BrJAC

Brazilian Journal of Analytical Chemistry

an International Scientific Journal

Design of Experiments (DoE) in Pharmaceutical Applications

Interaction Between University and Industry

Design of Experiments (DoE) Application in Two Cases of Study in Pharmaceutical Industries

Romero Moreira Souza, Luiz Americo Verginio Bonamichi, Edenir Rodrigues Pereira-Filho

April – June 2024 Volume 11 Number 43

Welcome to the 21st ENQA and 9th CIAQA to be held at Belém do Pará, one of the most prominent cities in the Brazilian Amazon

From September 15th to 18th, 2024

Venue: Hangar Convention and Exhibition Center of the Amazon, in Belém do Pará, Brazil

ENQA is a rotating conference organized by the Analytical Chemistry Division of the Brazilian Chemical Society with over 40 years of history. It is the largest and most significant scientific conference in Analytical Chemistry in Brazil, and one of the main Chemistry events in Latin America. The 21st ENQA will be a defining moment in the conference's history, as it will be the first edition held in the Northern region of Brazil.

The 21st ENQA and 9th CIAQA will take place at Belém do Pará, Brazil, a metropolis surrounded by rivers and islands with a distinctive culture and a rich gastronomy primarily based on ingredients from the Amazon rainforest's diverse flora and fauna.

The ENQA/CIAQA 2024 has the theme **"Analytical Chemistry and its Contributions to the Development of a Sustainable Society"**. In recent years, Analytical Chemistry has been strengthening itself as a more environmentally recommendable science, encouraging actions and works that align with sustainable development. Thus, gathering knowledge that contributes to the assurance of products and environments and to the development of instrumentation and methods to address upcoming demands and challenges.

21º ENQA
9º CIAQA

Brazilian Journal of Analytical Chemistry

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Aims & Scope

BrJAC is a double-blind peer-reviewed research journal, dedicated to the diffusion of significant and original knowledge in all branches of Analytical Chemistry and Bioanalytical Chemistry. It is addressed to professionals involved in science, technology, and innovation projects at universities, research centers and in industry. The **BrJAC welcomes** the submission of research papers reporting studies devoted to new and significant analytical methodologies, putting in evidence the scientific novelty, impact of the research, and demonstrated analytical or bioanalytical applicability. BrJAC **strongly discourages** those simple applications of routine analytical methodologies, or the extension of these methods to new sample matrices, unless the proposal contains substantial novelty and unpublished data, clearly demonstrating advantages over existing ones.

BrJAC is a quarterly journal that publishes original, unpublished scientific articles, reviews and technical notes. In addition, it publishes interviews, points of view, letters, sponsor reports, and features related to analytical chemistry.

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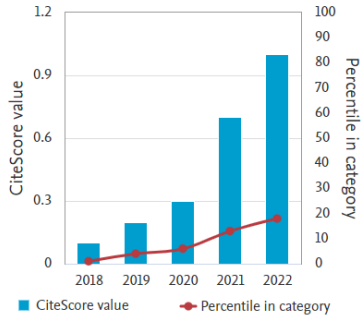

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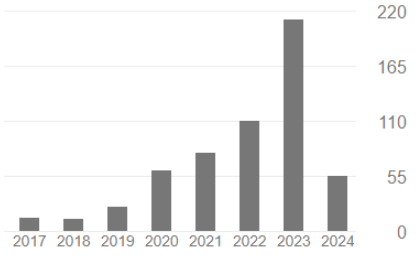

