniolo, R.; Bontempelli, G. *Talanta*, **2019**, *199*, pp 14-20 (https://doi.org/10.1016/j.talanta.2019.01.126).
54. Maria, J.; Noordin, M. I. *J. Therm. Anal. Calorim.*, **2014**, *115* (2), pp 1907-1914 (https://doi.org/10.1007/s10973-013-3413-8).
55. Moosavi, S. M.; Ghassabian, S. Linearity of Calibration Curves for Analytical Methods: A Review of Criteria for Assessment of Method Reliability. In: Stauffer, M. (Ed.). *Calibration and Validation of Analytical Methods – A Sampling of Current Approaches.* Intechopen, **2018**, Chapter 6, pp 109-127 (https://doi.org/10.5772/intechopen.72932).
56. Sartori, E. R.; Clausen, D. N.; Pires, I. M. R.; Salamanca-Neto, C. A. R. *Diam. Relat. Mater.*, **2017**, *77*, pp 153-158 (https://doi.org/10.1016/j.diamond.2017.07.001).
57. Tyszczuk, K.; Korolczuk, M. *Bioelectrochemistry*, **2010**, *78* (2), pp 113-117 (https://doi.org/10.1016/j.bioelechem.2009.08.005).

Supplementary Material

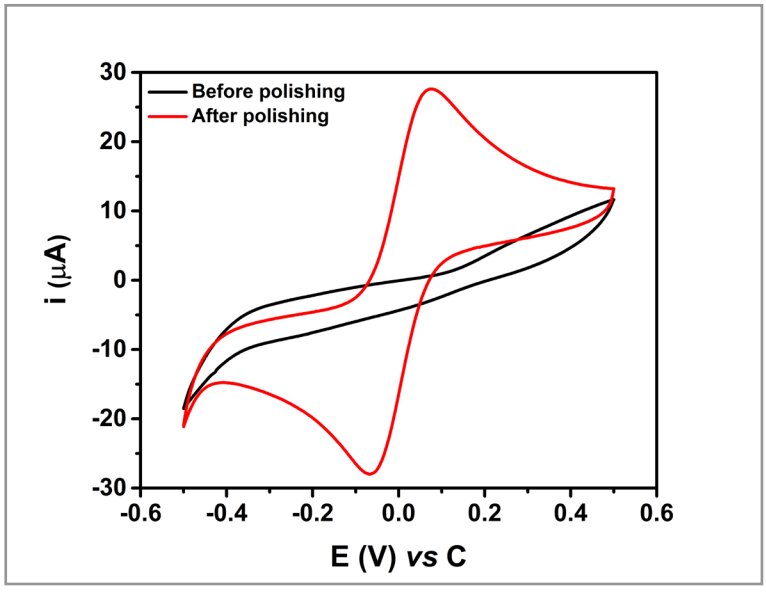


Figure S1. Cyclic voltammograms recorded in presence of 1.0 mmol L^{-1} $[Fe(CN)_6]^{4-/3-}$ prepared in KCl 0.1 mol L^{-1} using electrode without sanding and after polishing process with sandpaper. Electrochemical conditions: 50 mV s^{-1} ranging from -0.5 to 0.5 V *vs* C.

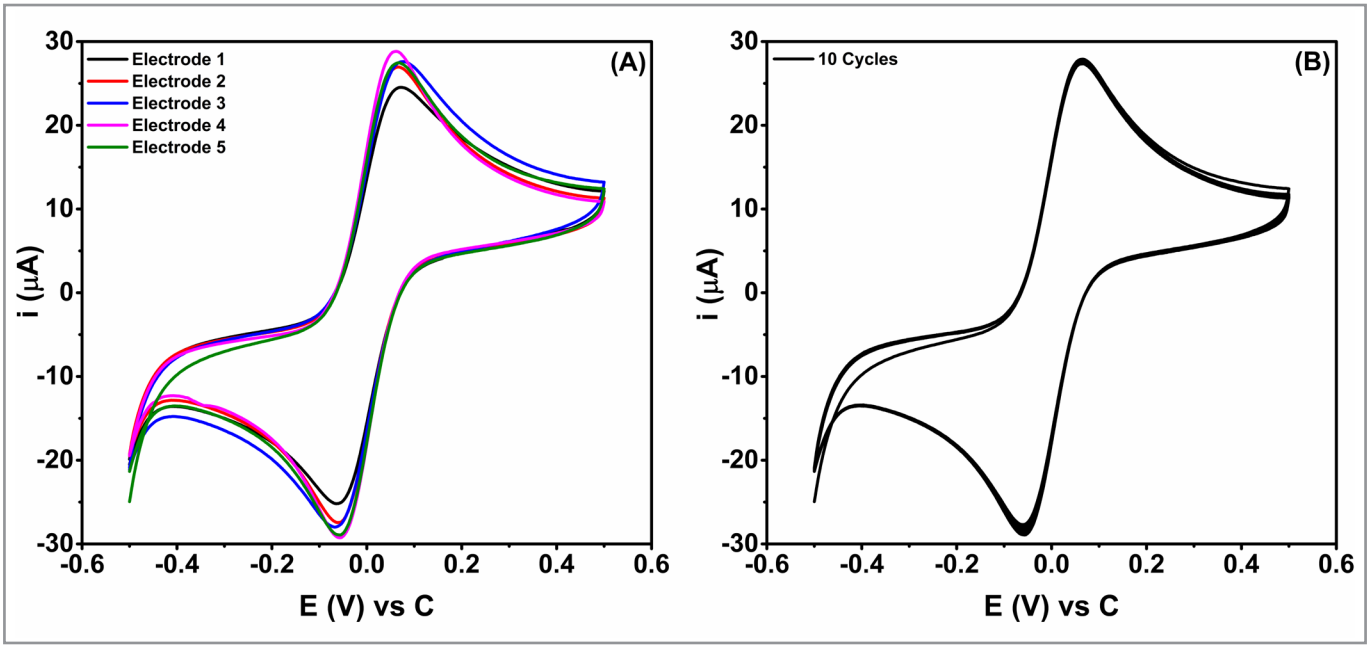


Figure S2. Cyclic voltammograms responses obtained for (A) reproducibility and (B) repeatability electrochemical experiments.

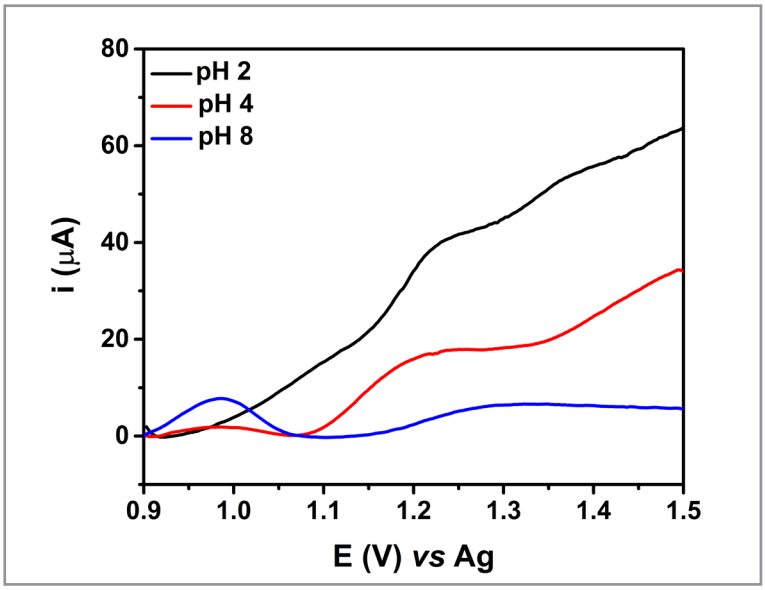


Figure S3. Square wave voltammograms measurements of sildenafil citrate involving the optimization study of buffer solution. Buffer solution conditions: 0.03 mol L^{-1} Britton-Robinson in presence of 500 μmol L^{-1} sildenafil citrate. Electrochemical conditions: 10 Hz frequency, 0.025 V amplitude and 0.003 V step ranging from 0.9 to 1.5 V *vs* Ag.

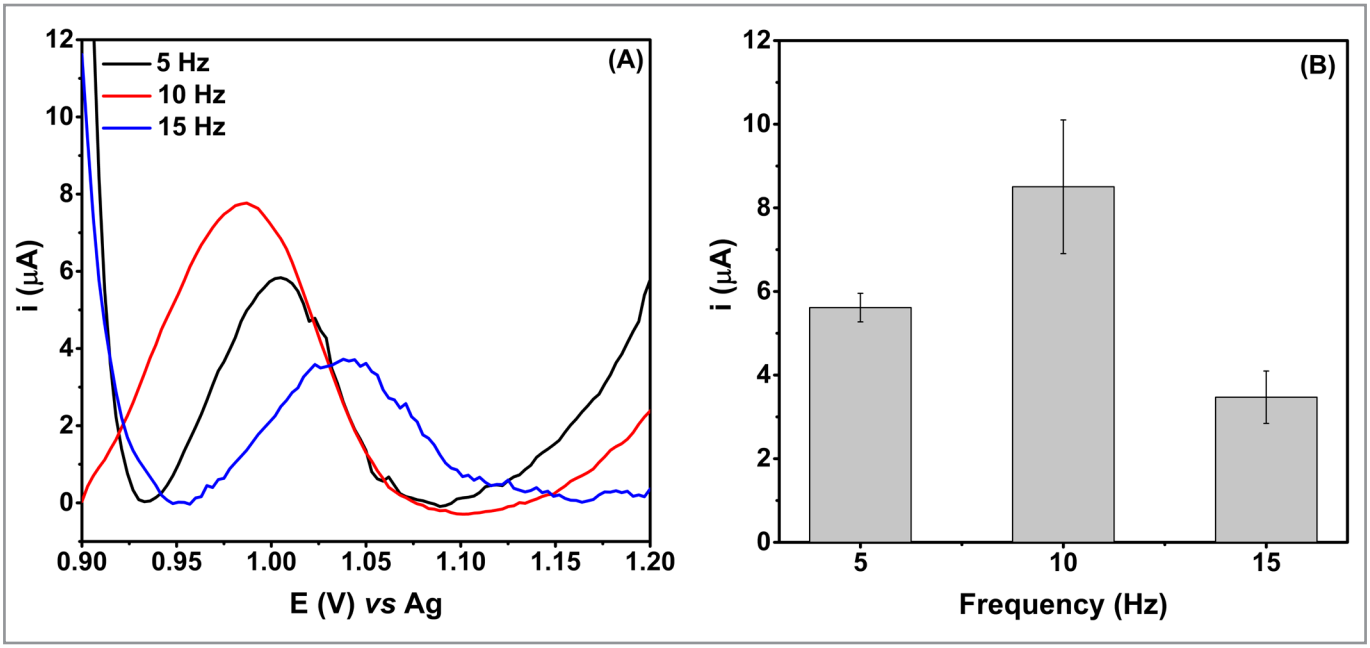


Figure S4. (A) SWV experiments of the frequency parameter optimization involving the sildenafil citrate oxidation. Buffer solution condition: 0.03 mol L^{-1} Britton-Robinson (pH = 8). (B) Peak current signal of sildenafil citrate oxidation. Optimized electrochemical conditions: 0.025 V amplitude, 0.003 V step and potential range 0.9 to 1.5 V.

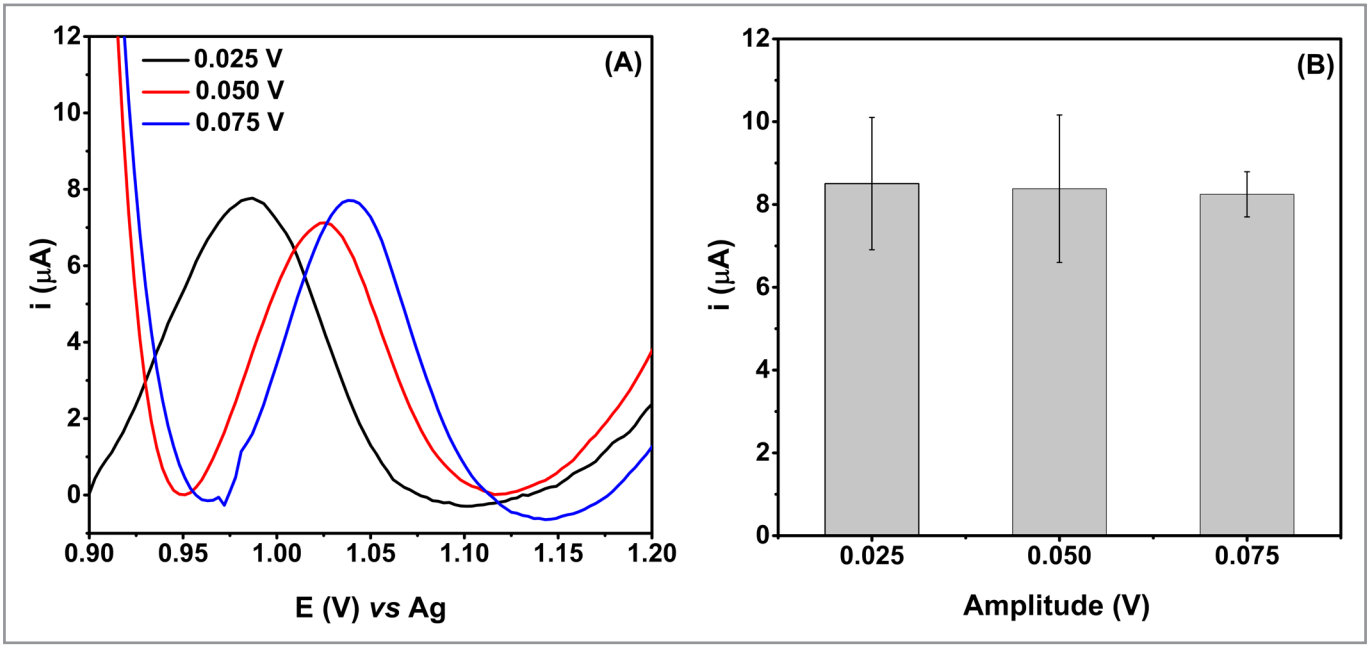


Figure S5. (A) SWV experiments of the amplitude parameter optimization involving the sildenafil citrate oxidation. (B) Peak current responses of sildenafil citrate oxidation. Fixed SWV conditions: 0.003 V step, 10 Hz frequency ranging from 0.9 to 1.5 V *vs* Ag.

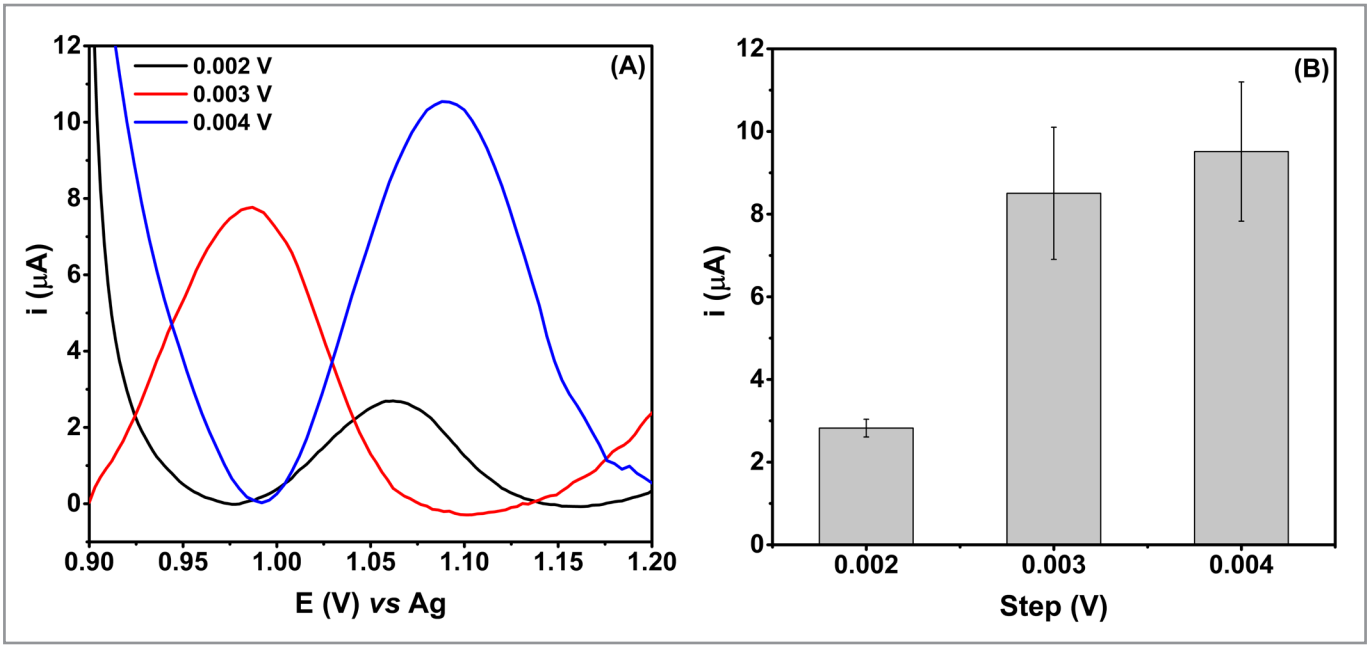


Figure S6. (A) SWV experiments of the step parameter optimization involving the sildenafil citrate oxidation. (B) Peak current responses of sildenafil citrate oxidation. Fixed SWV conditions: 0.025 V amplitude, 10 Hz frequency ranging from 0.9 to 1.5 V *vs* Ag.

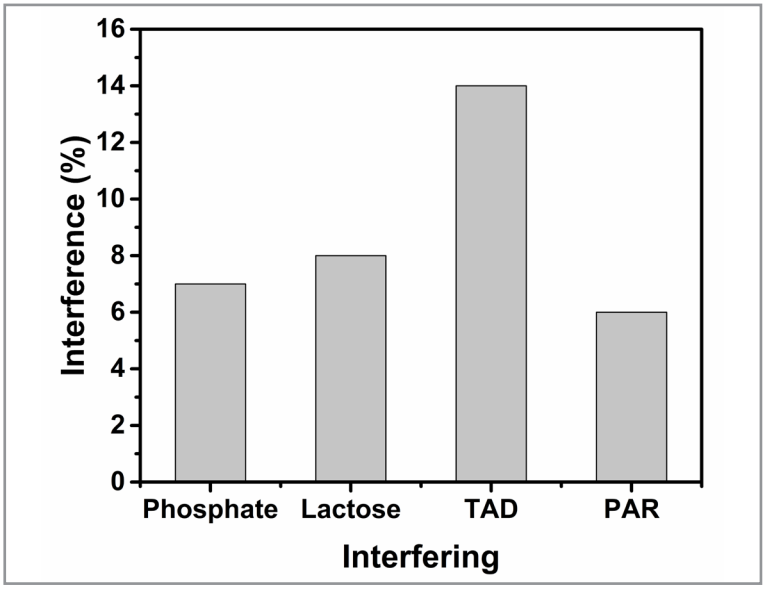


Figure S7. Obtained electrochemical results to sildenafil oxidation realized in presence of possible interfering species.

Table SI. Comparison of the type of electrode, electrochemical method, linear range and limit of detection (LOD) for sildenafil citrate detection

Electrode	Sample	Method	Linear range (mol L^{-1})	LOD (mol L^{-1})	Reference
Glassy carbon	Pharmaceutical formulations	SWV	$6.0x10^{-6}$ – $3.0x10^{-4}$ and $4.0x10^{-6}$ – $3.0x10^{-4}$	$6.3x10^{-7}$ and $1.1x10^{-6}$	[1]
Boron-doped diamond electrode	Pharmaceutical formulations	DPV	$7.3x10^{-7}$ – $7.3x10^{-6}$	$6.4x10^{-7}$	[2]
Scrren-printed carbon	Pharmaceutical formulations	BIA	$3.0x10^{-6}$ – $21x10^{-6}$	$5.2x10^{-8}$	[3]
Screen-printed glassy carbon	Pharmaceutical formulations	SWV	$1.0x10^{-6}$ – $1.4x10^{-5}$	$5.5x10^{-8}$	[4]
Screen-printed carbon electrode	Pharmaceutical formulations and adulterated tablet	SWV	$1.0x10^{-6}$ – $20x10^{-6}$	$2.0\ x10^{-7}$	This study

REFERENCES

1. Özkan, S. A.; Uslu, B.; Zuman, P. *Anal. Chim. Acta*, **2004**, *501*, pp 227-233 (https://doi.org/10.1016/j.aca.2003.09.033).
2. Batista, É. F.; Sartori, E. R.; Medeiros, R. A.; Rocha-Filho, R. C.; Fatibello-Filho, O. *Anal. Lett*., **2010**, *43* (6), pp 1046–1054 (https://doi.org/10.1080/00032710903491153.).
3. Backes, R. S.; Guedes, T. J.; dos Santos, W. T. P.; da Silva, R. A. B. *Quim. Nova*, **2013**, *40* (7), pp 1248-1255 (http://dx.doi.org/10.21577/0100-4042.20170047).
4. Farghali, R. A.; Ahmed, R. A. *Int. J. Electrochem. Sci*., **2012**, *7* (12), pp 13008-13019.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 229-239
doi: 10.30744/brjac.2179-3425.TN-59-2020

BrJAC

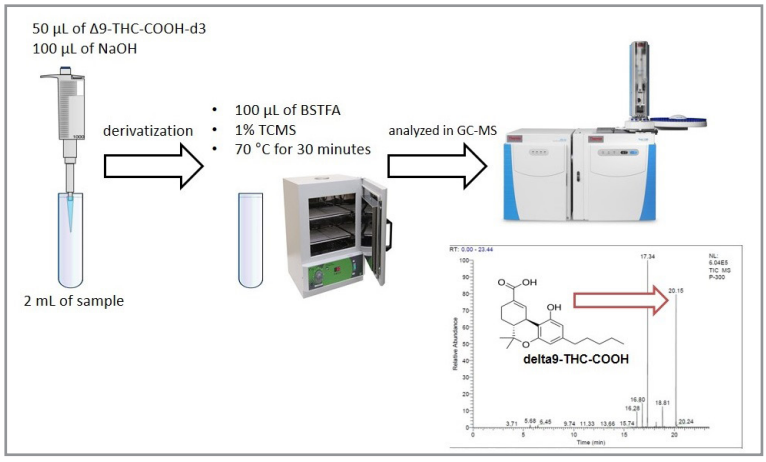
TECHNICAL NOTE

Quantitative analysis of Δ9-THC-COOH in Human Urine by the Liquid-Liquid Extraction technique and Gas Chromatography-Mass Spectrometry: Adaptation, Optimization and Validation

Kleber Ota de Oliveira[1], Deborah Thais Palma Scanferla[1], Jordana Quaglia Pereira da Silva[1], Mariana Aparecida Oliveira Madia[1], Erika Bando[1], Miguel Machinski Junior[1], Camila Marchioni[2], Simone Aparecida Galerani Mossini[1]*

[1]Universidade Estadual de Maringá, Avenida Colombo, 5700, Jardim Universitário, 87020-900, Maringá, PR, Brazil

[2]Universidade Federal de Santa Catarina, Rua Engenheiro Agronômico Andrei Cristian Ferreira, s/n, Trindade, 88040-900, Florianópolis, SC, Brazil



The main active compound of *Cannabis sativa* is Δ9-tetrahydrocannabinol, which is quickly transformed into 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (Δ9-THC-COOH) in the human body. This research aimed to validate an efficient, fast and low-cost technique for Δ9-THC-COOH analysis in urine with adaptations of existing analytical methods. The validation process was carried out in accordance with guidelines published by ANVISA and with international guidelines. The analyte was extracted by liquid-liquid extraction and identified/quantified by gas chromatography coupled to mass spectrometry. Linear curves ranges were from 5 to 300 ng mL^{-1} (r = 0.9993; y = 0.0269x – 0.0364). Intra and inter-day precision varied from 3.38 to 9.04% and accuracy was between 83 to 112.9%. The Δ9-THC-COOH remained stable after 15-30 days of storage at -20 °C (long-term test), after 5 freeze-thaw cycles and post-processing for up to 72 hours. The method is fast, low-cost, with detection limits and quantification below the cut-off (15 ng mL^{-1}), which makes it useful and efficient for routine use at toxicology laboratories, for drug addiction and doping control, for forensic purposes and also for controlling the use of drugs of abuse by vehicle drivers.