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BrJAC

EDITORIAL

Towards to Sustainability

Marco Aurélio Zezzi Arruda

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BrJAC Editor-in-Chief

The Horizon 2030 program of the United Nations presents a proposal for sustainable development and 17 goals for transforming our world.[1] Various actions and public policies are now striving to attain these objectives, with (bio)analytical chemistry, due to its nature, playing a fundamental role not only in industry but also in education and research.[2] Nowadays, within this concept, the appeal of a methodology continues to be sensitive, precise and accurate in terms of analytical chemistry but the analytical characteristics are also targeted towards the principles of environmental sustainability. Thus, in the literature, it is common to find that methods where the three Hs were used (i.e. High sample/reagent volumes, High risks and Hard work) are now being substituted by methods involving the three Rs (i.e. Reduce, Reuse and Recycle). It is easy to rationalize that the multitude of applications in different research areas (e.g. chemistry, human health, biology, environment, etc.) tend towards minimalist concepts that may involve all analytical sequences. Additionally, to attain the 'greenness' metrics of a method, different greenness assessment tools are available in the literature, such as GAPI or AGREE,[2] and metrology and chemometrics may help to attain this objective.

Within the context of sustainable development, this volume of the BrJAC is prone to a diversity of applications towards sustainability that involve methods employing small volumes, low-cost sensors, minimalist techniques and the importance of teaching metrology, among others. It is time to enjoy the reading of this issue and take into consideration the important aspects for the next application of our research work, which may involve environmental protection, circular economy and resource sustainability, the so-called sustainability triad (Environment, Economy and Society). Onwards to sustainability!

(1) United Nations. Dept. of Economic and Social Affairs. *Transforming our world: the 2030 Agenda for Sustainable Development*. Available at: https://sdgs.un.org/2030agenda [Accessed on March 15, 2024.].

(2) Płotka-Wasylka, J.; Mohamed, H. M.; Kurowska-Susdorf, A.; Dewani, R.; Fares, M. Y.; Andruch, V. Green analytical chemistry as an integral part of sustainable education development. *Curr. Opin. Green Sustainable Chem*. **2021**, *31*, 100508. http://dx.doi.org/10.1016/j.cogsc.2021.100508

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Marco Aurélio Zezzi Arruda, a Fellow of the Royal Society of Chemistry (FRSC), has a degree in Industrial Chemistry from the Methodist University of Piracicaba (1987), a Master's degree in 'Nuclear Energy in Agriculture' from the Center for Nuclear Energy in Agriculture at the University of São Paulo (1990), a Doctoral degree in 'Advanced Analytical Chemistry' from the University of Cordoba (1995) and a postdoctoral degree from the Center for Nuclear Energy in Agriculture (1995-1996). He is currently Full Professor at the Department of Analytical Chemistry of the Institute of Chemistry at the University of Campinas (IQ-Unicamp). He also coordinates the Sample Preparation, Spectrometry and Mechanization Group (GEPAM) and is a member of the advisory board of the Brazilian Institute of Science and Technology (INCT) for Bioanalytics.

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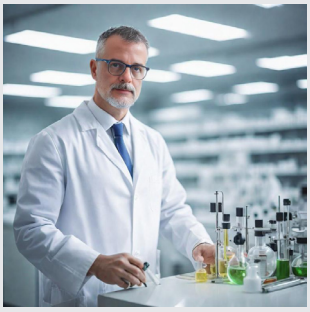
INTERVIEW



Professor José Luis Capelo Martinez, a researcher who believes that science and technology have a direct and tangible impact on human well-being kindly granted BrJAC an interview