Keywords: Δ9-THC-COOH; human urine; liquid-liquid extraction; gas chromatography-mass spectrometry.

Cite: de Oliveira, K. O.; Scanferla, D. T. P.; da Silva, J. Q. P.; Madia, M. A. O.; Bando, E.; Machinski Junior, M.; Marchioni, C.; Mossini, S. A. G. Quantitative analysis of Δ9-THC-COOH in Human Urine by the Liquid-Liquid Extraction technique and Gas Chromatography-Mass Spectrometry: Adaptation, Optimization and Validation. *Braz. J. Anal. Chem.,* 2022, *9* (34), pp 229–239. doi: http://dx.doi.org/10.30744/brjac.2179-3425.TN-59-2020

Submitted 15 October 2020, Resubmitted 14 December 2020, 2nd time Resubmitted 11 February 21, Accepted 11 February 21, Available online 08 March 2021.

INTRODUCTION

Cannabis is the most consumed drug in the world. In Brazil, even though it is illegal, the scenario is not different. Data from reports published by the Alcohol and Drug Research Unit (UNIAD) state that *Cannabis* is the most consumed illegal substance in Brazil. 5.8% of the adult population report having used *Cannabis* at least once in life and 2.5% report having used it at least once in 2012 [1]. The main psychoactive compound of *Cannabis sativa* is Δ9-tetrahydrocannabinol (Δ9-THC), which can be quickly bio-transformed by liver enzymes into several by-products. One of them is 11-hydroxy-Δ9-THC. The oxidation of 11-hydroxy-Δ9-THC originates the inactive product 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (Δ9-THC-COOH). It can be conjugated with glucuronic acid and excreted in urine. Therefore, the identification of this analyte in urine is the best analysis procedure to check for an individual's exposure to the drug [2]. Δ9-THC-COOH is a major biotransformation product that can be identified in urine, blood and hair analysis by chromatography techniques. Most of the methods described for extraction of this analyte include mainly Solid Phase Extraction (SPE) and Liquid-Liquid Extraction (LLE), with separation and detection by GC-MS and LC-MS or LC-MS/MS techniques [3-10]. LLE is a method for isolating many drugs from biological matrices based on drugs separation between the aqueous phase (biological) and an organic extraction solvent. LLE has advantages over other techniques, once it is a cheap and simple operation. It is also a fast technique that provides good repeatability and high recovery of most cannabinoids [9].

Andrenyak et al. [11] have achieved improved performance characteristics using LLE and MTSFA derivatization for the determination of cannabinoids in human plasma when compared with assays that used SPE as their main procedure. In another study, González-Mariño et al. [12] developed a LLE technique for the determination of cannabinoid and synthetic cannabinoid metabolites in wastewater as a simple and fast alternative to SPE protocols.

LLE procedures can be optimized in forensic analysis. Purshcke et al. [13] optimized a classical LLE technique for analysis of cannabinoids in human blood serum with a fully automated workflow, achieving fast and reliable results.

Most of these techniques used blood or hair as biological samples for drug analysis. Urine has several advantages over other matrices, such as the fact that its collection is less invasive. Besides, it can be obtained in large quantities and presents good conservation and stability of the analytes, which allows freezing [14].

When investigating a sample for psychoactive substances, the *National Institute on Drug Abuse (NIDA)* recommends associating screening methods with confirmatory techniques, such as gas chromatography coupled to mass spectrometry (GC-MS) [15] . Although most current methods use high performance liquid chromatography (HPLC), GC-MS is a standard Gold technique for the analysis of drugs. Its consumables are cheaper than in other techniques, and its specificity, sensitivity and limit of detection are good [9]. Moreover, GC-MS is available at many toxicology laboratories.

To provide a reliable determination of a certain analyte, the validation of methods is indispensable. It serves the purpose of demonstrating that a specific analytical technique has the performance characteristics required. In Brazil, the National Health Surveillance Agency (ANVISA) published the RDC Nº 27, May 17th, 2012, and the RCD N°166, Jun 27th, 2017, which are guidelines for the validation of analytical and bioanalytical techniques to be carried out properly [16,17]. The parameters assessed through validation are also in accordance with the United Nations Office on Drugs and Crime (UNODC) [18].

Thus, this work aimed to adapt, optimize and validate a method for the detection and quantification of Δ9-THC-COOH in human urine by GC-MS. The compound Δ9-THC-COOH was chosen for analysis because it is considered a biomarker of exposure to *Cannabis* products and the most prevalent metabolite in urine samples [19]. The LLE-GC-MS technique proposed in this work combines the simplicity, speed and low cost of LLE together with the availability, low cost and effectiveness of GC-MS.

MATERIALS AND METHODS

Materials

Standard solutions of Δ9-THC-COOH (1.0 mg mL^{-1} in methanol), along with their deuterated internal standards Δ9-THC-COOH-d_3 (1.0 mg mL^{-1} in methanol) were purchased from Cerilliant Corporation® (Round Rock, TX, USA). The sodium hydroxide solution was provided by Biotec-LabMaster Ltda® (Paraná, Brazil). Acetic acid was supplied by Labsynth® (Diadema, Brazil). Methanol, n-hexane, ethyl acetate were purchased from Merck® (Darmstadt, Germany). BSTFA (bis-trimetilsilil-trifluoroacetamida) and TCMS (trimetilclorossilano) were purchased from Sigma Aldrich®, Round Rock, Texas, USA. Deionized water was provided by Milli-Q system, Millipore® (Barueri, Brazil). Working solutions were prepared through adequate dilution of the stock solutions with methanol for final concentrations of 10 µg mL^{-1}. All solutions were stored in a freezer at -20 ºC.

GC-MS analysis

The analyses were carried out by using a TRACE 1300 GC System Gas Chromatograph coupled to a Thermo Scientific® ISQ Series quadrupole mass-selective detector (MSD) (Thermo Fisher Scientific, Milan, Italy), with the coupling of an AI 1310 automated analyzer. Separation of the analytes was done by using a capillary column with 5% of Phenyl Polysilphenylene-Siloxane (TR-5MS) (30 m x 0.25 mm x 0.25 µm), supplied by Thermo Scientific® (Milan, Italy). The temperature of the injector port was 280 °C, and the temperature of the interface was 250 °C. The oven ramp was set to initialize at 90 °C for 2 min, and then increase in 10 °C/min until reaching 220 °C, kept for 4 min and, then, increase again in 30 °C/min, reaching up to 290 °C, kept for 6 min. The whole process lasted approximately 23 min. The carrier gas (Helium) was adjusted to a constant flow rate of 1.0 mL/min, and 1 µL of the samples was injected in splitless mode. The mass spectrometer was operated in electron ionization mode (EI). Qualification and quantification of ions were performed in the selected ion monitoring (SIM) mode, and they were chosen based on selectivity and abundance, in order to maximize the signal-to-noise ratio in the extracts prepared. Three ions were monitored (the quantification ion is underlined): Δ9-THC-COOH *m/z* 371, 473 and 488.

Sample preparation

Urine samples free from the drugs (such as *Cannabis*) were provided by 10 volunteers who were nonusers. Five samples from drug abuse users were obtained by convenience sampling, as subjects were chosen according to sentinel events reports obtained via epidemiological monitoring programs at the University Hospital of Maringá (HUM) [20]. The study was approved by the Ethics Committee for Research on Human Beings from the State University of Maringá, under registration number 458.185.

Analytes were extracted by LLE. For that purpose, 2 mL of the sample, 50 ng mL^{-1} of internal standard Δ9-THC-COOH-d_3 and 100 µL of NaOH 10% (v/v) were placed in 15 mL propylene tubes and taken to an incubator for hydrolysis at 60 °C for 20 minutes. The tubes were taken out of the incubator until reaching room temperature. Then, 2 mL of deionized water, 2 mL of acetic acid 10% (v/v) and 8 mL of extractor solvent were added to the tubes (n-hexane: ethyl acetate, 9:1, v/v), submitted to mechanical agitation for 30 minutes and centrifuged at 700 x g for 5 minutes. The supernatants were transferred to glass conical tubes and led to evaporate at 50 °C in water bath. For derivatization, 100 µL of BSTFA (bis-trimethylsilyl-trifluoroacetamide) and 1% TCMS (trimethylchlorosilane) were added to the dried residues, which were kept in an incubator at 70 °C for 30 minutes. The tubes were centrifuged at 448 x g for 3 minutes, the volumes were transferred to 2 mL vials, and 1 µL were injected and analyzed on GC-MS (Figure 1).

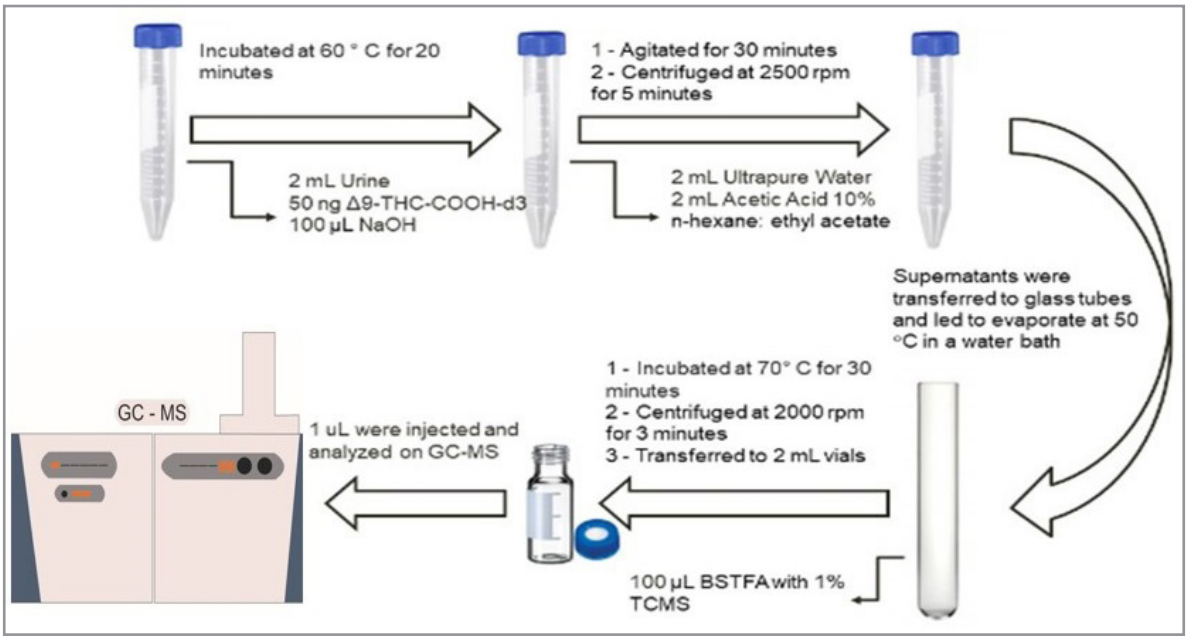


Figure 1. Sample treatment and LLE procedure for Δ9-THC-COOH analysis in urine.

Validation procedure

After the adaptations and optimization described, the method was validated in accordance with ANVISA and UNODC [16,18], comprising parameters such as selectivity, linearity, limit of detection, limit of quantification, precision, accuracy, matrix effect, carryover and stability.

Specificity / Selectivity

Specificity was assessed by evaluating the retention times (RT) of the peaks corresponding to the analytes evaluated. Two blank samples (with the addition of internal standards: 10 ng mL^{-1} of Δ9-THC-COOH-d_3) were analyzed, in addition to 10 urine samples from different sources without the addition of internal standards to verify the presence of possible interferences. The presence or absence of any interfering peaks (endogenous substances), at a significant level, close to the analyte retention time and the internal standard, were assessed. The responses of interfering peaks close to the internal standard retention time must be less than 5% of the internal standard response [16].

Linearity

Linearity was accomplished with blank urine (pool sample) spiked with Δ9-THC-COOH standard solutions at different concentrations (5 ng mL^{-1}, 10 ng mL^{-1}, 15 ng mL^{-1}, 50 ng mL^{-1}, 150 ng mL^{-1} and 300 ng mL^{-1}). The analyses of different concentrations, within the range stablished, were carried out in six repetitions. Calibration curves of the analytes were obtained by correlation between the signal response (area ratio of the analyte peak and the internal standard) and analyte concentration in the sample. Acceptance criteria included the correlation coefficient (r) above 0.99. A linear calibration model was used with 1/x weighting (inverse of the concentration), generally recommended for bioanalytical methods. When the error variance is not constant across the quantification range of the analytical method, it is necessary to use the weighting that has the lowest value to sum the relative errors of the nominal values of the calibration standards versus their values obtained by the curve equation. Application of the 1/x factor adequately compensates for the occurrence of heteroscedasticity [16].

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Determinations of the LOD and LOQ were done by fortifying different blank (urine) samples, analyzed in quadruplicates, using signal-to-noise ratio and visual evaluation methods, respectively [16]. The LOD was determined by verifying what was the lowest concentration assessed to have resulted in qualitatively chromatographic peaks (their magnitudes must be at least thrice higher than the noise peaks, signal/noise ratio ≥ 3:1) [16]. Samples were fortified with decreasing concentrations of the analyte and assessed until no qualitatively chromatographic peaks were obtained. The LOQ estimated was considered to be the lowest concentration capable of obtaining detection (signal/noise ratio ≥ 10:1), identification, accuracy and precision criteria in all fortified samples.

$$LOD = 3.3\sigma/S \qquad LOQ = 10\sigma/S$$

were σ = standard deviation and S = slope of the calibration curve

Intra-day and inter-day precision and accuracy

Intra-day precision was evaluated through the analysis, on the same day, of six replicates (n=6) of blank samples enriched with the analyte at three (3) control levels: low control (10 ng mL^{-1}), medium control (100 ng mL^{-1}) and high control (250 ng mL^{-1}). Inter-day precision was evaluated during three consecutive days, in five repetitions. Precision was calculated by using the coefficient of variation (CV). The acceptance criterion was ≤ ±15% for all concentrations, except at the LOQ, in which ≤ ±20% was accepted [16,18]. The values can be considered acceptable when varying ≤ ±20% for the LOQ and ≤ ±5% for all the other concentrations.

Accuracy was validated using 3 concentrations of the Δ9-THC-COOH standard (10 ng mL^{-1}, 100 ng mL^{-1} and 250 ng mL^{-1}, low, average and high concentrations, respectively), through the analysis during six consecutive days. It was calculated by using the percentage of the known concentration value (mean concentration measured/ theoretical concentration) x 100. The values can be considered acceptable when varying from 80% to 120% for the lower limit of quantification and from 85% to 115% for all the other concentrations [16,18].

Matrix effect

The matrix effect was determined by statistical evaluation of the slope coefficients of the calibration curves constructed with the analyte (standard) in solvent and with the sample (urine) fortified with the analyte (standard), with a level of significance of 5% (five percent) adopted in the hypothesis test [16]. The curve built in solvent was performed in deionized water, under the same conditions as the curve performed for linearity.

Stability

The stability parameter was evaluated in three situations: (1) long term, (2) freeze-thaw cycle and (3) post-processing [16]. In test (1), the time variation in which the matrix is stable when stored at -20 °C was evaluated. For that, samples were analyzed at two concentrations: low (10 ng mL^{-1}) and high (250 ng mL^{-1}), after an interval of 15 and 30 days of storage. In test (2), resistance of the analyte was evaluated for its degradation under freezing-thawing cycles. Then, the low (10 ng mL^{-1}) and high (250 ng mL^{-1}) concentrations of the analyte were evaluated in samples after five cycles. Finally, test (2) evaluated the extracted samples, injected after 24, 48 and 72 hours at room temperature and without resuspension. Again, the analyte concentrations in the sample were low (10 ng mL^{-1}) and high (250 ng mL^{-1}).

Carryover

For the carryover evaluation, three injections of a single blank sample were made, one before and two after the injection of a sample at the highest point of the calibration curve (300 ng mL^{-1}). The results of the blank sample injections were compared with those obtained from the LOQ. The signal should not be detected at a concentration higher than the LOQ [16].

RESULTS AND DISCUSSION

The methodology of this study was based on a simple LLE technique with adaptations of existing methods [3,13]. The retention time obtained for Δ9-THC-COOH was 20.15 minutes, and three ions were monitored (the quantification ion is underlined): Δ9-THC-COOH *m/z* 371, 473 and 488 (Figure 2).

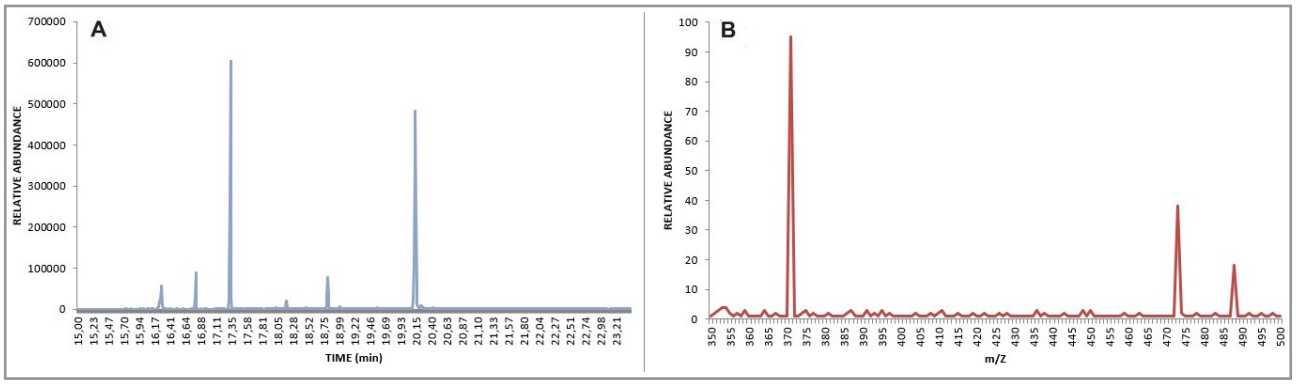


Figure 2. Chromatogram (A) and mass spectra (B) of Δ9-THC-COOH obtained by LLE/GC-MS. Where: (A) 20.15 min = retention time, (B) Quantifier and identification ion (*m/z*) of Δ9-THC-COOH (371, 473, 488).

The hydrolysis step was performed because over 80% of the THC-COOH excreted in the urine are conjugated with the glucuronic acid [19]. Therefore, hydrolysis prior to GC-MS analysis is required to better quantify the total of cannabinoids. Moreover, alkaline hydrolysis is the most effective one for the Δ9-THC-COOH glucuronide conjugate [2].

The chosen solvent mixture (n-hexane: ethyl acetate, 9:1, v/v) was the only one to have achieved quantifiable results in our extraction procedure. Other solvent mixtures tested, such as chloroform: ethyl acetate (80:20, v/v) did not present adequate sensitivity for chromatographic detection. With that mixture, it was not possible to quantify by GC-MS the analyte under study with precision and accuracy. Acetic acid was added to adjust the pH after the basic hydrolysis step. Derivatization using BSTFA and 1% TCMS proved to be more efficient than it is when using MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide), leading to better recovery rates for this technique. A good recovery rate was obtained using LLE. This technique is simple, cheap and fast to be performed at a laboratory of toxicological analyses.

The complete validation of LLE-GC-MS met a set of guidelines by national and international bodies [16-18,21]. Evaluation of samples with Δ9-THC-COOH standard addition and blank samples indicated no interference with the analysis of the analyte of interest, showing that there was adequate selectivity, with no interfering peak close to the retention time of all analytes and internal standards of interest. Figure 3 shows the chromatogram for an ultrapure water sample containing Δ9-THC-COOH standard at 300 ng mL^{-1} (A), a urine sample without the analyte (blank sample, B), and a positive urine sample (C).