J. L. Capelo Martinez, PhD, gets his bachelor's degree in chemistry by the University of Santiago de Compostela (Spain), his doctorate in Analytical Chemistry by University of Vigo, UVIGO (Spain, award to the best doctoral thesis 2002) and his Post-Doc from the Instituto Superior Técnico de Lisboa (Portugal). His academic career comprises assistant to staff and lecturer at the UVIGO; research fellow at the Chemistry Department of the New University of Lisbon, CD-FCT-UNL, research fellow at the CDUVIGO, and assistant professor at the CD-FCT- UNL. Currently he is Associate Professor at the CD-FCT-UNL. Dr. Capelo is co-head of the bioscopegroup (www.bioscopegroup.org) and his CV comprises (up to October 2023): 290 manuscripts; 250 congress communications (orals and posters); 23 projects; 2 Patents, 1 license agreement and 3 books (1 authored and 2 edited). He has chaired 65 international conferences and is presently involved in the direction of 7. He was a member of the advisory board of Talanta from 2006 to 2014 and is Editor in Chief of the on-line Journal JIOMICS (www.JIOMICS.com) since its creation in 2011. He is presently mentoring or co-mentoring a total of 3 doctoral theses, and he has mentored 3 post-doctoral grants, 17 doctoral grants, 9 masters and 8 final projects. His current research interest is devoted to developing new methodological approaches in personalised medicine using new proteomics approaches and unravelling bacterial resistance to antibiotics. He is a Fellow Member of the Royal Society of Chemistry, member of the American Chemical Society and member of the Portuguese Society of Chemistry. H index 45 (Scopus Scholar). 8300 Citations.
His skills include sampling and sample treatment for trace metals; metal speciation, proteomics, biomarker discovery; food chemistry; and development and validation of analytical procedures. Analytical techniques: HPLC-ICP-MS, ET-AAS, F-AAS, CVAAS, HG- AAS, HG-AFS, MALDI-TOF-MS/MS, RP-HPLC-ESI-IT-MS/MS. Teaching (theory and laboratory) in Analytical Biochemistry, Proteomics, and related disciplines.
Awards: Best 2002 Doctoral Thesis in Chemistry. University of Vigo. Spain. Rainbow Prize 2017.

BrJAC: How was your childhood?
Dr. Martinez: My childhood was marked by a challenging situation within my family. It was a period of turmoil and uncertainty, where I often grappled with complex emotions and circumstances beyond my control. Family issues cast a long shadow over those formative years, making it a time of profound introspection and self-discovery.

Amidst the chaos and adversity, I found solace and refuge in the realms of science and culture. These two pillars became my sanctuary, offering respite from the tumultuous environment surrounding me.

Cite: Professor José Luis Capelo Martinez, a researcher who believes that science and technology have a direct and tangible impact on human well-being kindly granted BrJAC an interview**.** *Braz. J. Anal. Chem.* 2024, *11* (43), pp 3-10. http://dx.doi.org/10.30744/brjac.2179-3425.interview.jlcmartinez

I discovered that delving into the world of knowledge provided a sense of purpose and direction, allowing me to channel my energies into something constructive and meaningful.

With its unending mysteries and the pursuit of understanding the natural world, science became a source of fascination and wonder. It provided me with a sense of order and logic, offering explanations for the phenomena I encountered in everyday life. Through books, experiments, and exploration, I embarked on a journey of intellectual curiosity that would shape my future aspirations.

"…culture offered me a window into diverse perspectives and experiences beyond my own. Literature, art, music, and history provided an escape into different worlds and eras, allowing me to broaden my horizons and cultivate empathy…"

On the other hand, culture offered me a window into diverse perspectives and experiences beyond my own. Literature, art, music, and history provided an escape into different worlds and eras, allowing me to broaden my horizons and cultivate empathy. Engaging with cultural expressions became a way to connect with humanity's collective wisdom and creativity.

While the family issues I faced during my childhood were undeniably challenging, they inadvertently propelled me toward the pursuit of knowledge and self-improvement. I learned that adversity can be a catalyst for growth and resilience, and it was during those difficult times, that I forged a deep-seated commitment to science and culture as vehicles for personal transformation.

In retrospect, my childhood struggles taught me that even in the darkest moments, there is always a beacon of light to be found in pursuing knowledge and appreciating human creativity. These experiences have shaped my character and fueled my passion for lifelong learning and a profound respect for the transformative power of science and culture.

BrJAC: What early influences encouraged you to study chemistry? Did you have any influencers, such as a teacher?