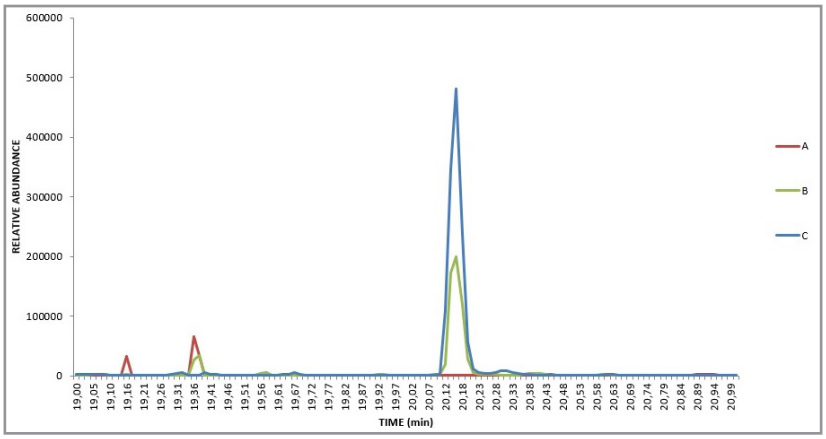


Figure 3. GC-MS Chromatogram obtained from LLE for samples containing Δ9-THC-COOH. Retention time of the analyte is 20.15. Where: (A) chromatogram of a urine sample not containing the analyte (blank sample), (B) concentration chromatogram of a positive urine sample at 121 ng mL^{-1}, and (C) chromatogram of an ultrapure water sample containing Δ9-THC-COOH standard at 300 ng mL^{-1}

As for the linearity of the method, the calibration curves were generated at the concentration range tested (5 to 300 ng mL^{-1}), and the coefficient of correlation was 0.9993 (linear regression equation: y = 0.0269x – 0.0364). Those were correctly adjusted by indicating the mathematical models at the level of 5% [21]. This linearity interval represents concentrations of Δ9-THC-COOH, usually found in urine samples of *Cannabis* users (5 to 300 ng mL^{-1}) [21,22].

The limits found in this study resulted in 5 and 10 ng mL^{-1} for LOD and LOQ, respectively. According to the Substance Abuse and Mental Health Services Administration (SAMHSA) [23], the suggested cut-off value in confirmatory assays for THC-COOH in urine is 15 ng mL^{-1} in routine analyses, usually performed by GC-MS. For LOD and LOQ values, the SAMHSA also preconizes that analytical methods for detection of Δ9-THC-COOH must be able to detect such analyte at values below the cut-off, which makes the method here tested in accordance with international guidelines and with other studies published in the literature [24,25].

The precision and accuracy parameters also presented satisfactory results. CV and RSD values were lower than 20% for LOQ concentration, and lower than 15% at other concentrations (Table I). Moreover, precision and accuracy were within acceptable ranges, as determined by the followed guidelines [16,18]. These parameters were also similar to those reported by other studies. Jamerson et al. (2005) obtained inter-assay precision ranging from 2.3 to 5.4% and intra-assay precision over 2% in their study with rapid quantification using GC-MS [26]. Nestić et al. (2013) obtained intra-assay precision ranging from 3.18 to 9.01% and inter-assay precision ranging from 0.99 to 8.80% [27].

Validation guidelines establish that linearity must be performed in the matrix [16,18]. This study was complemented in the evaluation of the matrix effect, once the linearity parameter in water was performed to verify the similarity between the curves in the presence and absence of the sample (urine). This evaluation consisted of comparing the values of the straight slope (0.0286 and 0.0269 for deionized water and urine, respectively). The parallelism of the straight lines is an indicator of the absence of interference from the constituents of the matrix, and its demonstration must be carried out by means of an adequate statistical evaluation, with a level of significance of 5% [16]. This matrix effect test proves that there is a parallelism of the lines, which indicates the absence of interference from the matrix. This does not mean that the lines are equal, since only the slope is considered (the linear coefficient is not). This fact proved the absence of interference from the components of the urinary matrix.

Table I. Analytical parameters of the method developed for detection and quantification of Δ9-THC-COOH, as assessed by LLE-GC-MS*

	Δ9-THC-COOH
Correlation coefficient (r)	0.993
LOD (ng mL^{-1})	5.00
LOQ (ng mL^{-1})	10.00
Intra-assay precision (CV%)	
Low concentration (10 ng mL^{-1})	3.66
Average concentration (100 ng mL^{-1})	4.46
High concentration (250 ng mL^{-1})	9.04
Inter-assay precision (CV%)	
Low concentration (10 ng mL^{-1})	3.38
Average concentration (100 ng mL^{-1})	4.46
High concentration (250 ng mL^{-1})	8.96
Accuracy (%)	
Low concentration (10 ng mL^{-1})	
Day 1	90.70
Day 2	112.90
Day 3	83.80
Day 4	93.80
Day 5	109.00
Day 6	83.00
Average concentration (100 ng mL^{-1})	
Day 1	94.60
Day 2	107.60
Day 3	96.40
Day 4	102.00
Day 5	95.50
Day 6	92.70
High concentration (250 ng mL^{-1})	
Day 1	100.70
Day 2	92.50
Day 3	91.80
Day 4	94.00
Day 5	95.90
Day 6	97.40

*LLE-GC-MS: liquid-liquid extraction-gas chromatography-mass spectrometry

The carryover and residual effect were not detected in the method we developed, even when we analyzed blank samples, injected after the last point of the calibration curve (300 ng mL^{-1}). Therefore, there was no need for comparison with the chromatograms obtained for blank samples fortified with concentrations corresponding to LOQ.

The analyte remained stable after 15 and 30 days of storage at -20 °C and after 5 freeze-thaw cycles. Post-processing stability was evaluated by re-injecting samples into the GC-MS apparatus after 24, 48 and 72 h. The results were compared with others obtained for freshly extracted samples that had a variation value lower than 15% (Table II). Stability results corroborate other results. Nestić et al (2013) used a spiked urine sample subjected to three freeze-thaw cycles and long-term stability. Those processed samples did not show significant change when analyzed [27].

Table II. Long-term, freeze-thaw cycle and post-processing stability of Δ9-THC-COOH in human urine assessed by LLE-GC-MS

Long-term stability (RSD%)		
	CB: 10 ng mL^{-1}	CA: 250 ng mL^{-1}
15 Days	3.21%	3.05%
30 Days	4.72%	4.61%
Freeze-thaw cycle stability (CV%)		
	CB: 10 ng mL^{-1}	CA: 250 ng mL^{-1}
After 5 Cycles	5.51%	8.32%
Post- Processing stability (CV %)		
	CB: 10 n g mL^{-1}	CA: 250 ng mL^{-1}
24 h	3.54%	4.40%
48 h	6.13%	9.93%
72 h	5.40%	10.98%

These results obtained by validation are similar to those described in other studies that also used urine as a biological matrix. Abraham et al. [28] described the following results: r = 0,999, intra-assay precision over 2.40% and inter-assay precision ranging from 2.60 to 7.40%. Nestić et al. [27] obtained the following results: r = 0,999, intra-assay precision ranging from 3.18 to 9.01% and inter-assay precision ranging from 0.99 to 8.80%. However, these techniques aforementioned used extractions by SPE, which can increase the cost of the analyses.

According to the method validation parameters and given the simplicity of the validated method, together with the ease of obtaining the urine matrix, the developed method meets the needs of toxicological analyses and can be applied in forensic routine, assisting in solving cases of violent deaths, traffic accidents, doping and drug addiction control.

The method was applied to authentic urine samples from five individuals diagnosed with trauma in association with the use of drugs, who were taken care of at the emergency service. The following results were obtained: [1] positive (43.80 ng mL^{-1}), [2] positive (46.70 ng mL^{-1}), [3] positive (121.23 ng mL^{-1}), [4] positive (73.95 ng mL^{-1}) and [5] positive (95.02 ng mL^{-1}). These results show that the LLE-GC-MS method could be used in laboratory routine, with reliable results.

Most current techniques use SPE as an extraction method [5-10]. The technique presented in this study was developed using an optimized LLE-GC-MS. It has proven to be a cheap, simple and fast alternative in routine laboratories and forensic analysis, with reproducible and reliable results. Using urine as a biological sample has advantages, such as the fact that its collection is less invasive. Besides, it can be obtained in large quantities and presents good conservation and stability of the analytes.

CONCLUSION

The LLE-GC-MS validated method was efficient, since it met all the required parameters, in accordance with international and national guidelines established for analysis of Δ9-THC-COOH in urine. Therefore, we provide a result that is applicable to the reality of most laboratories with scarce financial support. The use of a technique of extraction and identification that is easy to be put into practice, with low costs and reliability, as it is the case of LLE-GC-MS, is of great value for lower expenses in laboratories with few resources. Other previously mentioned techniques use more expensive instruments, such as HPLC, and also extraction methods that increase the costs of an analysis, which is unfeasible in laboratory routine. The search for the development of more modern techniques is a desirable requirement, but they often do not apply to the reality of many routine laboratories. In addition, we use one of the most requested samples for drug research, that is, urine.

Funding

This work was supported by Araucária Foundation (Brazil) [grant number 35197.406.32936.20112012], the National Council for Scientific and Technological Development (CNPq – Brazil) and the Coordination for the Improvement of Higher Education Personnel (CAPES – Brazil).

Acknowledgements

To Decit/SCTIE/MS; CNPq; Araucária Foundation and SESA-PR for the support and for funding the project. We are thankful to the National Council for Scientific and Technological Development (CNPq – Brazil) and the Coordination for the Improvement of Higher Education Personnel (CAPES – Brazil) for their financial support, as well as for granting the first author a scholarship. We also thank Rafael Lanaro, from the Laboratory of Toxicological Analysis of the Information and Toxicological Assistance Center, from the Faculty of Medical Sciences – Unicamp (CIATox), for technical assistance.

REFERENCES

1. Laranjeira, R.; Madruga, C. S.; Pinsky, I.; Caetano, R.; Mitsuhiro, S. S. *II LENAD Levantamento Nacional de Álcool e Drogas – O Consumo de Álcool no Brasil: Tendências entre 2006 e 2012* (http://www.mds.gov.br/webarquivos/arquivo/cuidados_prevencao_drogas/obid/publicacoes/pesquisas/LENAD_II_RVW.pdf).
2. Scheidweiler, K. B.; Desrosier, N. A.; Huestis, M. A. *Clin. Chim. Acta*, **2012**, *413* (23–24), pp 1839–1847 (http://dx.doi.org/10.1016/j.cca.2012.06.034).
3. Aamir, M.; Hafeez, A.; Ijaz, A.; Khan, S. A.; Chaudhry, N.; Ahmed, N. *Pakistan J. Pathol.*, **2016,** *27* (2), pp 61–70.
4. Fuchs, N.; Miljanić, A.; Katić, A.; Brajenović, N.; Micek, V.; Fuchs, R.; Karačonji, I. B. *Arh. Hig. Rada Toksikol.*, **2019**, *70* (4), pp 325–331 (http://dx.doi.org/10.2478/aiht-2019-70-3352).
5. Sánchez-González, J.; Salgueiro-Fernández, R.; Cabarcos, P.; Bermejo, A. M.; Bermejo-Barrera, P.; Moreda-Piñeiro, A. *Anal. Bioanal. Chem.*, **2017**, *409* (5), pp 1207–1220 (http://dx.doi.org/10.1007/s00216-016-0046-3).
6. Raharjo, T. J.; Verpoorte, R. *Phytochem. Anal.*, **2004**, *15* (2), pp 79–94.
7. Gasse, A.; Pfeiffer, H.; Köhler, H.; Schürenkamp, J. *Int. J. Legal. Med.*, **2016**, *130* (4), pp 967–974 (http://dx.doi.org/10.1007/s00414-016-1368-6).
8. Citti, C.; Braghiroli, D.; Vandelli, M. A.; Cannazza, G. *J. Pharm. Biomed. Anal.*, **2018**, *147,* pp 565–579 (https://doi.org/10.1016/j.jpba.2017.06.003).
9. Shah, I.; Al-Dabbagh, B.; Salem, A. E.; Hamid, S. A. A.; Muhammad, N.; Naughton, D. P. *BMC Chem.*, **2019**, *13* (1), Article number 106 (http://dx.doi.org/10.1186/s13065-019-0627-2).
10. Schillack, H. *A simultaneous quantitative determination of both natural and synthetic cannabinoids in bio-matrix by ultra-high pressure liquid chromatography tandem mass spectrometry*. Master thesis, **2019**, Faculty of Health Sciences, University of Pretoria, Pretoria.

11. Andrenyak, D. M.; Moody, D. E.; Slawson, M. H.; O'Leary, D. S.; Haney, M. *J. Anal. Toxicol*., **2017**, *41* (4), pp 277–288 (https://doi.org/10.1093/jat/bkw136).
12. González-Mariño, I.; Thomas, K. V.; Reid, M. J. *Drug Test. Anal*., **2018**, *10* (1), pp 222–228 (https://doi.org/10.1002/dta.2199).
13. Purschke, K.; Heinl, S.; Lerch, O.; Erdmann, F.; Veit, F. *Anal. Bioanal. Chem.*, **2016**, *408* (16), pp 4379–4388 (http://dx.doi.org/10.1007/s00216-016-9537-5).
14. Musshoff, F.; Madea, B. *Ther. Drug Monit.*, **2006**, *28* (2), pp 155–163 (http://dx.doi.org/10.1097/01.ftd.0000197091.07807.22).
15. De Brabanter, N.; Van Gansbeke, W.; Hooghe, F.; Van Eenoo, P. *Forensic Sci. Int.*, **2013**, *224* (1–3), pp 90–95 (https://doi.org/10.1016/j.forsciint.2012.11.004).
16. Agência Nacional de Vigilância Sanitária (ANVISA, Brasil). Resolução RDC nº 27, de 17 de maio de 2012. *Guia para Validação de Métodos Bioanalíticos,* **2012** (http://portal.anvisa.gov.br/documents/33880/2568070/rdc0027_17_05_2012.pdf/c6edeb56-200d-4482-8a19-99fa11c33fd3).
17. Agência Nacional de Vigilância Sanitária (ANVISA, Brasil). Resolução RDC nº 166, de 24 de julho de 2017. *Guia para Validação de Métodos Analíticos*, **2017** (https://www.in.gov.br/materia/-/asset_publisher/Kujrw0TZC2Mb/content/id/19194581/do1-2017-07-25-resolucao-rdc-n-166-de-24-de-julho-de-2017-19194412)
18. United Nations Office on Drugs and Crime (UNODC), *Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens*, **2009**, p 67.
19. Huestis, M.; Smith, M. *Marijuana and the Cannabinoids*. Humana Press, New Jersey, **2007**.
20. Santana, C. J.; Oliveira, M. L. F. *Revista da Rede de Enfermagem do Nordeste*, **2017**, *18* (5), pp 671–678 (https://doi.org/10.15253/2175-6783.2017000500015).
21. Peters, F. T.; Drummer, O. H.; Musshoff, F. *Forensic Sci. Int.*, **2007**, *165* (2–3), pp 216–224 (https://doi.org/10.1016/j.forsciint.2006.05.021).
22. Meier, U.; Dussy, F.; Scheurer, E.; Mercer-Chalmers-Bender, K.; Hangartner S. *Forensic. Sci. Int.*, **2018**, *291*, pp 62–67 (https://doi.org/10.1016/j.forsciint.2018.08.009).
23. Substance Abuse and Mental Health Services Administration (SAMHSA). *Analytes and their cutoffs, SAMHSA Guidelines*, **2008**, p 1 (https://www.samhsa.gov/sites/default/files/workplace/2010GuidelinesAnalytesCutoffs.pdf).
24. Bush, D. M. *Forensic Sci. Int.,* **2008**, *174* (2–3), pp 111–119 (https://doi.org/10.1016/j.forsciint.2007.03.008).
25. Pelição, F. S. *Avaliação da presença de drogas de abuso em amostras de sangue colhidas de vítimas fatais de acidentes de trânsito na Região Metropolitana de Vitória-ES*. Doctoral thesis, **2014,** University of São Paulo, São Paulo, Brazil (https://doi.org/10.11606/T.60.2014.tde-17042015-111424).
26. Jamerson, M. H.; Welton, R. M.; Morris-Kukoski, C. L.; Klette, K. L. *J. Anal. Toxicol.,* **2005**, *29* (7), pp 664–668 (https://doi.org/10.1093/jat/29.7.664).
27. Nestić, M.; Babić, S.; Pavlović, D. M.; Sutlović, D. *Forensic Sci. Int.,* **2013**, *231* (1–3), pp 317–324 (https://doi.org/10.1016/j.forsciint.2013.06.009).
28. Abraham, T. T.; Lowe, R. H.; Pirnay, S. O.; Darwin, W. D.; Huestis, M. A. *J. Anal. Toxicol.*, **2007**, *31* (8), pp 477–485 (https://doi.org/10.1093/jat/31.8.477).

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 240-243

FEATURE

PDF

National Institute of Criminalistics

The National Institute of Criminalistics (INC) is the central criminalistics body of the Brazilian Federal Police, located in Brasília, linked to the Technical-Scientific Directorate of the Federal Police.

The INC has several laboratories of various specialties installed in the services and thematic sectors, which are: Computer Expertise Service; Documentoscopy Expertise Service; Engineering Expertise Service; Accounting and Economic Expertise Service; Audiovisual and Electronics Expertise Service; Laboratory Expertise Service; Forensic Genetics Sector; Ballistic Expertise Sector; Environmental Expertise Sector; Forensic Medicine and Dentistry Sector; Crime Scene Investigation Sector; Geographic Information Sector; and Specialized Group on Bombs and Explosives.

Dr. Ricardo Guanaes Cosso, Federal Criminal Expert and Director of the National Institute of Criminalistics

The INC conducts, standardizes and disseminates techniques and methodologies for analysis of the most diverse types of traces of criminal actions, such as drugs of abuse, medicines, fuels, pesticides, food and others, and authenticity tests of works of art. Modern analytical techniques are used, such as chromatography, high resolution and isotope ratio mass spectrometry, atomic spectrometry, infrared spectrometry, X-ray spectrometry, scanning electron microscopy and other complementary techniques.

According to Dr. Jesus Antonio Velho, Federal Criminal Expert and Head of the Institutional Development Sector, several techniques are used for forensics authenticity analysis of artwork, with Raman spectroscopy being a nondestructive technique for the work of art itself.

Laboratory of Instrumental Analytical Chemistry of the National Institute of Criminalistics

With regard to documentoscopy, Elvio Botelho, MSc, Federal Criminal Expert and Head of the Laboratory Expertise Service, highlights the chemical analyses of different colorants (dyes and pigments) used commercially in inks used in handwritten documents. There are two types of document dating examinations through chemical analysis of dye degradation:

I. Comparison of inks: with the purpose of verifying whether graphic records are contemporary and/or produced by the same pen.

II. Date comparison: between the alleged date (or informed in the document) and the one suspected (presumed) by the investigation. Due to the speed of degradation observed experimentally in pen ink dyes, pen ink dating examination requests should only be processed if the difference between the date in the questioned document and the date suspected by the police investigation is greater than several years.

Some prominent cases studied at the National Institute of Criminalistics

Operation “Carne Fraca” was an operation triggered by the Federal Police in March 2017 to investigate an alleged scheme of adulteration of meat sold in the domestic and foreign markets. This operation investigated the Ministry of Agriculture, Livestock and Supply (MAPA) and the largest companies in the sector in Brazil, such as JBS S.A., owner of the Seara, Swift and Friboi Vigor brands, and BRF S.A., owner of the Sadia and Perdigão brands.

In October 2007, Operation “Ouro Branco” uncovered frauds practiced by two milk cooperatives in the State of Minas Gerais (MG): the “Cooperativa dos Produtores de Leite do Vale do Rio Grande (Coopervale)”, based in Uberaba, and the “Cooperativa Agropecuária do Sudoeste Mineiro (Casmil)”, based in Passos. The fraud consisted of adding a chemical solution of caustic soda, citric acid, sodium citrate, salt, sugar and water to the milk to increase its volume and shelf life. According to the expert Botelho, as fraud in milk was not a type of case commonly found in forensic chemistry investigations, it was necessary to establish partnerships with several public research institutions for the development and performance of analysis using different analytical techniques for detecting substances used for the adulteration of milk: with the Farming National Laboratory (LANAGRO, MG), the addition of whey to milk was identified by gel permeation chromatography, the addition of alkaline substances was identified by ash alkalinity and the addition of sodium citrate was identified by ultraviolet/visible spectroscopy; with the Thomson Laboratory of

the University of Campinas (Unicamp, Campinas, SP), techniques were developed to detect maltodextrin addition by ion mobility mass spectrometry and vegetable fat addition by MALDI-TOF (matrix-assisted laser desorption/ionization–time-of-flight) mass spectrometry; with the Ezequiel Dias Foundation (FUNED, MG), the addition of sucrose and lactic acid was identified by high-performance liquid chromatography; and with the Instituto Adolfo Lutz (IAL, SP), the fatty acid profile in milk was determined by gas chromatography with flame ionization detection.

Projects in development at the National Institute of Criminalistics

In its final implementation phase, the National Laboratory for Stable Isotopes (LANIF) was designed to have two integrated laboratory bases, one at the INC in Brasília and the other at the Federal Police Department in Manaus, Amazonas. Both have isotope ratio mass spectrometry (IRMS) equipment. Additionally, LANIF can count on the technological park of a network of partner institutions. At the INC, an isotope ratio mass spectrometer (model DELTA V Plus) coupled to a FlashSmart elementary analyzer and a Trace GC 1310 gas chromatograph coupled to an ISQ7000 quadrupole mass spectrometer from Thermo Fisher Scientific are already in the installation/training phase. In Manaus, the laboratory is already in operation.

Laboratory of Isotopic Analysis of the National Institute of Criminalistics

The isotopic ratio technique makes it possible to determine or exclude the origin of criminal evidence of different types, such as drugs of abuse, explosives and wood. It also enables the development of efficient solutions to assist in the identification of missing people or check whether commercialized animals come from captivity or from nature. The technique has a transversal character, is innovative at the national level and will assist in the integration of investigations that involve different scientific themes at the same time.

A great advantage of the isotopic technique is the speed and objectivity of the laboratory analysis. This technique makes it possible to make important inferences regarding various aspects of criminal conduct, such as the origin of the trace or trafficked material, routes of criminal action, adulteration or counterfeiting processes and authorship, among others. Following this trend, the INC instituted the creation of isotope databases and the use of isotopic analysis in police investigations and criminal proceedings as strategic guidelines.

Other projects developed at the National Institute of Criminalistics

- FARMONITOR – monitoring of legislation and trends in pharmaceutical counterfeiting.
- Drug chemical profiling (PeQui Project) – involving the seizure of cocaine and MDMA (3,4-methylenedioxymethamphetamine) followed by determination of the chemical profiles of the drugs seized across the country, establishing characteristics such as the origin of the drug, the products used for manufacture, the conditions of transport and the purity of each sample.
- Identification of new psychoactive substances (NPS) – characterization of NPS seized for the first time, using nuclear magnetic resonance spectroscopy, with the support of the Institute of Chemistry of the University of Brasília when necessary.
- Toxicology – validation of methodologies in the scope of ISO/IEC 17025:2017 accreditation.
- Preparation of certified reference materials and drug proficiency testing in partnership with the National Institute of Metrology, Quality and Technology of Brazil (Inmetro) and the National Secretariat for Drug Policy of the Ministry of Justice and Public Safety (SENAD/MJSP).
- CLOACIN Project – analysis of drugs in sewage, in partnership with the SENAD/MJSP.

Accreditation

In September 2014 the Laboratory Expertise Service (SEPLAB) of the National Institute of Criminalistics received the accreditation certificate for compliance with the ISO/IEC 17025:2005 Standard, which defines the requirements for quality management in analytical laboratories.

It was the National Accreditation Board/FQS Forensic Accreditation (ANSI-ASQ) that granted the accreditation certificate to SEPLAB, which became the first laboratory of forensic chemistry in Brazil to receive this international recognition and one of the few in Latin America. Currently, Inmetro is the body responsible for maintaining SEPLAB accreditation in ISO/IEC 17025:2017.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 244-255

BrJAC

SPONSOR REPORT

PDF

This section is dedicated for sponsor responsibility articles.

Analysis of Pharmaceutical Products for their Elemental Impurities with the Thermo Scientific iCAP RQ ICP-MS

Julian Wills and Daniel Kutscher
Thermo Fisher Scientific, Bremen, Germany

This report was extracted from the Thermo Scientific Application Note 43325

Keywords: FDA 21 CFR part 11, Microwave digestion, Pharmaceutical compliance, Pharmaceutical preparations, United States pharmacopeia, USP 232, USP 233

Goal

To demonstrate the use of the Thermo Scientific™ iCAP™ RQ ICP-MS to accurately determine concentrations of elemental impurities in pharmaceutical products brought into solution using microwave digestion. All sample preparation, measurement and data evaluation to be compatible with the guidelines defined in USP chapters <232> Elemental Impurities – Limits and <233> Elemental Impurities – Procedures.

INTRODUCTION

Impurities in pharmaceutical products are of great concern not only due to the inherent toxicity of certain contaminants, but also due to the adverse effects that contaminants may have on drug stability and shelf-life. This necessitates the monitoring of organic and inorganic impurities throughout the pharmaceutical manufacturing process, from raw ingredients to final products. United States Pharmacopeia (USP) General Chapter <231>, introduced in 1905, is a colorimetric test involving the co-precipitation of ten sulfide-forming elements and a visual color comparison to a 10 ppm lead standard. The limitations of this test are well understood (non-specificity, the test is based on limited understanding of trace metal toxicity, etc.) so that consequently the USP published two new general chapters to replace <231> starting January 1st, 2018.

- Chapter <232> Elemental Impurities [1] – Limits; defines the maximum limits of fifteen elements in pharmaceutical products.
- Chapter <233> Elemental Impurities [2] – Procedures; defines how the testing for these elements should be performed.

From that date onward, all elemental impurity testing and all elemental impurity testing must instead conform to the limits set out in Chapter <232>, using the procedures set out in Chapter <233>.

In addition to the requirements described in the USP documents, any analytical system used for the creation of analysis data for pharmaceuticals must also comply with the US Food and Drug Administration's (FDA) 21 CFR Part 11 regulations regarding electronic records and validation of electronic signatures. These regulations are concerned with ensuring the integrity and authenticity of any electronic records and electronic signatures that 'persons create, modify, maintain, archive, retrieve or transmit' [3]. Control software used by analytical instruments in pharmaceutical production must therefore incorporate tools to maintain the integrity of the analytical method and subsequent results. In order to provide a transparent pathway to data generation, the control software should include support for audit trails and electronic signatures as well as security features to ensure that alterations cannot be made without clear indication of what has been changed, who changed it and why.

This note describes the effective application of the Thermo Scientific™ iCAP™ RQ single quadrupole (SQ) ICP-MS, to the detection and quantification of the 15 target elements specified in USP <232>, in accordance with the ICP-MS procedures described in USP <233>. In order to generate data compliant with the procedures described in 21 CFR Part 11, the Thermo Scientific Qtegra™ Intelligent Scientific Data Solution™ (ISDS) Software includes comprehensive features for the pharmaceutical industry, such as user access levels, audit trails, support for electronic signatures as well as integrated, secure data management.

Sample preparation

It has been demonstrated that direct aqueous dissolution is suited for the preparation of water soluble pharmaceutical samples before subsequent USP <233> compliant ICP-MS analysis. Indirect dissolution via closed vessel microwave digestion, however, is recognized as the most universal sample preparation method for materials for subsequent elemental analysis by ICP-MS. An important advantage of the closed vessel microwave approach is the retention of volatile elements, in particular mercury that might otherwise be lost.

Three pharmaceutical products were selected for analysis as part of this study:

- Drug A: a phytotherapeutic (herbal) medicine
- Drug B: a vascular medicine
- Drug C: an antianxiety medicine

All three drugs were brought into solution via a microwave digestion procedure using an UltraWAVE closed vessel microwave digestion system (Milestone Inc., Shelton, CT, USA). Different microwave recipes are available to address specific sample matrices making this the most universal method of sample preparation for subsequent elemental analysis.

Samples of each drug (0.5 g) were weighed into 15 mL disposable glass vials. For Drugs A and B, 3 mL of HNO_3 was added to each tube. For Drug C, 2 mL of HNO_3 and 1 mL of H_2SO_4 was added to each vial. In compliance with the repeatability requirements defined in USP <233>, six separate preparations of each material were prepared.

Sample vials were transferred into the microwave digestion system which was then closed, pressurized with nitrogen at 40 bar and the temperature program shown in Table 1 was launched. High pressure digestions are recommended due to the use of lower temperatures minimizing the loss of volatile elements.

Table 1. Closed vessel microwave temperature program used for the dissolution of pharmaceutical products

Step	Time (min)	Temperature (ºC)	Power (W)
1	15	200	1500
2	10	200	1500

When sufficiently cooled, the clear, colorless digested material was transferred to polypropylene vials and made up to 50 mL with ultrapure water. Each sample was then diluted by a factor of five into 15 mL polypropylene autosampler vials in a matrix of 1.2% HNO_3 and 0.5% HCl + 200 µg L^{-1} of gold to give a total dilution factor of 500 from the original solid sample. This diluent was used to ensure stability of the target elements in solution and efficient washout of these elements between samples from the sample introduction system.

The samples were measured using an external calibration approach against calibration solutions prepared in the same diluent as the samples. The calibration solutions contained all of the elements listed under the Oral daily dose PDE (in µg g^{-1}) in USP <232>. Internal standardization was applied, using Ga, In and Tl internal standards at 5, 10 and 10 µg L^{-1} respectively, added online via a T-piece.

Calibration solution preparation

Sample analyses were carried out in accordance with the requirements described in USP <233> Elemental Impurities – Procedures. This document specifies that the elements to be measured should be calibrated against standard solutions at concentrations of blank, 0.5J and 2J where J = the concentration (w/w) of the element(s) of interest at the target limit, appropriately diluted to the working range of the instrument [2].

Target limits for each of the USP <232> controlled elements were calculated by dividing the permitted daily exposure based on a 50 kg person (PDE) by the maximum daily dose. For the three drugs used in this work, the maximum daily dose is 10 g.

Table 2. Target limits (J) for the fourteen elements specified in USP <232>

Element	Oral daily dose PDE* (µg day^{-1})	Target limit J (µg g^{-1})
Cadmium	5	0.5
Lead	5	0.5
Inorganic arsenic	15	1.5
Inorganic mercury	30	3
Iridium	100	10
Osmium	100	10
Palladium	100	10
Platinum	100	10
Rhodium	100	10
Ruthenium	100	10
Chromium	11000	1100
Molybdenum	3000	300
Nickel	200	50
Vanadium	100	20
Copper	3000	300

*PDE = permitted daily exposure based on a 50 kg person

With this target limit taken into account, and as the samples were diluted by a factor of 500 from the original sample, two multielemental calibration solutions were prepared at the concentration levels 0.5J and 2J in 2% HNO_3.

RESULTS

Calibration Curves

Linear calibrations with low (sub ng g^{-1}) blanks were obtained for all elements. Example calibration lines for the ‘big four’ elements are shown in Figure 1.

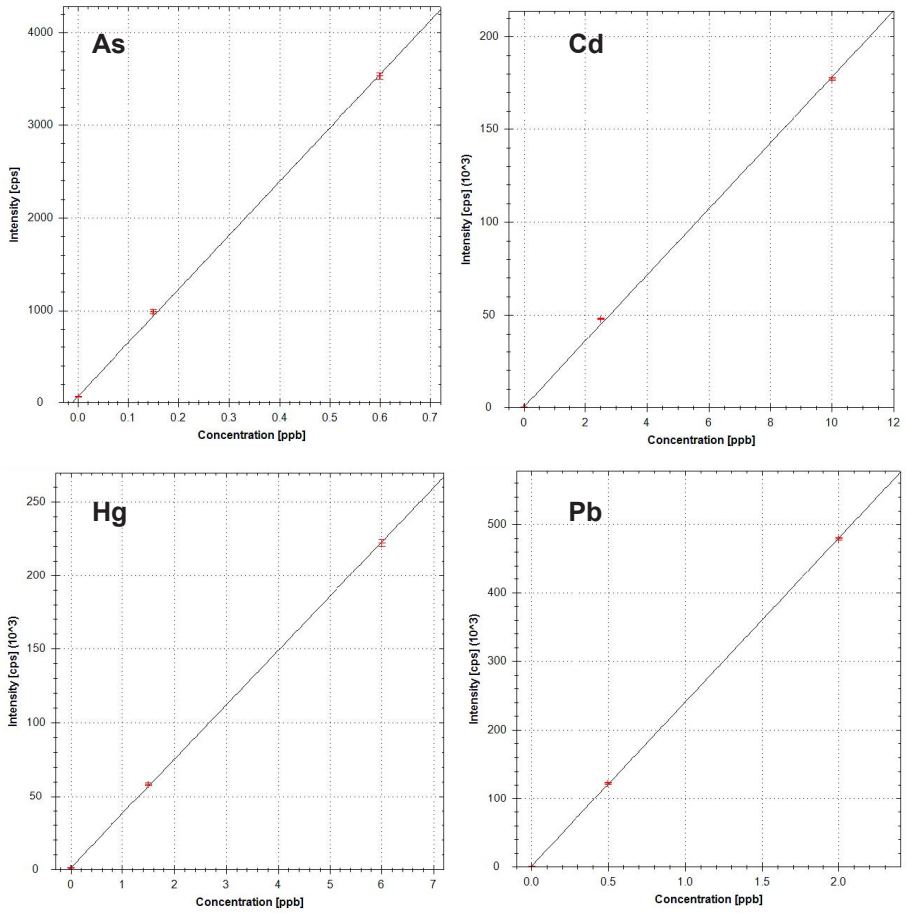


Figure 1. Example calibrations for the 'big four' elements: As, Cd, Hg and Pb.

Instrumental and Method Detection Limits

Single digit pg g^{-1} instrumental detection limits (LoD) are typically obtained for all of the USP <232> defined elements (Table 3). Background equivalent concentrations (BEC) for the 1.2% HNO_3 and 0.5% HCl calibration solution were also calculated. Low or sub pg g^{-1} detection limits (LOD) highlight the excellent detection power of the iCAP RQ ICP-MS for single mode He KED analysis for the USP <232> required elements.

However, while the instrumental detection limits in Table 3 illustrate the detection capabilities of the iCAP RQ ICP-MS for the analysis of the USP <232> required elements, they are not representative of what can practically be achieved on a routine basis. In order to assess this, method detection limits (MDL) were determined from the analysis of three (microwave digestion) procedural blanks from three separate analytical runs performed on different days. Three times the standard deviation of the mean of the blanks from each day was calculated, corrected for dilution and are compared to the Target Limit (J) in the solid (from Table 2). The comparison shows that the attainable MDLs for all elements are at least 50 times lower than the target limit in the solid.

Table 3. Instrumental detection limit (LOD, based on 3 x the standard deviation of the calibration blank), background equivalent concentration (BEC) (reported as ng g^{-1}) and resulting MDLs (reported as µg g^{-1}) for the USP <232> defined elements

Isotope	LOD (ng g^{-1})	BEC (ng g^{-1})	MDL (µg g^{-1})	Target limit J (µg g^{-1})
^{51}V	0.0035	0.0629	0.014	10
^{52}Cr	0.007	0.042	0.008	1100
^{60}Ni	0.0012	0.0163	0.100	20
^{63}Cu	0.0049	0.0910	0.186	300
^{75}As	0.0009	0.0087	0.0005	1.5
^{95}Mo	0.0026	0.0013	0.027	300
^{101}Ru	0.0003	0.00005	0.025	10
^{103}Rh	0.0001	0.00005	0.026	10
^{105}Pd	0.0036	0.0351	0.044	10
^{111}Cd	0.00001	0.00009	0.006	0.5
^{189}Os	0.0007	0.0003	0.043	10
^{193}Ir	0.0005	0.0045	0.023	10
^{195}Pt	0.0001	0.0002	0.024	10
^{202}Hg	0.0099	0.0290	0.018	3
^{208}Pb	0.0009	0.0035	0.009	0.5

Sample analysis results

The final concentrations determined for each target element in the pharmaceutical products tested (six repeat analyses per sample) are shown in Table 4. MDL and target limit (J) values are provided for comparison. Determined concentrations found to be less than the MDL are marked as '<MDL'.

Table 4. Final concentrations obtained for each target element from the six replicate analyses of the three drugs tested

Element	Drug A (µg g^{-1})	Drug B (µg g^{-1})	Drug C (µg g^{-1})	MDL (µg g^{-1})	Target Limit J (µg g^{-1})
Cadmium	<MDL	<MDL	<MDL	0.006	0.5
Lead	0.134	0.171	0.017	0.009	0.5
Inorganic arsenic	0.056	0.091	0.065	0.001	1.5
Inorganic mercury	0.032	<MDL	<MDL	0.018	3
Iridium	<MDL	<MDL	<MDL	0.023	10
Osmium	<MDL	0.107	0.161	0.043	10
Palladium	0.073	<MDL	<MDL	0.044	10

Table 4 continuation. Final concentrations obtained for each target element from the six replicate analyses of the three drugs tested

Element	Drug A (µg g^{-1})	Drug B (µg g^{-1})	Drug C (µg g^{-1})	MDL (µg g^{-1})	Target Limit J (µg g^{-1})
Platinum	<MDL	<MDL	<MDL	0.024	10
Rhodium	<MDL	<MDL	<MDL	0.026	10
Ruthenium	<MDL	<MDL	<MDL	0.025	10
Chromium	<MDL	<MDL	<MDL	0.008	1100
Molybdenum	0.121	0.647	0.073	0.027	300
Nickel	0.780	1.92	12.8	0.100	50
Vanadium	0.224	0.402	0.509	0.014	20
Copper	29.2	5.53	0.965	0.186	300

In each sample some elements were found to be below the calculated MDL but no element was found to be above the Target Limit, J.

Drift

Following the requirement detailed in USP <233>, the read back concentrations for one of the calibration standards analyzed before and after the sample solutions were compared. This comparison is made to ensure that the initial calibration remains valid over the entire analysis. The test is deemed to pass if the relative difference between two analyses of the calibration solution is less than 20%. All elements were found to be reproducible over the complete analysis period (three hours in total) with relative standard deviation (RSD) between 0.1% to maximum 4%, and hence well within the USP <233> defined limit for the calibration solution containing a 2J spike.

Validation procedure

The USP requires that the analytical procedure used to determine elemental impurities in each individual pharmaceutical product passes a series of validation tests before being accepted as suitable. In order to demonstrate the applicability of the iCAP RQ ICP-MS based method described above, its performance was assessed by testing the USP <233> defined criteria (accuracy, precision (repeatability), and ruggedness) [2] for the analysis of the three drugs used in this test.

Accuracy test

In order to assess the accuracy of the method, a series of spike recovery tests were made following the guidelines set out in USP <233>. The spike recoveries for each repeat of all three samples at the 0.5J and 1.5J spike levels are given in Figures 2a and 2b.

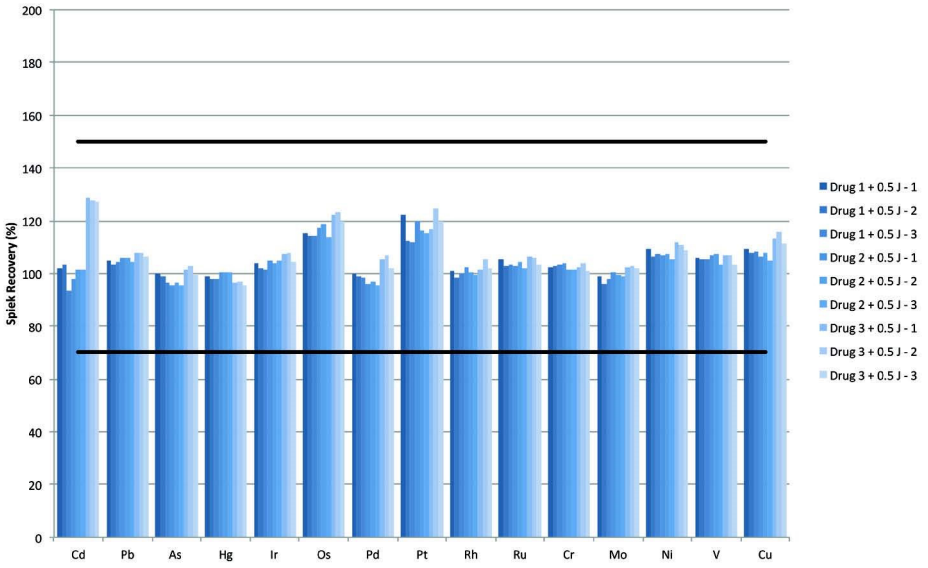


Figure 2a. Recoveries (in %) for the 0.5J spike level.

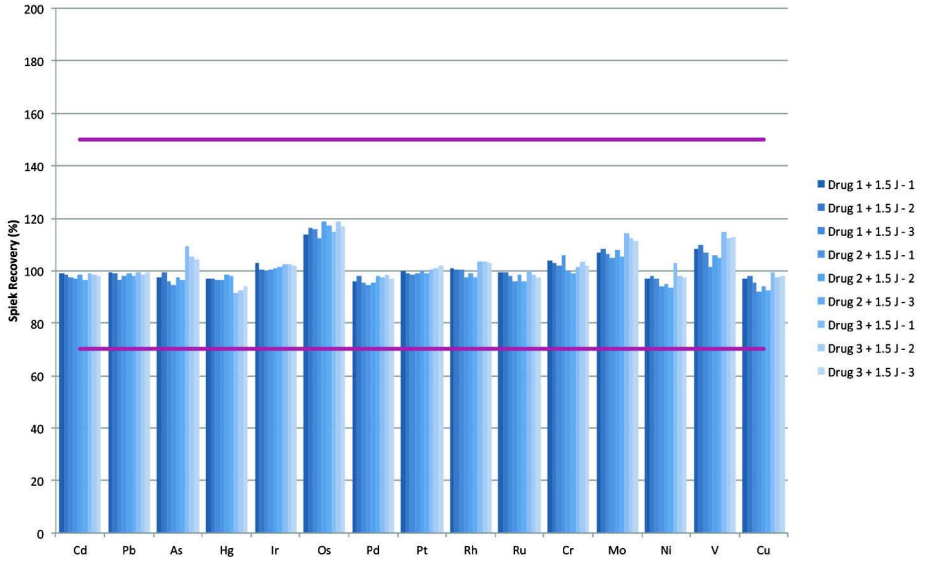


Figure 2b. Recoveries (in %) for the 1.5J spike level.

USP <233> states that the acceptance criteria for this test are recoveries of between 70 and 150% for the mean of the three repeat analyses of each sample at both spike levels.

Figures 2a and 2b show that these criteria are easily met using the iCAP RQ ICP-MS, with average recoveries at both spike levels ranging from 92 to 128%.

Precision test

The precision (repeatability) of the method was assessed by measuring six independent aliquots of each of the three materials tested spiked with the fourteen USP defined elements at the target limit (J). The results from these tests are shown in Tables 5a, 5b and 5c.

USP <233> defines that the precision (% RSD) from the six repeat analyses should not be greater than 20%.