Dr. Martinez: I consider myself incredibly fortunate to have experienced a formative period in my life that was enriched by the guidance of a truly remarkable individual – a woman whose passion for science was both contagious and inspiring. From the ages of 15 to 18, during three consecutive years that were instrumental in shaping my academic journey, she imparted to me the wonders of physics and chemistry. This remarkable educator, her name was Cármen, driven by an unwavering dedication to her craft, ignited in me a deep curiosity and fascination for the world of science. Her teaching transcended the boundaries of textbooks and classroom walls; it was an immersive experience that brought abstract scientific concepts to life. With her guidance, I ventured into the realms of physics and chemistry, unraveling the mysteries of the universe at a level that I had never before imagined.

But the influence of great teachers extended beyond this singular mentor. I was fortunate to be part of an academic environment that boasted an excellent cohort of educators, all contributing to nurturing young minds. My educational journey unfolded at the best public institute in my hometown of Vigo (Santa Irene), where knowledge was valued and intellectual growth was nurtured.

Reflecting upon those transformative years, I have realised that my secondary school education was perhaps the most influential and pivotal phase of my life. It was a period when the seeds of curiosity were sown, my passion for science was ignited, and the foundations of lifelong learning were firmly laid.

Education at the secondary school level carries the potential to shape the course of one's life profoundly. During these critical years, we are not only introduced to the subject matter but also to the ways of thinking, problem-solving, and questioning that will accompany us throughout our academic and personal journeys. The teachers who guide us during this time play an important role in nurturing our intellect, curiosity, and ambition.

Looking back, I am deeply grateful for the incredible educators who crossed my path, their dedication, and their unwavering belief in the potential of their students. Their influence, combined with my passion for science and the nurturing environment of my school, propelled me in the right direction and set me on a path of intellectual discovery and growth.

As I continue my academic and professional journey, I carry with me the profound impact of those years and the invaluable lessons learned from exceptional educators. It is a testament to the enduring power of education, mentorship, and the pivotal role that teachers can play in shaping the future of their students.

BrJAC: How was the beginning of your career in chemistry?
Dr. Martinez: The beginning of my career in the field of chemistry was marked by two remarkable mentors who left an indelible mark on my academic journey – Professor Carlos Bendicho, my doctoral mentor, and Professor Ana Mota, my Postdoctoral mentor. Their unwavering guidance and support were instrumental in shaping my path and instilling in me a profound passion for applied chemical analysis.

Professor Carlos Bendicho (University of Vigo, Spain), my esteemed doctoral mentor, was not only a source of knowledge but also a beacon of inspiration. Under his tutelage, I delved into the intricate world of chemistry, exploring its depths and applications. His mentorship went far beyond the confines of the laboratory, as he encouraged me to think critically, to question assumptions, and to approach scientific challenges with creativity and innovation. Professor Bendicho's dedication to his students and his commitment to fostering a love for chemistry were evident in his tireless efforts to nurture our intellectual growth. His mentorship equipped me with the necessary skills and ignited a deep-seated passion for the field.

After completing my doctoral studies, I had the privilege of working alongside Professor Ana Mota during my Postdoctoral research (Instituto Superior Técnico, Portugal). Professor Mota's expertise and guidance further enriched my understanding of applied chemical analysis. Her mentorship was characterized by an unwavering commitment to excellence, a meticulous approach to research, and a genuine enthusiasm for scientific discovery. Under her mentorship, I honed my research skills, delving into the intricacies of advanced analytical techniques and their real-world applications.

Professor Carlos Bendicho and Professor Ana Mota imparted valuable knowledge and served as role models of dedication and passion for their respective fields. Their mentorship extended beyond the laboratory, as they encouraged me to embrace challenges, persevere in the face of obstacles, and continuously strive for excellence. Their unwavering support and belief in my potential instilled in me the confidence to pursue a career in applied chemical analysis with zeal and determination.

BrJAC: What has changed in your profile, ambitions, and performance since the time you started your career?
Dr. Martinez: Throughout my journey as a researcher, I have not only witnessed growth in my academic and professional endeavours but, more importantly, in my development as a human being. This evolution has led me to redirect my research efforts towards areas where intellect and knowledge can be harnessed to make a profound impact on saving lives and improving the human condition.