Table 5a. Precision for six separate measurements of Drug A spiked at the target limit (J), expressed as percent recovery

Element	Drug A - 1	Drug A - 2	Drug A - 3	Drug A - 4	Drug A - 5	Drug A - 6	Mean	RSD (%)
Cadmium	99.0	100.0	98.6	97.0	97.8	99.2	98.6	1.1
Lead	112.6	110.6	109.6	113.6	112.6	109.6	111.4	1.5
Inorganic arsenic	118.0	114.6	114.6	114.6	111.3	114.6	114.6	1.8
Inorganic mercury	99.3	98.7	98.0	98.0	96.3	97.7	98.0	1.0
Iridium	101.5	100.0	100.5	100.5	99.0	100.0	100.3	0.8
Osmium	115.0	112.5	112.5	114.0	112.5	114.0	113.4	0.9
Palladium	98.5	99.0	98.5	97.0	97.0	97.2	97.9	0.9
Platinum	96.5	94.5	96.5	94.0	93.5	93.0	94.7	1.6
Rhodium	101.5	102.5	102.0	99.0	101.0	100.5	101.1	1.2
Ruthenium	100.0	101.5	101.0	99.5	100.0	99.5	100.3	0.8
Chromium	102.6	101.9	103.1	102.8	101.7	103.4	102.6	0.7
Molybdenum	109.0	112.0	110.5	109.0	109.5	109.0	109.8	1.1
Nickel	99.4	101	97.7	99.4	98.0	98.4	99.0	1.2
Vanadium	108.0	107.0	106.5	106.5	105.5	107.0	106.8	0.8
Copper	112.4	112.4	110.2	110.2	106.8	110.7	110.5	1.9

Table 5b. Precision for six separate measurements of Drug B spiked at the Target Limit (J), expressed as percent recovery

Element	Drug B - 1	Drug B - 2	Drug B - 3	Drug B - 4	Drug B - 5	Drug B - 6	Mean	RSD (%)
Cadmium	100.6	101.6	101.4	100.4	99.4	98.6	100.3	1.2
Lead	117.9	114.9	116.9	115.9	115.9	115.9	116.2	0.9
Inorganic arsenic	117.3	118.5	116.9	118.1	117.8	117.2	117.6	0.5
Inorganic mercury	98.0	97.7	98.0	98.7	98.0	98.2	98.1	0.3
Iridium	100.5	100.5	101.5	103.5	1001.0	101.2	101.2	1.2
Osmium	116.5	114.5	117.5	118.0	117.5	117.8	117.0	1.1
Palladium	97.5	98.5	99.5	98.0	97.5	97.5	98.1	0.8
Platinum	97.2	97.0	97.5	99.2	98.5	97.4	97.8	0.9
Rhodium	101.5	101.0	100.7	101.2	100.0	100.8	100.9	0.5
Ruthenium	100.8	101.1	101.4	100.6	99.8	100.9	100.8	0.5
Chromium	104.6	103.5	103.8	102.9	103.6	104.1	103.8	0.6

Table 5b continuation. Precision for six separate measurements of Drug B spiked at the Target Limit (J), expressed as percent recovery

Element	Drug B - 1	Drug B - 2	Drug B - 3	Drug B - 4	Drug B - 5	Drug B - 6	Mean	RSD (%)
Molybdenum	117.5	117.2	116.8	116.5	115.9	116.1	116.7	0.5
Nickel	98.5	97.5	99.5	100.0	98.2	97.6	98.6	1.0
Vanadium	105.8	108.0	108.6	107.7	107.4	106.8	107.4	0.9
Copper	99.2	98.5	100.2	99.8	98.0	96.7	98.7	1.3

Table 5c. Precision for six separate measurements of Drug C spiked at the Target Limit (J), expressed as percent recovery

Element	Drug C - 1	Drug C - 2	Drug C - 3	Drug C - 4	Drug C - 5	Drug C - 6	Mean	RSD (%)
Cadmium	100.1	98.6	99.4	99.6	99.8	99.6	99.5	0.5
Lead	100.4	99.8	100.7	100.5	100.5	101.3	100.5	0.5
Inorganic arsenic	116.9	117.5	117.9	115.5	118.1	117.1	117.2	0.8
Inorganic mercury	91.3	90.7	93.0	92.7	91.0	93.0	92.0	1.2
Iridium	100.1	100.9	104.5	102.8	102.1	102.5	102.2	1.5
Osmium	115.5	117.1	119.4	117.5	119.9	118.7	118.0	1.4
Palladium	96.5	97.8	100.4	99.8	100.6	99.9	99.2	1.7
Platinum	95.5	96.7	99.1	97.2	97.4	98.5	97.4	1.3
Rhodium	102.3	102.8	105.1	103.7	105.3	104.8	104.0	1.2
Ruthenium	98.0	99.1	100.0	99.4	100.8	99.7	99.5	0.9
Chromium	101.8	102.5	102.0	103.1	101.5	102.3	102.2	0.6
Molybdenum	112.4	113.8	114.2	113.6	114.8	114.6	113.9	0.8
Nickel	108.2	109.0	111.2	111.8	114.1	112.2	111.1	2.0
Vanadium	110.8	111.1	114.2	113.8	114.2	114.7	113.1	1.5
Copper	96.1	95.5	99.2	99.0	98.7	99.8	98.1	1.8

Tables 5a, 5b and 5c show that a precision of < 20% is easily achieved.

Ruggedness test

The ruggedness of the method was assessed by measuring six independent aliquots of each of the three materials tested spiked with the fourteen USP defined elements at the target limit (J), on three separate days. A final average and % RSD were calculated from the averages of the values obtained on each day. The results from these tests are shown in Tables 6a, 6b and 6c.

USP <233> defines that the ruggedness (% RSD) from three repeat analyses on different days should not be greater than 25%.

Table 6a. Ruggedness for three repeat measurements of Drug A spiked at the target limit (J), expressed as percent recovery

Element	Drug A - 1	Drug A - 2	Drug A - 3	Mean	RSD (%)
Cadmium	98.4	96.8	98.0	97.7	0.9
Lead	97.2	95.2	93.2	95.2	2.1
Inorganic arsenic	95.0	96.0	97.0	96.0	1.0
Inorganic mercury	98.0	97.3	96.0	97.1	1.0
Iridium	100.0	100.0	98.0	99.3	1.2
Osmium	113.0	10.2	97.0	103.4	8.2
Palladium	97.6	95.8	96.5	96.6	0.9
Platinum	94.4	95.8	95.6	95.3	0.8
Rhodium	101.0	99.0	100.0	100.0	1.0
Ruthenium	99.9	98.7	99.0	99.2	0.6
Chromium	102.1	103.2	102.9	102.7	0.6
Molybdenum	109.0	107.0	106.0	107.3	1.4
Nickel	98.6	95.6	94.6	96.3	2.2
Vanadium	106.0	98.0	98.0	100.7	4.6
Copper	95.8	92.0	89.8	92.5	3.3

Table 6b. Ruggedness for three repeat measurements of Drug B spiked at the target limit (J), expressed as percent recovery

Element	Drug B - 1	Drug B - 2	Drug B - 3	Mean	RSD (%)
Cadmium	99.2	98.0	98.0	98.4	0.7
Lead	97.8	95.8	93.8	95.8	2.1
Inorganic arsenic	92.7	93.2	92.8	92.9	0.3
Inorganic mercury	97.3	96.7	95.3	96.4	1.1
Iridium	101.0	102.0	99.0	100.7	1.5
Osmium	116.0	99.0	104.0	106.3	8.2
Palladium	97.0	95.7	95.5	96.1	0.8
Platinum	96.7	97.8	96.6	97.0	0.7
Rhodium	100.0	99.0	98.0	99.0	1.0
Ruthenium	99.6	98.6	98.1	98.8	0.8
Chromium	103.5	102.9	103.2	103.2	0.3

Table 6b continuation. Ruggedness for three repeat measurements of Drug B spiked at the target limit (J), expressed as percent recovery

Element	Drug B - 1	Drug B - 2	Drug B - 3	Mean	RSD (%)
Molybdenum	115.0	113.0	111.0	113.0	1.8
Nickel	97.4	95.2	94.4	95.7	1.6
Vanadium	106.0	98.1	99.0	101.0	4.3
Copper	97.9	95.6	94.1	95.9	2.0

Table 6c. Ruggedness for three repeat measurements of Drug C spiked at the target limit (J), expressed as percent recovery

Element	Drug C - 1	Drug C - 2	Drug C - 3	Mean	RSD (%)
Cadmium	98.8	95.2	97.6	97.2	1.9
Lead	100.0	98.1	96.7	98.3	1.7
Inorganic arsenic	116.7	115.2	116.4	116.1	0.7
Inorganic mercury	91.3	89.3	90.7	90.4	1.1
Iridium	102.0	103.1	98.9	101.3	2.1
Osmium	117.1	107.8	99.2	108.0	8.3
Palladium	98.5	95.1	97.0	96.9	1.8
Platinum	96.7	99.5	95.6	97.3	2.1
Rhodium	102.8	102.1	99.7	101.5	1.6
Ruthenium	98.8	97.6	96.2	97.5	1.3
Chromium	101.5	102.8	103.5	102.6	1.0
Molybdenum	113.0	110.8	105.6	109.8	3.5
Nickel	123.4	117.4	119.6	120.1	2.5
Vanadium	113.7	105.0	102.2	107.0	5.6
Copper	97.5	94.3	92.2	94.7	2.8

Tables 6a, 6b and 6c show that a precision of < 25% across three days is easily achieved. The excellent measurement stability for µg L^{-1} levels of Mercury in each drug (< 1% precision over 3 days) is a result of the sample preparation method described and the stability of the iCAP RQ ICP-MS.

CONCLUSION

This application note has shown that the iCAP RQ ICP-MS is an ideal tool for elemental determination in pharmaceutical products after dissolution by microwave digestion. For the three drugs tested, method detection limits fifty times lower than the target limits were produced showing that the iCAP RQ ICP-MS is easily capable of accurately and precisely measuring all fourteen of the specified elements at the target limits listed in USP <232>. Based on this, when considering the continual change in regulations defined

by USP and other National and International bodies, ICP-MS represents a future-proof investment for pharmaceutical laboratories embarking on elemental impurity analyses. The described method exceeds the analytical performance criteria described in USP <233> by a wide margin.

Finally, the range of security features, data management and audit trailing tools included in the advanced and flexible Qtegra ISDS Software provides the necessary support to meet the demands of 21 CFR Part 11 in the highly regulated pharmaceutical industry environment.

REFERENCES

1. General Chapter <232> Elemental Impurities - Limits, United States Pharmacopeia.
2. General Chapter <233> Elemental Impurities - Procedure, United States Pharmacopeia.
3. 21CFR11 2017, Food and Drug Administration.

Find out more at thermofisher.com/SQ-ICP-MS

This Sponsor Report is the responsibility of Thermo Fisher Scientific.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 256-261

BrJAC

SPONSOR REPORT

PDF

This section is dedicated for sponsor responsibility articles.

Detection of controlled substances in blood samples using the VeriSpray ion source with TSQ Altis MS for clinical research and forensic toxicology

Greta Ren[1], Nicholas Manicke[1], Cornelia Boeser[2], Neloni R. Wijeratne[2]

[1]Indiana University - Purdue University Indianapolis, Indianapolis, IN
[2]Thermo Fisher Scientific, San Jose, CA

This report was extracted from the Thermo Scientific Technical Note 65420

Keywords: Illicit drugs, opiates, opioids, amphetamines, PS-MS/MS, TSQ Altis MS, VeriSpray PaperSpray ion source, TraceFinder, forensic toxicology, benzodiazepines

Application benefits

- Quick turn-around time
- Reduced cost per sample, increased ease-of-use and robustness
- Six drugs of abuse analytes in single quantitative method

Goal

To develop a robust, sensitive, reliable, and reproducible PaperSpray-mass spectrometry workflow for detection of illicit drugs in blood for clinical research and forensic toxicology using the Thermo Scientific™ TSQ Altis™ mass spectrometer connected with the Thermo Scientific™ VeriSpray™ PaperSpray system.

INTRODUCTION

The abuse of controlled substances is a serious ongoing problem worldwide, causing significant societal disruption and economic damage. One part of the overall strategy to mitigate the effects from the abuse of drugs requires high-performance methodologies for the screening and quantitation of these substances in biological matrices. Modern forensic toxicological and clinical research laboratories need simpler methods that provide higher throughput and faster analysis for the screening and quantitation of drugs of abuse.

PaperSpray technology combined with triple quadrupole mass spectrometry is an ideal choice for rapid drug screening and quantitation in clinical research and forensic toxicology applications for two main reasons. First, studies have demonstrated that low ng/mL or lower detection limits are obtainable directly from blood, which is sufficient for the detection of relevant drugs at target concentrations. Second, reduce the burden on the laboratory by simplifying method development, reducing the amount of bench work and thus decreasing time to result. Reports in the literature for screening by PaperSpray MS include the detection of amphetaminelike designer drugs in oral fluid [1], agrichemicals in fruit [2], targeted triple quadrupole based screening [3], and use of HR-MS/MS for urine [4] and blood screening [5].

Triple quadrupole mass spectrometers are unit-resolution instruments that achieve high selectivity by monitoring characteristic collision induced dissociation (CID) fragment ions. When operated in selected reaction monitoring (SRM) mode, the instruments give high sensitivity and robust quantitation.

In this study, we investigated the VeriSpray PaperSpray ion source coupled to a triple quadrupole mass spectrometer as a drug screening tool for applications in clinical research and forensic toxicology. Experiments were carried out using the VeriSpray PaperSpray ion source on a TSQ Altis triple-stage

quadrupole mass spectrometer. The VeriSpray system enables robust, rapid, and automated PaperSpray analysis. Sample storage, extraction, and ionization all take place on VeriSpray sampling plates. Biofluids are spotted and dried directly on the sampling plate. The plates contain 24 individual PaperSpray tips, each of which analyzes a separate sample (Figure 1A). Analysis of the plate is carried out automatically via the VeriSpray ion source (Figure 1B) in a matter of minutes. To demonstrate proof-of-concept, controlled substances commonly encountered in clinical research and forensic toxicology were tested (cocaine, diazepam, fentanyl, hydrocodone, methamphetamine, and zolpidem).

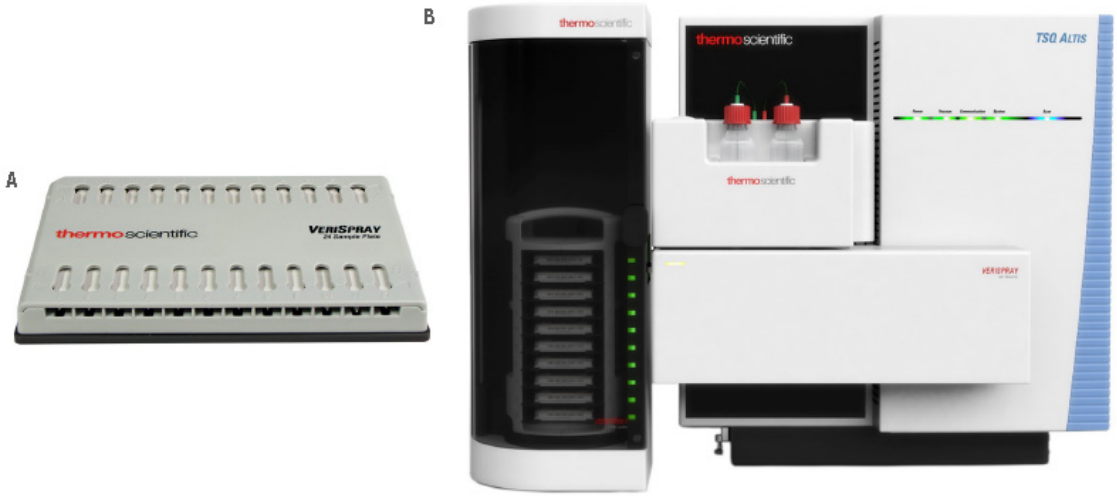


Figure 1. (A) VeriSpray sample plate and (B) VeriSpray PaperSpray system mounted to TSQ Altis triple quadrupole mass spectrometer

EXPERIMENTAL

Sample preparation

The method was adapted from a previous PaperSpray method for drug detection [5]. Calibration standards were prepared in pooled human blood. Working solutions at 20x concentration were prepared in methanol by serial dilution and spiked into blood the day of analysis. Blood samples (100 µL) were mixed with 300 µL of aqueous internal standard solution containing isotopically labeled analogs of each of the analytes. A 6 µL aliquot of each blood sample was then spotted onto a VeriSpray cartridge and allowed to dry at room temperature for 1 hour or in an incubator for 20 minutes at 40 ºC.

PaperSpray and MS conditions

The PaperSpray solvents (both sample rewet and spray solvents) were acetonitrile/acetone/water 0.01% acetic acid (85:10:5), applied according to the settings in Table 1. The TSQ Altis triple quadrupole mass spectrometer was used for detection. The experimental conditions were optimized with a time dependent spray voltage of 3.8 kV, a cycle time of 0.8 s, and resolution of 0.7 Da FWHM for both Q1 and Q3. The source parameters and SRM table along with the critical MS features for all target analytes are listed in Tables 2 and 3, respectively. The optimum RF lens settings and collision energies for the product ions were determined by infusion of the individual standards into the mass spectrometer.

Table 1. VeriSpray solvent application parameters. Each rewetting and solvent dispense is 10 µL

Rewetting dispense delay	
Dispense	Delay (s)
1	1
Solvent dispense delay	
Dispense	Delay (s)
1	1
2	1
3	1
4	1
5	3
6	3
7	5
8	5
9	5
10	5
11	7
12	7
13	7
14	7
15	7

Table 2 (A). Source parameters for analysis of Illicit drugs on the TSQ Altis triple quadrupole mass spectrometer

Ion Source Parameter	**Value**
Spray Voltage	Time Dependent
Positive Ion	3800 V
Sweep Gas	0 Arb
Ion Transfer Tube Temperature	300 °C
CID Gas	2 mTorr

Table 2 (B). Time dependent spray voltage

Time (min)	**Voltage (V)**
0	0
0.1	3800
1.1	0

Table 3. Optimized mass spectrometer transitions for the Illicit drugs in blood with acquisition time of 1.2 min and positive polarity for each sample

Compound	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	RF Lens (V)
Methamphetamine	150.1	91.1	21	91
	150.1	119.1	12	91
Methamphetamine-D5	155.1	92.1	21	91
	155.1	121.1	12	91
Diazepam	285.1	193.1	33	223
	285.1	222.0	28	223
Diazepam-D5	290.1	198.1	33	223
	290.1	227.0	28	223
Hydrocodone	300.1	171.1	40	207
	300.1	199.1	31	207
Hydrocodone-D6	306.1	174.1	40	207
	306.1	202.1	31	207
Cocaine	304.1	150.1	26	172
	304.1	182.2	21	172
Cocaine-D3	307.1	153.1	26	172
	307.1	185.2	21	172
Zolpidem	308.2	235.2	36	228
	308.2	263.2	27	228
Zolpidem-D6	314.2	235.2	36	228
	314.2	263.2	27	228
Fentanyl	337.4	105.1	38	200
	337.4	188.1	24	200
Fentanyl-D5	342.4	105.1	38	200
	342.4	188.1	24	200
Buprenorphine	468.4	396.2	40	299
	468.4	414.3	35	299
Buprenorphine-D4	472.4	400.2	40	299
	472.4	415.3	35	299

Data acquisition and analysis

Data acquisition and processing were conducted using Thermo Scientific™ TraceFinder™ software version 4.1. Limits of detection were calculated by the formula $3*s_b/m$, where s_b is the standard error of the intercept and m is the slope of the calibration line.

RESULTS AND DISCUSSION

The seven controlled substances were successfully quantitated simultaneously as shown in Figure 2.

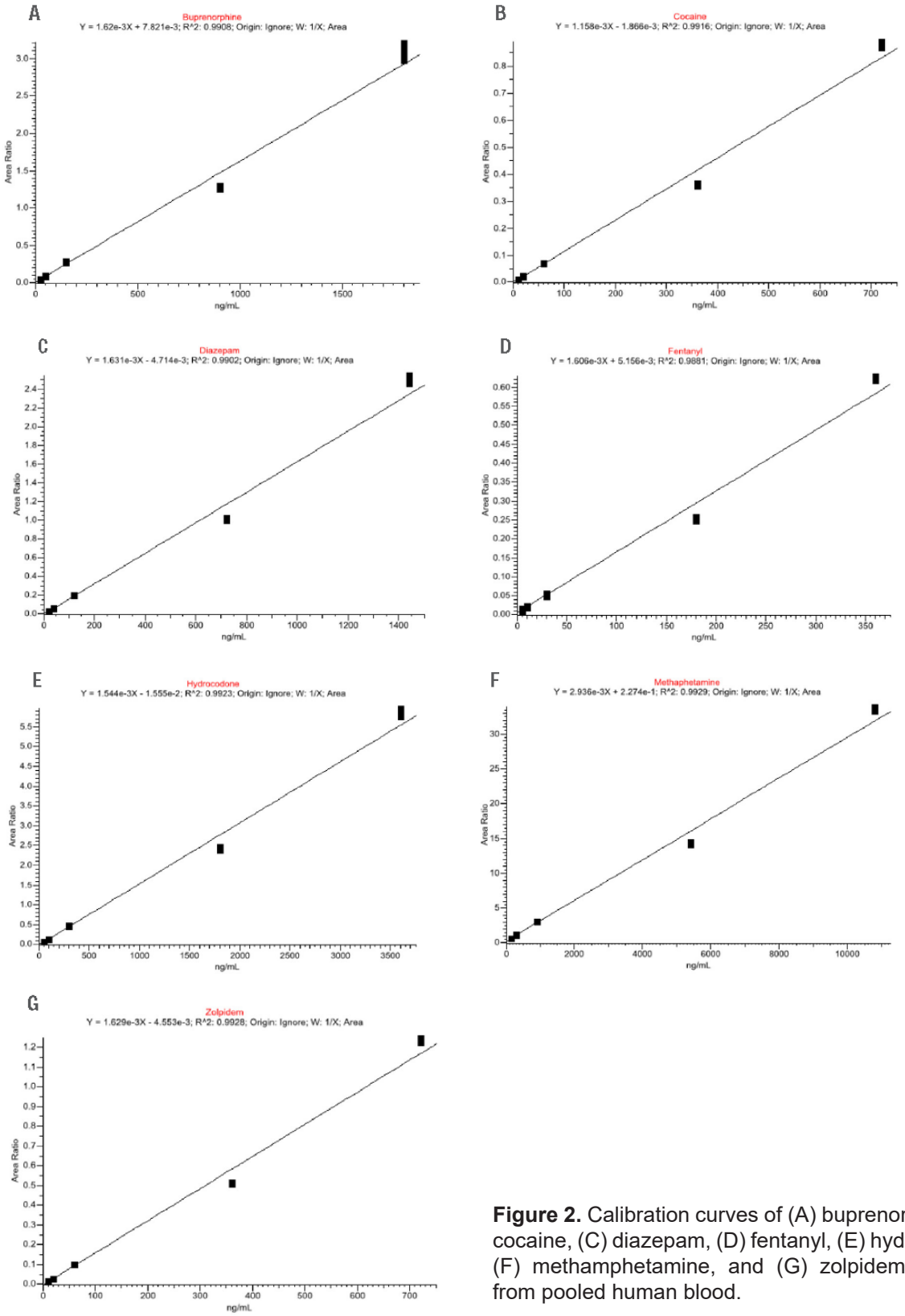


Figure 2. Calibration curves of (A) buprenorphine, (B) cocaine, (C) diazepam, (D) fentanyl, (E) hydrocodone, (F) methamphetamine, and (G) zolpidem obtained from pooled human blood.

The correlation coefficient (R^2) for each calibration curve was greater than 0.98, indicating good linearity. The detection limits (Table 4) are below the concentrations normally encountered in forensic toxicology with the exception of buprenorphine. Total analysis time for the dried blood spots was approximately two minutes. This included the extraction step as well as the mass spectrometric detection, both of which take place automatically using the VeriSpray sample plate.

Table 4. Limits of detection (LOD) and calibration curve correlation coefficient (R^2) from human blood obtained using the VeriSpray system

Compound	LOD (ng/mL)	R^2
Buprenorphine	13	0.9909
Cocaine	5	0.9916
Diazepam	11	0.9902
Fentanyl	3	0.9881
Hydrocodone	23	0.9923
Methamphetamine	68	0.9928
Zolpidem	5	0.9928

CONCLUSIONS

PaperSpray MS on the VeriSpray sampling plates and ion source was capable of accurate quantitation of controlled substances in human blood for clinical research and forensic toxicology. Analysis was fast and simple, requiring no sample pretreatment or separations.

REFERENCES

1. Lee, H.; Jhang, C. S.; Liu, J. T.; Lin, C. H., Rapid screening and determination of designer drugs in saliva by a nib-assisted paper spray-mass spectrometry and separation technique. *J. Sep. Sci*. **2012**, *35* (20), 2822–2825.
2. Soparawalla, S.; Tadjimukhamedov, F. K.; Wiley, J. S.; Ouyang, Z.; Cooks, R. G., In situ analysis of agrochemical residues on fruit using ambient ionization on a handheld mass spectrometer. *Analyst* **2011**, *136* (21), 4392–4396.
3. Jett, R.; Skaggs, C.; Manicke, N. E., Drug screening method development for paper spray coupled to a triple quadrupole mass spectrometer. *Analytical Methods* **2017**, *9* (34), 5037-5043.
4. Michely, J. A.; Meyer, M. R.; Maurer, H. H., Paper Spray Ionization Coupled to High Resolution Tandem Mass Spectrometry for Comprehensive Urine Drug Testing in Comparison to Liquid Chromatography-Coupled Techniques after Urine Precipitation or Dried Urine Spot Workup. *Analytical Chemistry* **2017**, *89* (21), 11779–11786.
5. McKenna, J.; Jett, R.; Shanks, K.; Manicke, N. E., Toxicological Drug Screening using Paper Spray High-Resolution Tandem Mass Spectrometry (HR-MS/MS). *Journal of Analytical Toxicology* **2018**, *42* (5), 300–310.

Find out more at thermofisher.com/verispray

This Sponsor Report is the responsibility of Thermo Fisher Scientific.

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 34, pp 262-266

BrJAC

SPONSOR REPORT

PDF

This section is dedicated for sponsor responsibility articles.

Determination of Total Mercury in Clinical Matrices Utilizing Direct Analysis for Mercury Detection in Blood, Hair and Urine Samples

This report was extracted from the Milestone Industry Report DMA–80 *evo* / Clinical

SUMMARY

Excessive exposure to mercury has been linked to, among others, neurological and developmental disorders in children, as well as, cardiovascular disease, neurological and other problems in adults. Individuals at a high risk of mercury exposure are typically monitored through the analysis of blood, urine and, occasionally, hair samples.

Many laboratories have testing procedures in place to analyze for mercury in clinical samples. These procedures have typically included the use of Cold Vapor Atomic Absorption (CVAA) or ICP-MS. Both of these techniques, although effective, require costly and time-consuming sample digestion prior to analysis. Direct mercury analysis, an alternative to these methods, has been used successfully to determine total mercury in clinical matrices. This technique requires no sample preparation and delivers results in as little as six (6) minutes per sample.

INTRODUCTION

Methyl mercury is a well-known neurotoxin that has been shown to effect brain development. Studies have shown that, when ingested by pregnant women, the methyl mercury can cross the placenta and effect development of the central nervous system. Even small amount of methyl mercury can impact the time it takes a child to walk, talk, hear and write. The most common mechanism for humans to be exposed to this neurotoxin is through the consumption of fish and seafood. However, mercury can also enter the body via inhalation and absorption through skin. The most effective way to monitor people who are suspected of mercury intoxication is through the analysis of blood, urine and hair samples. Because methyl mercury is not excreted from the body, blood analysis is the preferred method to determine total mercury in the human body.

Several methods exist for the determination of mercury in clinical matrices. Traditional analytical methods such as Cold Vapor Atomic Absorption (CVAA) and ICP-MS both require sample preparation prior to analysis. This results in both techniques being costly, labor-intensive and subsequently, having a long turnaround time. Direct mercury analysis, as described in EPA Method 7473 and ASTM Method 6722-01, is a cost-effective, proven alternative to these labor-intensive, wet chemistry techniques.

Direct analysis affords the laboratory many benefits including:

- Reduced Sample Turnaround (6 minutes)
- No Sample Preparation
- Reduced Hazardous Waste Generation
- Reduction of Analytical Errors
- General Cost Savings (70% versus CVAA)

EXPERIMENTAL

Instrument

The DMA-80 *evo*, Direct Mercury Analyzer, as referenced in EPA Method 7473, from Milestone (www.milestonesrl.com) was used in this study (Figure 1). The DMA-80 *evo* features a circular, stainless steel,

interchangeable 40 position autosampler for virtually limitless throughput and can accommodate both nickel (500 mg) and quartz boats (1500 µL) depending on the requirements of the application.

Figure 1. Milestone's DMA-80 *evo*.

It operates from a single-phase 110/220 V, 50/60 Hz power supply and requires regular grade oxygen as a carrier gas. As the process does not require the conversion of mercury to mercuric ions, both solid and liquid matrices can be analyzed without the need for acid digestion or other sample preparation. The fact that zero sample preparation is required also eliminates all hazardous waste generation. All results, instrument parameters including furnace temperatures, are controlled and saved with easy export capabilities to Excel or LIMS.

Principles of operation

Direct mercury analysis incorporates the following sequence: Thermal Decomposition, Catalytic, Conversion, Amalgamation, and Atomic Absorption Spectrophotometry. Controlled heating stages are implemented to first dry and then thermally decompose a sample introduced into a quartz tube. A continuous flow of oxygen carries the decomposition products through a hot catalyst bed where halogens, nitrogen, and sulfur oxides are trapped. All mercury species are reduced to Hg(0) and are then carried along with reaction gases to a gold amalgamator where the mercury is selectively trapped. All non-mercury vapors and decomposition products are flushed from the system by the continuous flow of gas. The amalgamator is subsequently heated and releases all trapped mercury to the single beam, fixed wavelength atomic absorption spectrophotometer. Absorbance is measured at 253.7 nm as a function of mercury content.

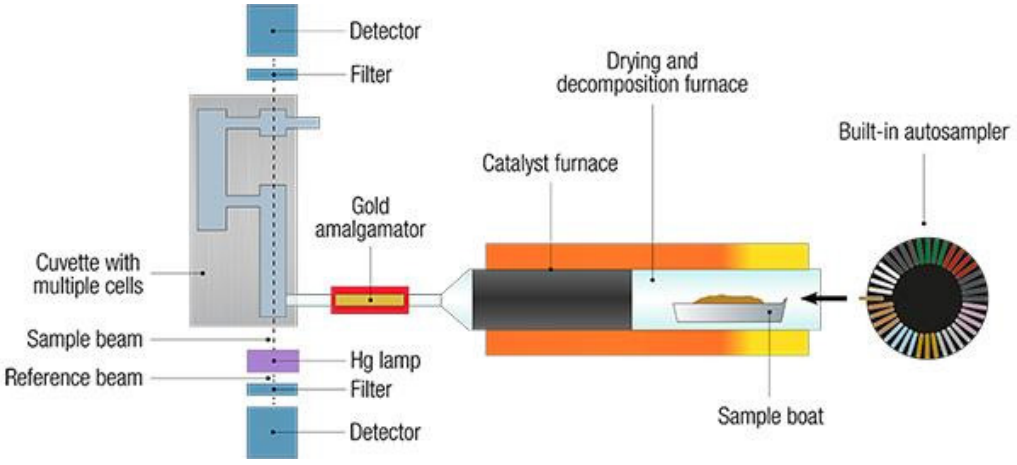


Figure 2. A schematic of Milestone's DMA-80 *evo*.

EXPERIMENTAL DISCUSSION

This study focuses on the effectiveness of the DMA-80 to analyse clinical matrices like blood, hair and urine. Ten blood Proficiency Test (PT) samples were obtained from a state agency. Samples were centrifuged and 100 L aliquots were pipetted into the quartz sample boats (1500 µL) for analysis on the DMA-80. Six urine samples were also analysed. Approximately 200 µL – 300 µL of urine was pipetted directly into the quartz samples boats for analysis. Finally, hair samples of approximately 5 – 10 mg, in powder form, were weighed out and placed into nickel sample boats for analysis.

Calibration

Calibration standards were prepared using a NIST traceable stock solution of 1000 ppm Hg preserved in 5% HNO_3. Working standards of 100 ppb and 1 ppm were prepared and preserved in 37% HCl and stored in amber glass vials. By injecting increasing sample volumes of standard into the quartz sample boats, calibration graphs of 0 – 20 ng (Figure 3) and 20 – 500 ng (Figure 4) of mercury were created using the 100 ppb and 1 ppm standards respectively.

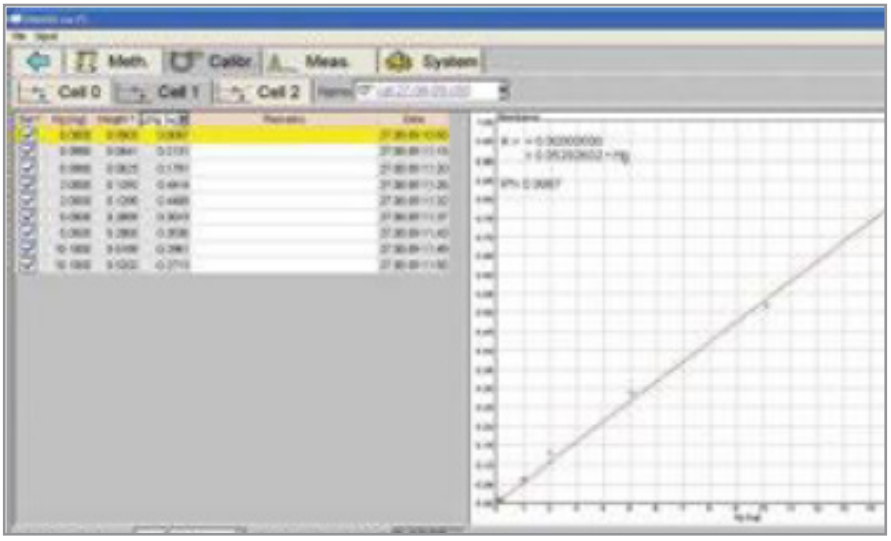


Figure 3. 0 ng – 20 ng Calibration Graph for ultra-level.

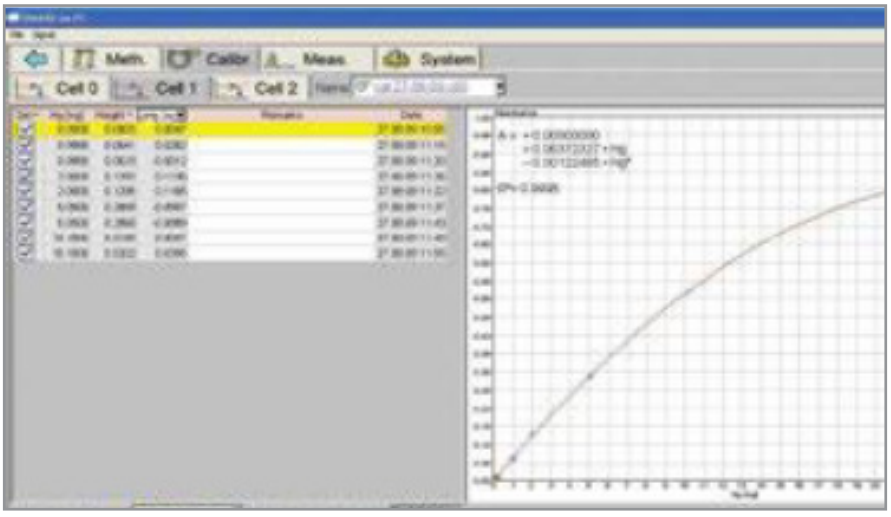


Figure 4. 20 ng – 1000 ng Calibration Graph for low to mid-level analysis (ppb, ppm).

Operating conditions

The DMA-80's operating conditions for all analyses are shown in Table 1.

Table 1. Analysis Operating Parameters

Parameter	Setting
Drying Temp/Time	30 seconds to 200 °C
Decomposition Ramp	90 seconds to 650 °C
Decomposition Hold	90 seconds at 650 °C
Catalyst Temp	565 °C
Purge Time	60 seconds
Amalgamation Time	12 seconds at 900 °C
Recording Time	30 seconds
Oxygen Flow	120 mL/min

RESULTS

Results of the blood proficiency samples are shown in Table 3. It is important to point out that all samples were within their certified range. Tables 2 and 4 describe the results of urine and hair analysis respectively. All samples were analysed in triplicate. Good reproducibility was achieved on the hair analysis with most samples having an RSD < 5%.

Table 2. Urine Analysis

Sample	Concentration	% RSD
1	5.82±0.06 ppb	1.11
2	21.1±0.4 ppb	1.71
3	47 ± 2 ppb	5.65
4	98 ± 4 ppb	4.18
5	180 ± 4 ppb	2.03
6	200 ± 10 ppb	5.76

Table 3. Hair Analysis

Sample	Concentration	% RSD
1	212 ± 3 ppb	3.5

Table 4. Blood Analysis: DMA-80 *vs* Known Concentrations

Sample	Weight (g)	Assigned Target (ng/g)	DMA-80 (ng/g)
1	0.1015	32.7	39.3
2	0.1999	1.9	1.3
3	0.1024	5.4	4.5
4	0.1020	17.3	19.8
5	0.1002	12.0	13.1
6	0.1002	25.6	31.0
7	0.1036	2.9	1.2
8	0.1009	1.8	0.0
9	0.1049	10.4	11.3
10	0.1013	6.4	5.8

CONCLUSION

The DMA-80 *evo*, direct mercury analyzer, successfully processed all three clinical matrices and provides a fast, accurate and reliable alternative to wet chemistry techniques.

No sample preparation is required meaning results are obtained within six minutes. This is ideal for clinical laboratories looking for quick turnaround of their in-house samples.

Further reading

Please visit our Hg info center for complete access to application notes, technical papers, as well as links to valuable resources for mercury testing.

Go to www.milestonesrl.com/dma-80

To learn more about mercury and other related topics, feel free to visit these websites:

EPA Method 7473: http://www.epa.gov/waste/hazard/testmethods/sw846/pdfs/7473.pdf

ASTM Method D6722: http://www.astm.org/Standards/D6722.htm

EPA Mercury: http://www.epa.gov/mercury/

Methylmercury: http://en.wikipedia.org/wiki/Methylmercury

Mercury in Fish: http://www.epa.gov/waterscience/fish/advice/mercupd.pdf

This Sponsor Report is the responsibility of Milestone.

JOINT FORENSIC SCIENCE MEETING

8° ENQFOR
NATIONAL MEETING OF FORENSIC CHEMISTRY

5° SBCF
MEETING OF THE BRAZILIAN SOCIETY OF FORENSIC SCIENCE

Connecting Science and Justice

September 05-08 | 2022

Ribeirão Preto Convention Center
Ribeirão Preto/SP - Brazil

THEMATIC SESSIONS

SHORT COURSES

CRIME SCENE

The universe of FORENSIC SCIENCES

AWARDS

SPONSOR EXPOSURE

Take part!

SBCF Sociedade Brasileira de Ciências Forenses

USP

USP QUÍMICA RIBEIRÃO PRETO

For more information:
www.enqfor.org.br

RELEASE

Thermo Scientific iCAP RQ ICP-MS

Simplicity, productivity and robustness for routine labs

Expand your analytical capabilities with this complete trace elemental analysis solution for your high-throughput lab. User-inspired hardware and software combine in the Thermo Scientific™ iCAP™ RQ ICP-MS to deliver maximized productivity and robustness. Simplicity and ease-of-use work in concert to streamline work-flows and achieve 'right-first-time' results; essential to all busy labs.

This innovative single quadrupole (SQ) ICP-MS is the ideal trace elemental analyzer for 24/7 routine analysis to advanced applications in research.

Simplicity guarantees user-friendly operation

- Quick connect sample introduction system
- Easy access cones via bench-height drop-down door
- Open geometry architecture for easy peripheral connection
- Intuitive platform software for seamless workflows

Productivity delivers more analysis in less time

- Less training for software and hardware
- Compatibility with automation and sample handling systems
- Comprehensive interference removal in single measurement mode
- Reduced drift and operator intervention

Robustness ensures high up-time and low maintenance

- Improved matrix tolerance interface
- All new state-of-the-art electronics
- New sturdy design RF generator
- Reliable hot and cold plasma operation

Comprehensive interference removal assures data accuracy, while our innovative helium Kinetic Energy Discrimination (He KED) technology enables measurement of all analytes in a single mode.

Our highly effective QCell collision/reaction cell, combined with unique flatapole design reduces BECs even further than He KED alone, through the clever, dynamic application of low mass cut off (LMCO).

Intuitive Thermo Scientific™ Qtegra™ Intelligent Scientific Data Solution™ (ISDS) software delivers all the support features essential to any lab, while containing all the flexibility needed to achieve the most challenging applications.

ThermoFisher SCIENTIFIC

Complete trace elemental analysis

Innovation inspired by real laboratory needs lies at the core of our trace elemental analysis instruments. The iCAP RQ ICP-MS part of the new Thermo Scientific™ iCAP™ Qnova™ Series ICP-MS, delivers the accuracy, robustness and ease of use essential to every high-throughput lab. Driven by our user-friendly Qtegra™ Intelligent Scientific Data Solution™ (ISDS) software, this powerful new system is ideal for labs up-grading to ICP-MS for the first time. The new Element Finder™ plug-in for Qtegra ISDS software, automates wavelength selection, simplifying method development for interference free analysis with the iCAP 7000 Plus Series ICP-OES.

maximum productivity

VIDEO **WEBSITE**

- powerful solutions at thermofisher.com/TEA

Thermo Scientific™ iCE™ 3000 Series AAS

Thermo Scientific™ iCAP™ 7000 Plus Series ICP-OES

Thermo Scientific™ iCAP™ RQ ICP-MS

Thermo Scientific™ ELEMENT 2™ & XR™ HR-ICP-MS
ELEMENT GD™ PLUS GD-MS

RELEASE

Thermo Scientific™ VeriSpray™ PaperSpray Ion Source

The faster path to MS analysis

The VeriSpray ion source combined with next-generation Thermo Scientific™ mass spectrometers reduces time to results and cost per test with automated, high-throughput, direct mass spectrometry (MS)-based sample analysis.

The VeriSpray ion source allows use of “dilute and shoot” methods to minimize solvent consumption and time-consuming sample preparation steps like derivatization, while avoiding needs for expert operators and instrument downtime.

For laboratories currently performing LC or GC methods, or for those that want to add the robust sensitivity and selectivity of MS-based tests, the VeriSpray ion source provides an ideal alternative to the complexity of chromatography-based solutions.

Achieve remarkable simplicity

For many tests, the VeriSpray ion source solution eliminates time-consuming, complicated off-line sample preparation and chromatographic separation, simplifying MS analyses for any user.

Reduce sample turnaround time

The VeriSpray ion source solution permits highthroughput analysis of unprocessed samples in just one step. With the VeriSpray ion source, there is no chromatographic downtime or maintenance.

Lower costs per test

Compared to LC, the VeriSpray ion source substantially reduces solvent consumption and disposal, and eliminates LC or GC instrument maintenance costs and extra derivatization steps.

Automate analyses

A VeriSpray ion source solution can run up to 240 samples unattended. For automated sample spotting, the VeriSpray sample plates are compatible with robotic spotters. Barcode reading capabilities enable integration into Laboratory Information Systems (LIS).

Go green

Environmentally friendly, the VeriSpray ion source reduces solvent consumption. The VeriSpray ion source itself consumes very little solvent and produces minimal waste.

State-of-the-art design automates analyses for high throughput and lower costs

With an advanced design, the VeriSpray ion source automates direct MS analyses, helping to improve turnaround time and reduce sample analysis costs.

Find out more at VeriSpray PaperSpray Ion Source for Mass Spectrometry

thermoscientific

VeriSpray™ PaperSpray Ion Source

Improve turnaround time, reduce cost per test and minimize sample preparation with the Thermo Scientific™ VeriSpray™ PaperSpray ion source, a fully automated, high-throughput, direct ionization technique used with our newest Thermo Scientific™ TSQ triple quadrupole mass spectrometers. The VeriSpray system is fully integrated into instrument control software and application-specific software, and it is capable of running proprietary VeriSpray PaperSpray sample plates, for single or high-throughput applications.