"...research is not just an academic pursuit but a powerful tool for addressing pressing societal issues. It's a commitment to using my abilities to contribute to the greater good, to extend a helping hand to those in need, and to make a meaningful difference in the lives of individuals and communities...".

The quest for knowledge has been a constant driving force as a researcher. I have delved deep into various academic domains, expanded my expertise, and contributed to the scientific community. However, beyond the pursuit of academic excellence, I have come to realize that research holds the potential to bring about real-world change, particularly in the realm of healthcare and life sciences. Thus, my journey has led me to refocus my research on areas where advancements in science and technology have a direct and tangible impact on human well-being. Whether it's developing innovative medical treatments, creating sustainable solutions to global health challenges, or pioneering breakthroughs in biochemistry, my goal is to apply my intellect and skills to save lives and enhance the quality of life for people worldwide.

This transition reflects a deeper understanding of the responsibility that comes with knowledge and expertise. It's a recognition that research is not just an academic pursuit but a powerful tool for addressing

pressing societal issues. It's a commitment to using my abilities to contribute to the greater good, to extend a helping hand to those in need, and to make a meaningful difference in the lives of individuals and communities.

In conclusion, my growth as a researcher has been intrinsically linked to my growth as a human being. I have come to appreciate that research is not merely about expanding the boundaries of knowledge; it's about applying that knowledge for the betterment of humanity. Moving forward, I am dedicated to channeling my intellect, skills, and passion into research endeavors that have the potential to save lives and create a brighter, healthier future for all.

BrJAC: Could you comment briefly on the recent evolution of analytical chemistry, considering your contributions?

Dr. Martinez: Analytical chemistry, a field known for its precision and innovation, is poised to profoundly impact the world of medicine through a groundbreaking concept known as "prescriptomics." Coined by a visionary group of researchers, among whom I have the privilege to participate, this revolutionary approach is set to transform the landscape of medical diagnostics and treatment.

Applying cutting-edge analytical techniques, particularly mass spectrometry, at the heart of this transformation to analyse urine and blood samples. Mass spectrometry, with its unparalleled ability to provide detailed molecular information, is rapidly advancing in its capacity to revolutionise medical diagnostics.

"…imagine a future where artificial intelligence (AI) seamlessly integrates with mass spectrometry-based analysis. In this not-so-distant reality, AI algorithms can rapidly process vast amounts of data from patient samples and generate detailed reports for physicians…"

The promise of prescriptomics lies in its potential to supersede conventional diagnostic methods, including enzyme-linked immunosorbent assays (ELISAs). With the precision and sensitivity of mass spectrometry, prescriptomics can offer a comprehensive analysis of biological samples, yielding an intricate molecular profile that extends far beyond what traditional methods can provide.

Imagine a future where artificial intelligence (AI) seamlessly integrates with mass spectrometry-based analysis. In this not-so-distant reality, AI algorithms can rapidly process vast amounts of data from patient samples and generate detailed reports for physicians. These reports would not only reveal the current health status of individuals but also prescribe tailored treatment approaches with incredible precision.

Prescriptomics holds the potential to revolutionise patient care by providing healthcare professionals with real-time insights into each patient's unique molecular makeup. This information allows for highly precised personalised treatment plans, minimising the risk of adverse reactions and optimising therapeutic outcomes.

The impact of prescriptomics extends beyond diagnosis and treatment. It has the potential to reshape our approach to medicine, ushering in an era of proactive healthcare. Physicians will have the tools to detect diseases at their earliest stages, intervene before symptoms manifest, and even prevent illnesses altogether.

In conclusion, prescriptomics, driven by the power of analytical chemistry and mass spectrometry, promises to be a game-changer in medicine. It heralds a future where the analysis of urine and blood samples will not only diagnose conditions but also prescribe precise treatment strategies, ultimately advancing the goal of improving patient health and well-being.

BrJAC: What are your lines of research? You have published many scientific papers. Would you highlight any?