WEBINAR **WEBSITE**

ThermoFisher SCIENTIFIC

RELEASE

Save Time — Go Direct for Mercury Analysis

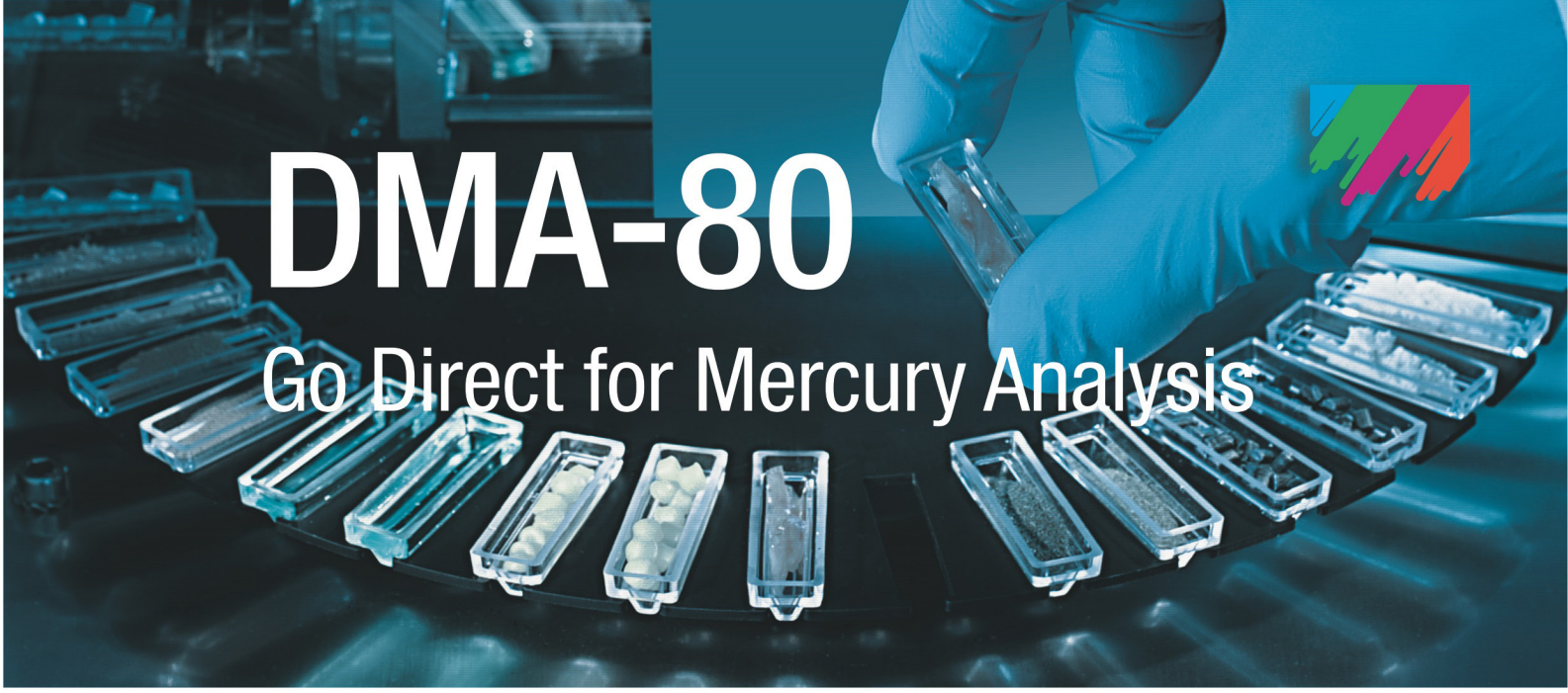
The Milestone DMA-80 has been at the forefront of direct total mercury analysis for almost two decades

The challenges in mercury determination are well known to analysts who often face a number of issues connected with tedious sample preparation process or the mercury analysis steps causing memory effect even after long cleaning cycles performed using latest generation of ICP-MS or cold vapor systems.

The DMA-80 mercury determination system can analyze any matrix (solid, liquid or gas) without any pre-treatment or chemical additions in as few as 6 minutes in full compliance with EPA method 7473. With thousands of units installed, Milestone defines the benchmark for direct mercury determination in a wide range of industries: environmental, food energy, cement, cosmetics, agriculture, mining and petrochemical.

- **Improved design** from the outside out for superior performance event at the ppt level.
- **Easy-to-use technology** with a single long-lasting calibration for all matrices.
- **No memory effect** thanks to the revolutionary autoblank feature.
- **Milestone Connect** for 30-years of experience at your fingertips
- **Fully compliant** with US EPA Method 7473, ASTM method D-6722-01 and D-7623-10.
- **Maximize your ROI** with an easy-to-maintain system and reliable components.

READ MORE



DMA-80

Go Direct for Mercury Analysis

SIX MINUTE, PREP-FREE MERCURY ANALYSIS

If youre looking for unsurpassed processing speed, efficiency and precision for mercury samples, its time for the Milestone DMA-80offering more than double the productivity of traditional cold vapor techniques at a fraction of the cost.

NO SAMPLE PREP REQUIRED

Eliminating the need for any wet chemistry sample treatment prior to analysis.

WIDEST DYNAMIC RANGE

Capable of achieving a detection limit of 0.001 to 30,000 ng of mercury

RESULTS IN JUST 6 MINUTES

Dramatically improves your laboratorys turnaround time and productivity.

UP TO 70% COST REDUCTION

Compared to traditional mercury analysis techniques such as CV-AAS.

Watch Videos, Download Brochures & Application Notes at:

VIDEO

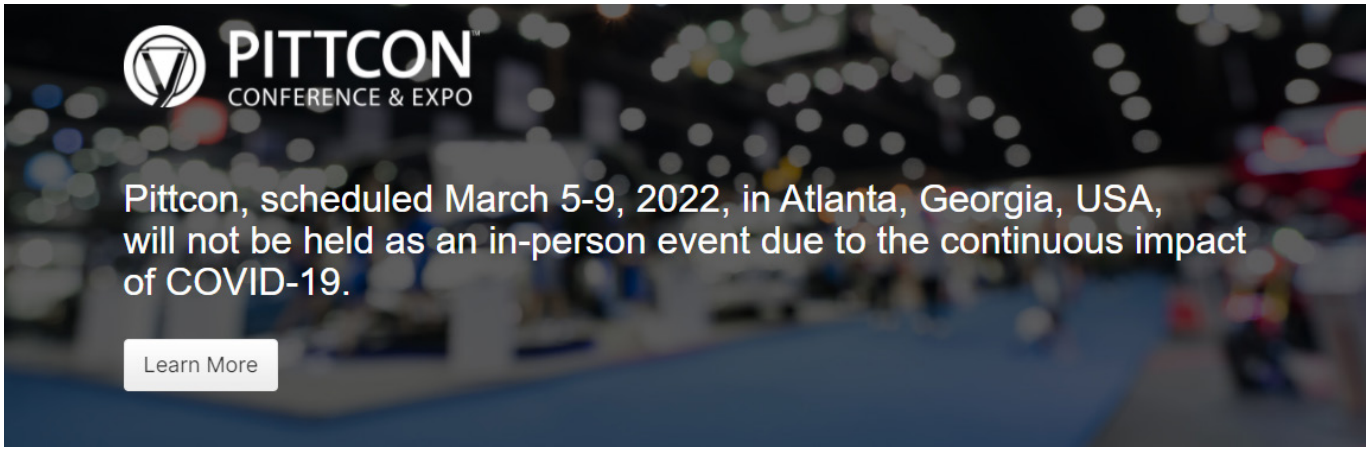
WEBSITE

DMA-80

DMA-1

MILESTONE

PITTCON®
CONFERENCE & EXPO



In the face of health and safety risks resulting from the ever-increasing communicability of emerging COVID-19 strains, the decision to cancel the in-person Pittcon was made.

Currently, alternate options for presenting Pittcon 2022 are being explored. More details on this will be released as soon as they are finalized.

For more information visit https://pittcon.org/#learn-more

RELEASE

SelectScience® Pioneers online Communication and Promotes Scientific Success

SelectScience® promotes scientists and their work, accelerating the communication of successful science. Through trusted lab product reviews, virtual events, thought-leading webinars, features on hot scientific topics, eBooks and more, independent online publisher SelectScience® provides scientists across the world with vital information about the best products and techniques to use in their work.

Some recent contributions from SelecScience® to the scientific community

Webinars

The 2020 opioids epidemic and the importance of drug screening

COVID-19 has led to a reversal of the downward trend in opioid misuse globally — watch this on-demand webinar to explore the crisis in depth. Prof. Dr. Marilyn A. Huestis, Senior Fellow, Thomas Jefferson University, and President, Huestis & Smith Toxicology, will break down how this global opioid epidemic emerged, its continued prevalence in 2020, as well as detail the current approaches for drug testing and the success of available treatment regimens for opioid dependance. Access this webinar here

Real-time forensic drug analysis using direct ionization mass spectrometry

Peter Cain, Scientific Advisor at Eurofins Forensic Services, discusses the analysis of seized drug material using a low-cost, compact mass spectrometer. This new approach utilizes ambient ionization to analyze seized drug material without any prior chromatographic separation, therefore providing results in near real time. The improved selectivity of this analysis results in fewer false positives and therefore fewer samples requiring confirmatory GC/MS or LC/MS analysis. Access this webinar here

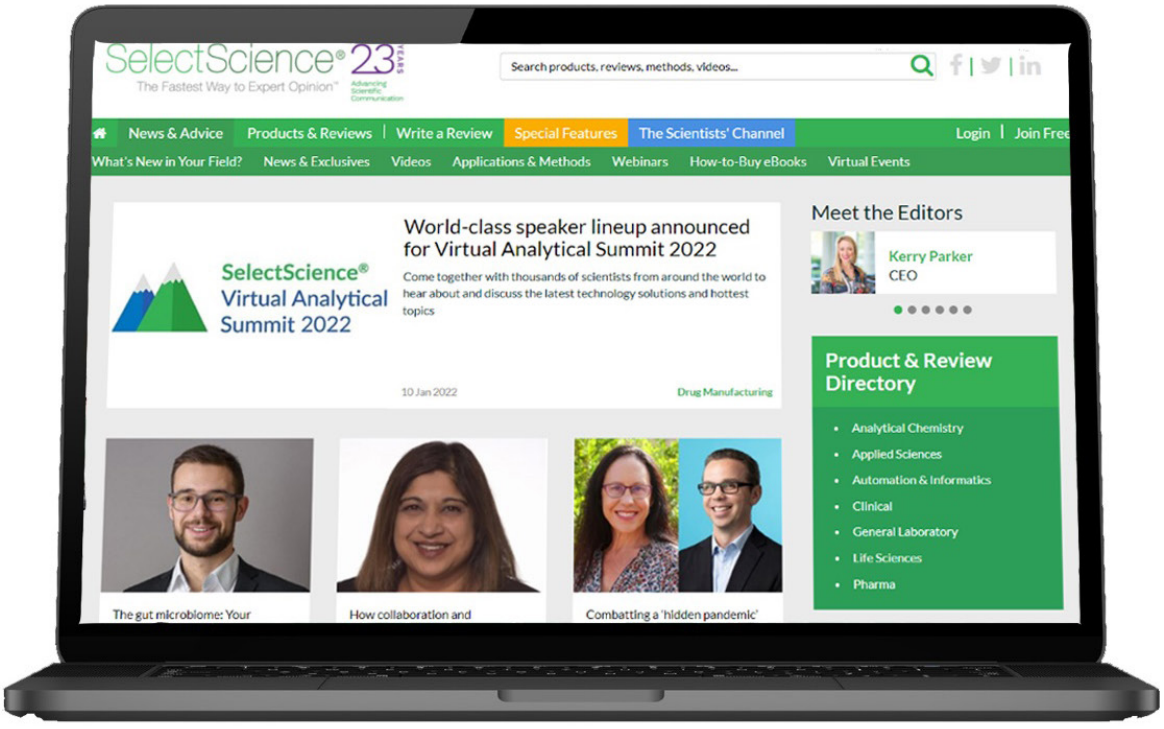
Video: ***How NGS enables mass food fraud screening***

Sofia Nogueira, Molecular Biology Laboratory Manager at Jeronimo Martins, discusses the power of next-generation sequencing (NGS) in food fraud testing and explains how NGS enables full screening of all ingredients within a sample. Watch this video here

Editorial Article: ***The use of probabilistic genotyping software in forensic DNA analysis***

In this guest editorial, Dr. Michael Coble discusses how probabilistic genotyping (PG) software has revolutionized the ability of forensic labs to interpret DNA profiles. Dr. Michael Coble is a fellow of the American Academy of Forensic Sciences and a member of the International Society for Forensic Genetics. Read this article here

SelectScience® is the leading independent online publisher connecting scientists to the best laboratory products and applications.



- Working with Scientists to Make the Future Healthier.
- Informing scientists about the best products and applications.
- Connecting manufacturers with their customers to develop, promote and sell technologies.

SelectScience®
The Fastest Way to Expert Opinion

RELEASE

CHROMacademy is the leading provider of eLearning for analytical science

CHROMacademy *helps scientific organizations acquire and maintain excellence in their laboratories*.

For over 10 years, CHROMacademy has increased knowledge, efficiency and productivity across all applications of chromatography. With a comprehensive library of learning resources, members can improve their skills and knowledge at a pace that suits them.

CHROMacademy covers all chromatographic applications – HPLC, GC, mass spec, sample preparation, basic lab skills, and bio chromatography. Each paradigm contains dozens of modules across theory, application, method development, troubleshooting, and more. Invest in analytical eLearning and supercharge your lab.

For more information, please visit www.chromacademy.com/

LC GC's CHROMacademy
powered by crawford scientific

CHROMacademy Lite members have access to less than 5% of our content. Premier members get so much more !

Video Training courses

Fundamental HPLC
Fundamental GC
Fundamental LCMS
Fundamental GCMS
HPLC Method Development
GC Method Development

Ask the Expert

We are always on hand to help fix your instrument and chromatographic problems, offer advice on method development, help select a column for your application and more.

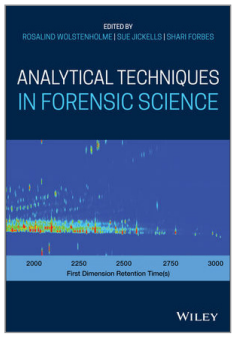
To find out more about Premier Membership contact:

Glen Murry: +1 732.346.3056 | Glen.Murry@ubm.com
Peter Romillo: +1 732.346.3074 | Peter.Romillo@ubm.com

www.chromacademy.com

The worlds largest e-Learning website for analytical scientists

NOTICES OF BOOKS

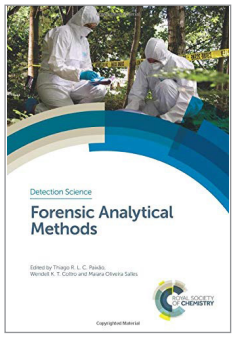


Analytical Techniques in Forensic Science

Rosalind Wolstenholme, Sue Jickells, Shari Forbes, Editors

October 2020. Publisher: Wiley

An in-depth text that explores the interface between analytical chemistry and trace evidence in forensic science. With contributions from noted experts on the topic, the text features a detailed introduction analysis in forensic science and then subsequent chapters explore the laboratory techniques grouped by shared operating principles. The applications reviewed include evidence types such as fibers, paint, drugs and explosives. Read more



Forensic Analytical Methods

Thiago R. L. C. Paixão, Wendell K. T. Coltro, Maiara Oliveira Salles, Editors

August 2019. Publisher: Royal Society of Chemistry

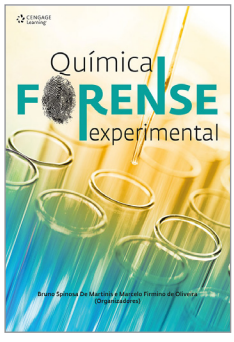
This is the first book that brings together the understanding of the analytical techniques and how these influence the outcome of a forensic investigation. Starting with a brief introduction of the chemical analysis for forensic application, some forensic sampling and sample preparation, the book then describes techniques used in forensic chemical sensing in order to solve crimes. Read more

"Locais de Crime. Dos vestígios à Dinâmica Criminosa"

Jesus A. Velho, Karina A. Costa, Clayton T. Damasceno, Editors

January 2013. Publisher: Millenium

This book covers concepts, techniques and procedures applied to the expert processing of crime scenes with schematic drawings of procedures, case analyses, images and color photos. It is a real treaty on Crime Scenes. It is essential reading for those who work or intend to work in the forensic area. Read more



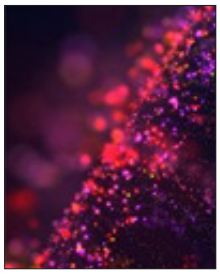
"Química Forense Experimental"

Bruno S. de Martinis, Marcelo F. de Oliveira, Editors

January 2016. Publisher: Cengage Learning

This book presents several chemical analysis techniques aimed at the forensic area, such as: colorimetric, spectrometric and electrochemical methods, separation techniques, among others. The chapters consist of case studies and a proposal for an experimental script for laboratory practice. The authors intend to encourage a detailed discussion of chemical analysis techniques in the context of Forensic Chemistry, discussing their operational advantages and intrinsic limitations. Read more

PERIODICALS & WEBSITES

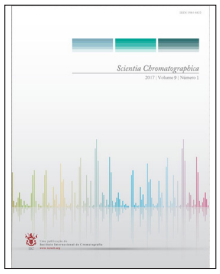


American Laboratory

American Laboratory® is a platform that addresses basic research, clinical diagnostics, drug discovery, environmental, food and beverage, forensics and other markets, and combines in-depth articles, news, and video to deliver the latest advances in their fields. ***LABTips – Particle Characterization and Testing:*** Characterization is critical in a vast number of industries. Regardless of application, knowing the size and shape of particles allows manufacturers to control product effectiveness and maintain customer satisfaction. Read more

LCGC

Chromatographyonline delivers practical, nuts-and-bolts information to help scientists and lab managers become more proficient in the use of chromatographic techniques and instrumentation, thereby making laboratories more productive and businesses around the world more successful. **Article:** ***The Role of Two-Dimensional Gas Chromatography in Arson Investigations***. Boegelsack, N. & O'Sullivan, G. from Mount Royal University in Calgary, CA, created a GC×GC workflow to analyse ignitable liquid residues in arson investigations. Read more



Scientia Chromatographica

Scientia Chromatographica is the first and to date the only Latin American scientific journal dedicated exclusively to Chromatographic and Related Techniques. With a highly qualified and internationally recognized Editorial Board, it covers all chromatography topics in all their formats, in addition to discussing related topics such as "The Pillars of Chromatography", Quality Management, Troubleshooting, Hyphenation (GC-MS, LC-MS, SPE-LC-MS/MS) and others. It also provides columns containing general information, such as: calendar, meeting report, bookstore, etc. Read more

Select Science

SelectScience® has transformed global scientific communications and digital marketing over the last 23 years. Informing scientists about the best products and applications. Connecting manufacturers with their customers to develop, promote and sell technologies promotes scientists and their work, accelerating the communication of successful science. Scientists can make better decisions using independent, expert information and gain easy access to manufacturers. SelectScience® informs the global community through Editorial, Features, Video and Webinar programs. Read more

Spectroscopy

With the Spectroscopy journal, scientists, technicians, and lab managers gain proficiency through unbiased, peer-reviewed technical articles, trusted troubleshooting advice, and best-practice application solutions.
Article: ***A Simple Introduction to Raman Spectral Identification of Organic Materials*** By Fran Adar. Innovative database search technology can help Raman spectroscopists identify molecular vibrations; here, we show how to use these tools more effectively. Read more

20ºENQA | 8ºCIAQA

Encontro Nacional de Química Analítica
20th Brazilian Meeting on Analytical Chemistry

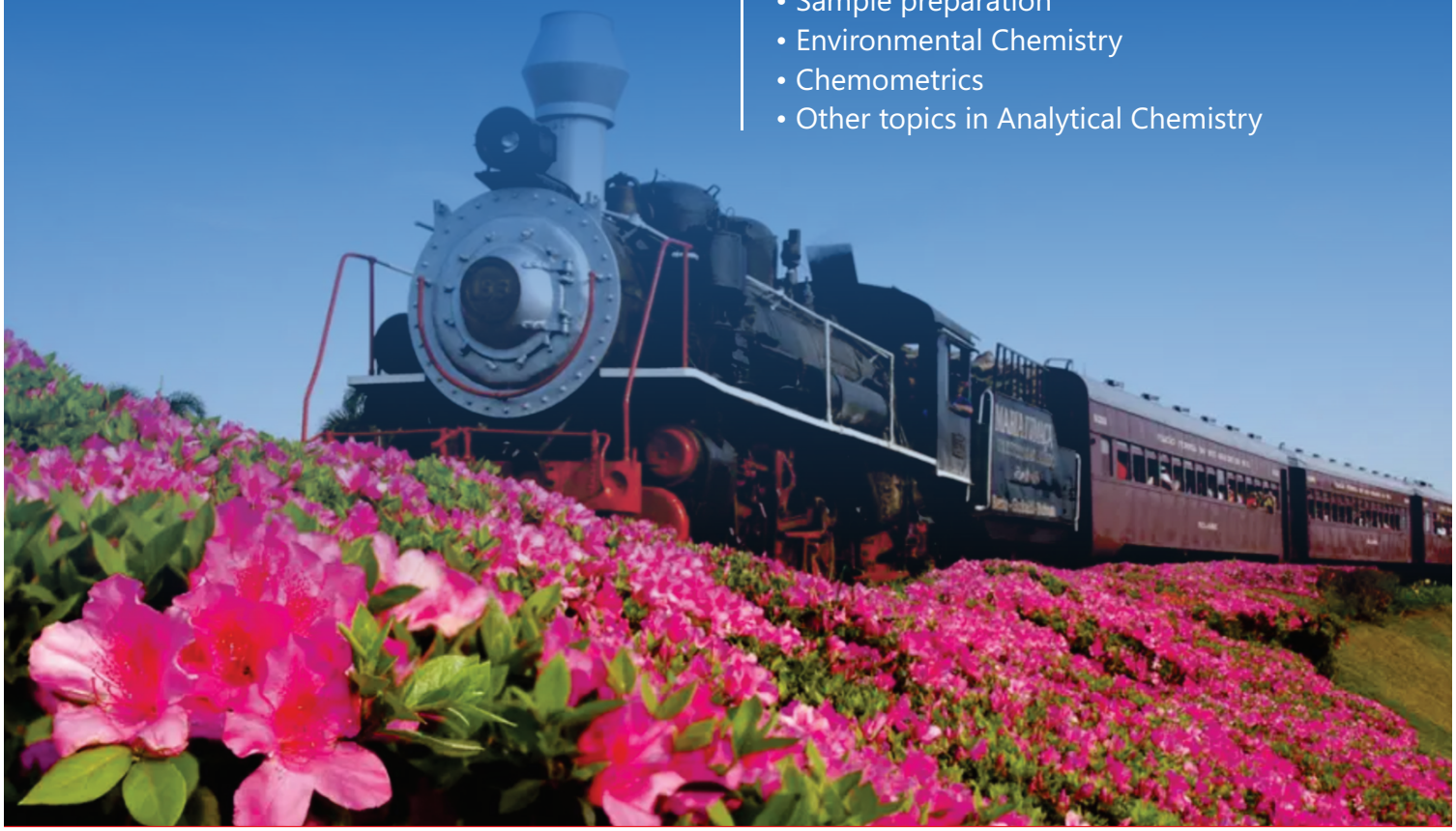
Congresso Ibero-Americano de Química Analítica

September • 25 to 28, 2022 • Dall'Onder Grande Hotel • Bento Gonçalves • RS • Brazil

These events are organized with short courses, conferences, oral and poster sessions, workshops, awards and commercial exhibitions.

Event topics

- Electrochemistry and Electroanalysis
- Atomic Spectrometry
- Mass Spectrometry
- Analytical Instrumentation
- Separation methods
- Sample preparation
- Environmental Chemistry
- Chemometrics
- Other topics in Analytical Chemistry



For more information: www.enqa.com.br

Held by: Sociedade Brasileira de Química | UFSM

Sponsorship – Diamond: Anton Paar, ThermoFisher Scientific

Sponsorship - Gold: Agilent, Astro 34, União, Metrohm, analítica

Sponsorship – Silver: HORIBA, Waters

Sponsorship - Bronze: ALLCROM, SHIMADZU

Organized by acontece

EVENTS in 2022 – It is suggested to consult the event's official website for updates.

January 9 – 10
Gordon Research Conference Electrochemistry "Fundamental to Applied Electrochemistry: New Frontiers in Charge Transfer Theory, Electrocatalysis, Materials for Energy Conversion/Storage, Sensing and Separations"
Ventura, CA, USA
http://www.grc.org/electrochemistry-conference/2022/

March 5 – 9
PITTCON In-person Conference & Expo was Canceled
Alternate options for presenting Pittcon 2022 are being explored
More details on this will be released as soon as they are finalized
For more information visit https://pittcon.org/#learn-more

April 19 – 22
EuroAnalysis 2022
Linnaeusgebouw, Nijmegen, Netherlands
https://10times.com/e1z5-d0hf-31sf

May 25 – 28
XXII Brazilian Congress of Toxicology (CBTox 2022)
Balneário Camboriú, SC, Brazil
www.cbtox2021.com.br

May 29 – June 2
19th International GCxGC Symposium
Canmore, Alberta, Canada
https://www.gcxgc-symposium.com

May 29 – June 2
241st Meeting of the Electrochemical Society (ECS)
Vancouver, BC, Canada
https://www.electrochem.org/241

May 30 – June 3
Colloquium Spectroscopicum Internationale (CSI XLII 2022)
Gijon, Spain
https://www.csi2022spain.com/en/

May 31 – June 3
45th Annual Meeting of the Brazilian Chemical Society (RASBQ)
Maceió, AL, Brazil
http://www.sbq.org.br/reunioes-anuais

June – ***strongly hoping that it will be possible to have a fully in person conference***
XVIII Chemometrics in Analytical Chemistry (CAC)
Courmayeur, Italy / Chamonix, France
http://cac2020.sciencesconf.org

EVENTS in 2022 – It is suggested to consult the event's official website for updates.

June 5 – 9
18th International Conference on Electroanalysis (ESEAC 2022)
Vilnius, Lithuania
http://www.eseac2020.com/

June 21 – 23
Analitica Latin America Expo & Conference
São Paulo, SP, Brazil
https://www.analiticanet.com.br/

July 18 – 22
8th International Caparica Conference on Analytical Proteomics
Caparica, Portugal
https://www.icap2022.net/

August 26 – 28
Journées de Chimie Analytique 2022 (JCA2022)
Libreville, Gabon
https://jca-2021.sciencesconf.org

September 5 – 8
Meeting of the Brazilian Society of Forensic Sciences & National Meeting on Forensic Chemistry (EnQFor)
Ribeirão Preto, SP, Brazil
https://www.sbcf.org.br/

September 25 – 28
20th National Meeting of Analytical Chemistry (ENQA) & 8th Ibero-American Congress of Analytical Chemistry (CIAQA)
Bento Gonçalves, RS, Brazil
https://enqa.com.br/

September 25 – 29
XX Brazilian Materials Research Society Meeting (SBPMat)
Foz do Iguaçu, PR, Brazil
https://www.sbpmat.org.br/pt/

October 15 – 18
Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq)
Foz do Iguaçu, PR, Brazil
https://www.sbbq.org.br/

December 10 – 15
III Ibero American Conference on Mass Spectrometry (IBERO 2022)
Rio de Janeiro, RJ, Brazil
https://www.ibero2022.com/

Acknowledgments

BrJAC editors are grateful to all those who have reviewed papers in 2021 using significant time and effort to provide constructive inputs.

Adalgisa Rodrigues de Andrade, Universidade de São Paulo, Brazil
Adriano de Araújo Gomes, Universidade Federal do Rio Grande do Sul, Brazil
Aldo Eliano Polettini, Università di Verona, Verona, Italy
Aline Thais Bruni, Universidade de São Paulo, Brazil
Amal Traces, University of Babylon, Iraq
Amanda Guiraldelli Mahr, United States Pharmacopeia, Brazil
Anderson Rodrigo Moraes de Oliveira, Universidade de São Paulo, Brazil
Andre Luis dos Santos, Universidade Federal de Uberlândia, Brazil
Andre Marcelo de Souza, Empresa Brasileira de Pesquisa Agropecuária, Brazil
Anna Jánosi, Hungarian University of Agriculture and Life Sciences, Hungary
Anna W. Sobanska, Medical University of Lodz, Poland
António O. S. S. Rangel, Escola Superior de Biotecnologia, Portugal
Ashraf Mozayani, Texas Southern University, United States
Balázs Zsirka, University of Pannonia, Hungary
Barry K. Logan, Center for Forensic Science Research and Education, United States
Bhawan Patel, Rowan University, United States
Bruno Spinosa de Martinis, Universidade de São Paulo, Brazil
Burkhard Horstkotte, Charles University, Czech Republic
Carlos Augusto Fernandes de Oliveira, Universidade de São Paulo, Brazil
Cassiana Carolina Montagner Raimundo, Universidade Estadual de Campinas, Brazil
Cecilia de Carvalho Castro Silva, Universidade Presbiteriana Mackenzie, Brazil
Cesar Ricardo Teixeira Tarley, Universidade Estadual de Londrina, Brazil
Cláudio Celestino Oliveira, Universidade Estadual de Maringá, Brazil
Claudio Martin Pereira de Pereira, Universidade Federal de Pelotas, Brazil
Daniel Santos, Universidade Federal de Santa Maria, Brazil
Diego Pereira dos Santos, Universidade Estadual de Campinas, Brazil
Dirce Pozebon, Universidade Federal do Rio Grande do Sul, Brazil
Douglas Neil Rutledge, AgroParisTech, France
Edina Baranyai, University of Debrecen, Hungary
Edson Irineu Muller, Universidade Federal de Santa Maria, Brazil
Eduardo Carasek, Universidade Federal de Santa Catarina, Brazil
Eduardo Costa de Figueiredo, Universidade Federal de Alfenas, Brazil
Eduardo Geraldo de Campos, Universidade de São Paulo, Brazil
Elcio Cruz de Oliveira, Pontifícia Universidade Católica, Brazil
Emanuel Carrilho, Universidade de São Paulo, Brazil
Emanuela Gionfriddo, University of Toledo, United States
Fabio Augusto, Universidade Estadual de Campinas, Brazil
Fábio Ferreira Gonçalves, Universidade Federal do Rio Grande, Brazil
Fábio Rodrigo Piovezani Rocha, Universidade de São Paulo, Brazil
Fabíola Manhas Verbi Pereira, Universidade Estadual Paulista, Brazil
Felipe Bachion de Santana, Universidade Estadual de Campinas, Brazil
Felipe Rebello Lourenço, Universidade de São Paulo, Brazil
Fernando Fabriz Sodré, Universidade de Brasília, Brazil
Flávio Venâncio Nakadi, Universidad de Zaragoza, Spain

Acknowledgments (Continuation)

George L. Donati, Wake Forest University, United States
Geórgia Christina Labuto Araújo, Universidade Federal de São Paulo, Brazil
Gildo Girotto Junior, Universidade Estadual de Campinas, Brazil
Guilherme Post Sabin, Scientific OpenScience, Brazil
Hadi Parastar, Sharif University of Technology, Iran
Haq Nawaz, College of Material Science and Technology, China
Hery Mitsutake, Universidade Estadual de Campinas, Brazil
Igor Miranda Santana, Universidade Estadual de Campinas, Brazil
Janice Zulma Francesquett, Faculdade Horizontina, Brazil
Jarbas José Rodrigues Rohwedder, Universidade Estadual de Campinas, Brazil
Jez Willian Batista Braga, Universidade de Brasília, Brazil
Joana de Jesus de Andrade, Universidade de São Paulo, Brazil
Joanna Suliburska, Poznan University of Life Sciences, Poland
João Alexandre Velho Prior, Universidade do Porto, Portugal
João Raul Belinato, Apex Science, Brazil
Jorge César Masini, Universidade de São Paulo, Brazil
Joseany de Moraes Santos Almeida, Pontifícia Universidade Católica, Brazil
Josué Carinhanha Caldas Santos, Universidade Federal de Alagoas, Brazil
Juliana Azevedo Lima Pallone, Universidade Estadual de Campinas, Brazil
Juliana Naozuka, Universidade Federal de São Paulo, Brazil
Katalin Zih-Perényi, Eötvös Loránd University, Hungary
Kelliton José Mendonça Francisco, Universidade de São Paulo, Brazil
Leandro Wang Hantao, Universidade Estadual de Campinas, Brazil
Letícia Malta Costa, Universidade Federal de Minas Gerais, Brazil
Licarion Pinto, Universidade Federal de Pernambuco, Brazil
Lilian Rodrigues Rosa Souza, Universidade de São Paulo, Brazil
Luciana Assis Terra, Agilent Technologies, Brazil
Magali Kemmerich, Universidade Federal do Pampa, Brazil
Maha K. Al-Tameemi, University of Central Florida, United States
Maiara Salles, Universidade Federal do Rio de Janeiro, Brazil
Marcelino Varona, Iowa State University, United States
Marcelo Firmino de Oliveira, Universidade de São Paulo, Brazil
Marcelo Martins de Sena, Universidade Federal de Minas Gerais, Brazil
Márcia Cristina Breitkreitz, Universidade Estadual de Campinas, Brazil
Márcia Miguel Castro Ferreira, Universidade Estadual de Campinas, Brazil
Márcio da Silva Coutinho, Fundação de Apoio à Escola Técnica, Brazil
Marco Flores Ferrão, Universidade Federal do Rio Grande do Sul, Brazil
Marco Tadeu Tadeu Grassi, Universidade Federal do Paraná, Brazil
Margareth Borges Coutinho Gallo, Fundação Oswaldo Cruz, Brazil
Maria C. Hespanhol, Universidade Federal de Viçosa, Brazil
Maria Eliana Lopes Ribeiro de Queiroz, Universidade Federal de Viçosa, Brazil
Maria Eugenia Queiroz Nassur, Universidade de São Paulo, Brazil
Maria Valnice Boldrin, Universidade Estadual Paulista, Brazil
Mariana Ramos de Almeida, Universidade Federal de Minas Gerais, Brazil
Mario Sergio Palma, Universidade Estadual Paulista, Brazil
Márk Horváth, Szent Istvan University, Hungary
Masoom Raza Siddiqui, King Saud University, Saudi Arabia

Acknowledgments (Continuation)

Melina Borges Teixeira Zanatta, Universidade Estadual Paulista, Brazil
Milton Katsumi Sasaki, Universidade Federal do Rio Grande do Sul, Brazil
Mónica Benicia Mamián-López, Universidade Federal do ABC, Brazil
Naiara Mariana Fiori Monteiro Sampaio, Universidade Federal do Paraná, Brazil
Osmar Damian Prestes, Universidade Federal de Santa Maria, Brazil
Pablo Richter, Universidad de Chile, Chile
Patrícia Valderrama, Universidade Tecnológica Federal do Paraná, Brazil
Paulo Henrique Gonçalves Dias Diniz, Universidade Federal do Oeste da Bahia, Brazil
Paulo Henrique Março, Universidade Tecnológica Federal do Paraná, Brazil
Paweł Świt, University of Silesia, Poland
Pedro Vitoriano Oliveira, Universidade de São Paulo, Brazil
Rafael Melo, Empresa Brasileira de Pesquisa Agropecuária, Brazil
Renata Amais, Universidade Estadual de Campinas, Brazil
Renato Lajarim Carneiro, Universidade Federal de São Carlos, Brazil
Rodrigo Moretto Galazzi, Analytik Jena – Analytical Instrumentation, Brazil
Ronaldo Censi Faria, Universidade de São Carlos, Brazil
Roy Edward Bruns, Universidade Estadual de Campinas, Brazil
Sarmento J. Mazivila, Universidade do Porto, Portugal
Sen Kamalika, University of Calcutta, India
Solange Cadore, Universidade Estadual de Campinas, Brazil
Suellen Alves, Universidade Estadual de Ponta Grossa, Brazil
Suresh Jain, Jodhpur National University, India
Tatiana Dillenburg SaintPierre, Pontifícia Universidade Católica, Brazil
Tatiana Vianna Francisco, Pontifícia Universidade Católica, Brazil
Thiago Regis Longo Cesar da Paixão, Universidade de São Paulo, Brazil
Tiele Medianeira Rizzetti, Universidade de Santa Cruz do Sul, Brazil
Valderi Luiz Dressler, Universidade Federal de Santa Maria, Brazil
Victor Gustavo Kelis Cardoso, Universidade Estadual de Campinas, Brazil
Victor Hugo Cavalcanti Ferreira, Universidade Estadual de Campinas, Brazil
Vijay Kotra, Quest International University, Malaysia
Viktor G. Mihucz, Eötvös Loránd University, Hungary
Wanderson Romão, Universidade Federal do Espírito Santo, Brazil
Wanessa Melcher Mattos, Universidade de São Paulo, Brazil
Wendell K. T. Coltro, Universidade Federal de Goiás, Brazil
William Reis de Araújo, Universidade Estadual de Campinas, Brazil

GUIDELINES FOR AUTHORS

Scope

The *Brazilian Journal of Analytical Chemistry* (BrJAC) is dedicated to the diffusion of significant and original knowledge in all branches of Analytical Chemistry and Bioanalytics. The BrJAC is addressed to professionals involved in science, technology and innovation projects at universities, research centers and in industry.

Professional Ethics

Manuscripts submitted for publication in BrJAC cannot have been previously published or be currently submitted for publication in another journal.

The BrJAC does not consider submissions of manuscripts that have been posted to preprint servers prior to submission, and will withdraw from consideration any papers posted to those servers prior to publication.

The submitted manuscripts are the full responsibility of the authors. Manipulation/invention/omission of data, duplication of publications, the publication of papers under contract and confidentiality agreements, company data, material obtained from non-ethical experiments, publications without consent, the omission of authors, plagiarism, the publication of confidential data and undeclared conflicts of interests are considered serious ethical faults.

The BrJAC discourages and restricts the practice of excessive self-citation by the authors.

The BrJAC does not practice coercive citation, that is, it does not require authors to include references from BrJAC as a condition for achieving acceptance, purely to increase the number of citations to articles from BrJAC without any scientific justification.

Misconduct will be treated according to the COPE's recommendations (https://publicationethics.org/) and the Council of Science Editors White Paper on Promoting Integrity in Scientific Journal Publications (https://www.councilscienceeditors.org/).

For more detailed information on the BrJAC's ethics and integrity policy, please see the "About us" menu at www.brjac.com.br

Manuscripts that can be submitted to BrJAC

- **Articles:** Full descriptions of an original research finding in Analytical Chemistry. Articles undergo double-blind full peer review.
- **Reviews:** Articles on well-established subjects, including critical analyses of the bibliographic references and conclusions. Manuscripts submitted for publication as Reviews must be original and unpublished. Reviews undergo double-blind full peer review.
- **Technical Notes:** Concise descriptions of a development in analytical methods, new techniques, procedures or equipment falling within the scope of the BrJAC. Technical notes also undergo double-blind full peer review.
- **Letters:** Discussions, comments, suggestions on issues related to Analytical Chemistry, and consultations to authors. Letters are welcome and will be published at the discretion of the BrJAC editor-in-chief.

Documents Preparation

It is highly recommended that authors download and use the templates to create their four mandatory documents to avoid the suspension of a submission that does not meet the BrJAC guidelines.

Download templates here

Cover Letter

In addition to the usual content of a Cover Letter to the Editor-in-Chief of the journal, the submitting author must declare any conflicts of interest on behalf of all co-authors and their agreement with the copyright policy of the BrJAC. Please read about conflicts of interest and copyright in the About us menu at www.brjac.com.br

It is the duty of the submitting author to inform their collaborators about the manuscript content and obtain their permission for submission and eventual publication.

The Cover Letter must be signed by the submitting author.

Title Page

The Title Page must contain information for each author: full name, affiliation and full postal address in the original language, and information on the contribution of each author to the work. Acknowledgments must be entered on the Title Page. The submitting author must sign the Title Page.

Novelty Statement

The Novelty Statement must contain clear and succinct information about what is new and innovative in the study in relation to previously related works, including the works of the authors themselves.

Manuscript

It is highly recommended that authors download the Manuscript template and create their manuscript in this template, keeping the layout of this file.

- **Language**: **English** is the language adopted by BrJAC. The correct use of English is of utmost importance. In case the Editors and Reviewers consider the manuscript to require an English revision, the authors will be required to send an English proofreading certificate before the final approval of the manuscript by BrJAC.
- **Required items**: the manuscript must include a title, abstract, keywords, and the following sections: Introduction, Materials and Methods, Results and Discussion, Conclusion, and References.
- **Identification of authors**: as the BrJAC adopts a double-blind review, the manuscript file must **NOT** contain the authors' names, affiliations nor acknowledgments. Full details of the authors and their acknowledgements should be on the Title Page.
- **Layout**: the lines in the manuscript must be numbered consecutively and double-spaced.
- **Graphics and Tables**: must appear close to the discussion about them in the manuscript. For **figures** use **Arabic** numbers, and for **tables** use **Roman** numbers.
- **Permission to use content already published**: to use figures, graphs, diagrams, tables, etc. identical to others previously published in the literature, even if these materials have been published by the same submitting authors, a publication permission from the publisher or scientific society holding the copyrights must be requested by the submitting authors and included among the documents uploaded in the manuscript management system at the time of manuscript submission.
- **Chemical nomenclature, units and symbols**: should conform to the rules of the International Union of Pure and Applied Chemistry (IUPAC) and Chemical Abstracts Service. It is recommended that, whenever possible, the authors follow the International System of Units, the International Vocabulary of Metrology (VIM) and the NIST General Table of Units of Measurement. Abbreviations are not recommended except for those recognized by the International Bureau of Weights and Measures or those recorded and established in scientific publications. Use L for liters. Always use superscripts rather than /. For instance: use mg mL^{-1} and NOT mg/mL. Leave a space between numeric values and their units.
- **References throughout the manuscript:** the references must be cited as **numbers in square brackets [ ]**. It is recommended that references older than 5 (five) years be avoided, except in relevant cases. Include references that are accessible to readers.
- **References item:** This item must be thoroughly checked for errors by the authors before submission. From 2022, BrJAC is adopting the American Chemical Society's Style in the Reference item. Mendeley Reference Manager users will find the Journal of American Chemical Society citation style in the Mendeley View menu. Non-users of the Mendeley Reference Manager may refer to the ACS Reference Style Quick Guide DOI: https://doi.org/10.1021/acsguide.40303

Manuscript Submission

The BrJAC uses an online manuscript manager system for the submission of manuscripts. This system guides authors stepwise through the entire submission process.

Submit manuscripts at www.brjac.com.br

The submitting author must add all co-authors to the Authors section of the manuscript manager system. After submission, all co-authors will receive an alert and will have the opportunity to confirm whether or not they are co-authors.

Four documents are mandatorily uploaded by the submitting author: Cover letter, Title Page, Novelty Statement and the Manuscript. Templates for these documents are available at www.brjac.com.br

The four documents mentioned above must be uploaded into the manuscript manager system as Word files. The manuscript Word file will be converted by the system to a PDF file which will be used in the double-blind peer review process.

All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail to the submitting author through the manuscript manager system.

Review process

Manuscripts submitted to the BrJAC undergo an initial check for compliance with all of the journal's guidelines. Submissions that do not meet the journal's guidelines will be suspended and an alert sent to the corresponding author. The authors will be able to resend the submission within 30 days. If the submission according to the journal's guidelines is not made within 30 days, the submission will be withdrawn on the first subsequent day and an alert will be sent to the corresponding author.

Manuscripts that are in accordance with the journal's guidelines are submitted for the analysis of similarities by the iThenticate software.

The manuscript is then forwarded to the Editor-in-Chief who will check whether the manuscript is in accordance with the journal's scope and will analyze the similarity report issued by iThenticate.

If the manuscript passes the screening described above, it will be forwarded to an Associate Editor who will also analyze the iThenticate similarity report and invite reviewers.

Manuscripts are reviewed in double-blind mode by at least 2 reviewers. A larger number of reviewers may be used at the discretion of the Editor. As evaluation criteria, the reviewers employ originality, scientific quality, contribution to knowledge in the field of Analytical Chemistry, the theoretical foundation and bibliography, the presentation of relevant and consistent results, compliance with the BrJAC's guidelines, clarity of writing and presentation, and the use of grammatically correct English.

Note: In case the Editors and Reviewers consider the manuscript to require an English revision, the authors will be required to send an English proofreading certificate, by the ProofReading Service or equivalent service, before the final approval of the manuscript by the BrJAC.

The 1st-round review process usually takes around 5-6 weeks. If the manuscript is not rejected but requires corrections, the authors will have one month to submit a corrected version of the manuscript. In another 3-4 weeks, a new decision on the manuscript may be presented to the corresponding author.

The manuscripts accepted for publication are forwarded to the article layout department. Minor changes to the manuscripts may be made, when necessary, to adapt them to BrJAC guidelines or to make them clearer in style, respecting the original content. The articles are sent to the authors for approval before publication. Once published online, a DOI number is assigned to the article.

Copyright

When submitting their manuscript for publication, the authors agree that the copyright will become the property of the Brazilian Journal of Analytical Chemistry, if and when accepted for publication. The submitting author declares in the Cover Letter, on behalf of all other co-authors, to consent to this transfer.

The copyright comprises exclusive rights of reproduction and distribution of the articles, including reprints, photographic reproductions, microfilms or any other reproductions similar in nature, including translations.

Final Considerations

Whatever the nature of the submitted manuscript, it must be original in terms of methodology, information, interpretation or criticism.

With regard to the contents of published articles and advertisements, the sole responsibility belongs to the respective authors and advertisers; the BrJAC, its editors, editorial board, editorial office and collaborators are fully exempt from any responsibility for the data, opinions or unfounded statements.