Dr. Martinez: Throughout my research journey, I've traversed a diverse landscape of analytical techniques, delving into various facets of science. I have explored many analytical methodologies, from atomic absorption spectrometry to mass spectrometry, from the luminescence of molecules to the intricacies of

chromatography. Today, my research is strategically focused on several compelling and impactful areas, each with unique significance in science and medicine. Recent publications in prestigious journals, such as our work featured in Nature Medical Communications, have showcased the innovative applications of our research in this domain.

One of the primary directions that has captured my attention revolves around the utilization of nanoparticles as powerful tools in medicine. These minuscule structures hold immense potential for applications in both diagnostics and therapy. Whether it's harnessing nanoparticles for targeted drug delivery, enhancing medical imaging, or combating antibiotic resistance, these tiny agents are poised to make significant contributions to healthcare.

In the realm of personalized medicine, I've been delving into the analysis of personalized proteomes. This cutting-edge approach involves deciphering the intricate web of proteins unique to each individual. By unraveling these proteomic profiles, we gain invaluable insights into an individual's health, paving the way for precision medicine. The ability to tailor medical treatments and interventions based on a patient's specific proteomic signature represents a paradigm shift in healthcare that holds great promise for improving patient outcomes.

My research portfolio has also expanded to include the analysis of archaeological and forensic proteomes, a relatively recent endeavor. This exciting line of inquiry has the potential to unlock secrets from the past and provide essential tools for modern forensic investigations.

Moreover, our contributions to the field of analytical chemistry have been notable. We've explored novel techniques, such as applying ultrasound to expedite proteome digestions with exceptional precision. These advancements enhance the speed and efficiency of proteomic analysis and contribute to the broader landscape of analytical chemistry.

In addition to these main research lines, I'm proud of our achievements in nanoparticle synthesis for biomarker discovery in diseases like myeloma and lymphomas. Our research has shed light on groundbreaking early disease detection and monitoring methods, potentially revolutionising diagnostic approaches.

Furthermore, our investigations into bacterial resistance have unveiled crucial insights. We've documented the spread of resistance to remote locations, even in areas with minimal human presence. This research underscores the urgency of addressing antibiotic resistance on a global scale and highlights the interconnectedness of human health and the environment.

As I reflect on my journey through these diverse research avenues, I am reminded of the ever-evolving nature of science and the limitless possibilities it offers. Each discovery, paper, and innovation has contributed to our collective understanding of the world and our quest to impact human health and well-being positively. In pursuing knowledge and improving society, my research endeavours continue to evolve, guided by the spirit of curiosity and discovery.

BrJAC: What is your opinion about Brazil's current chemistry research progress? What are the recent advances and challenges in scientific research in Brazil?

Dr. Martinez: Brazil has a rich history in chemistry research and has made significant contributions to various fields, including organic and natural product chemistry, materials science, and environmental chemistry. Brazilian universities and institutions have produced high-quality research and actively participated in international scientific collaborations.

The Brazilian government has also invested in scientific research through agencies like the National Council for Scientific and Technological Development (CNPq) and the Coordination for the Improvement of Higher Education Personnel (CAPES). These agencies provide funding and support for research projects, scholarships for students and researchers, and international collaboration opportunities.

This has led to one fact: from the best 10 Iberoamerican universities, 6 are from Brazil, the first is the University of São Paulo and the second one the University of Campinas.

However, challenges exist in the Brazilian research landscape, including funding constraints, infrastructure limitations, and administrative hurdles. These challenges can impact the progress and sustainability of research initiatives.

Something also worries us in Europe: the influence religion (evangelism) is gaining in science. The intelligent design, a terrible misunderstanding create by religious interest, is risking Brazilian science credit.

BrJAC: For you, what have been the most important recent achievements in analytical chemistry research? What are the landmarks? What has changed in this scenario with the COVID-19 pandemic?
Dr. Martinez: Prescriptomics is the most important one. Driven by analytical chemistry, in less than five years, artificial intelligence will be able to diagnose a disease, follow it up and check if the treatment is working. It will, in many instances, replace the physician.

Analytical chemistry is a diverse field with continuous advancements. Some recent important achievements and landmarks include advancements in mass spectrometry. Mass spectrometry techniques have become more powerful and accessible. High-resolution MS, tandem MS (MS/MS), and ion mobility MS have enabled researchers to detect and identify compounds at previously unimaginable levels of sensitivity and specificity.

Analytical chemistry has also made significant progress in the analysis of single cells, allowing for a better understanding of cellular heterogeneity and its role in various diseases, including cancer. Microfluidic devices and lab-on-a-chip technologies have advanced, making it possible to conduct complex analytical experiments on a miniaturized scale. These devices are being used for applications ranging from medical diagnostics to environmental monitoring.

Integration of different “omics” technologies like genomics, proteomics, and metabolomics has led to a more comprehensive understanding of biological systems, with implications for personalized medicine and drug discovery. The use of AI and machine learning in data analysis has become increasingly important, helping researchers make sense of the vast amount of data generated by analytical techniques.

There’s also a growing emphasis on environmentally friendly analytical techniques that minimize waste generation and use fewer hazardous reagents, known as Green Analytical Chemistry. The COVID-19 pandemic accelerated the development of diagnostic tests and the deployment of analytical methods for monitoring the virus. It highlighted the importance of rapid and accurate analytical techniques in public health.

Analytical chemistry played a crucial role in the development and quality control of COVID- 19 vaccines, ensuring their safety and efficacy. Furthermore, analytical chemistry continues to contribute to environmental monitoring, particularly in the detection of pollutants and the assessment of their impact on ecosystems.

These achievements underscore the ever-evolving nature of analytical chemistry and its impact on various scientific disciplines and industries. The COVID-19 pandemic has highlighted the critical role of analytical chemistry in public health and has accelerated the adoption of new technologies and methods in response to global health challenges.

BrJAC: What is the importance of awards for the development of science and new technologies?
Dr. Martinez: Awards play a pivotal role in the development of science and technology. They serve as a means of recognizing and motivating exceptional achievements, inspiring further innovation in various fields. Additionally, these accolades often come with valuable financial support, providing researchers with the necessary resources to advance their work. Furthermore, awards facilitate knowledge sharing and collaboration within the scientific community, fostering a spirit of cooperation that accelerates progress. Moreover, they help raise public awareness about significant advancements, garnering increased support and interest in science and technology. Lastly, these honors significantly boost the careers of researchers, attracting top talent and investment to further drive innovation and discovery.

BrJAC: For you, what is the importance of the national funding agencies for the scientific development of Brazil?
Dr. Martinez: National funding agencies play a crucial role in the scientific development of Brazil. They provide financial support for research projects, enabling scientists and researchers to conduct essential studies, experiments, and investigations across various disciplines. This funding is instrumental in advancing knowledge, promoting innovation, and addressing critical issues facing the country. Additionally, these agencies often foster international collaboration, allowing Brazilian researchers to connect with their global counterparts and contribute to the global scientific community. Furthermore, funding agencies help build research infrastructure and support the training of future generations of scientists, ensuring a sustainable and vibrant scientific ecosystem in Brazil.

BrJAC: At the moment, the situation for scientific research in Brazil is one of decreasing investment. How do you see this situation, and what would you say to young researchers?
Dr. Martinez: The decreasing investment in scientific research in Brazil is undoubtedly a concerning trend. It hampers the country's ability to remain competitive in the global scientific arena, hinders innovation, and affects the growth of knowledge-based industries. To young researchers, I would say, "Stay resilient and committed to your passion for science." Despite the challenges, your work can make a difference. Seek international collaborations, explore funding opportunities beyond national agencies, and advocate for the importance of science and research in your community and on a larger scale. Your dedication and contributions can help shape a brighter future for Brazilian science.

BrJAC: What advice would you give to a young scientist who wants to pursue a career in chemistry?
Dr. Martinez: I would offer advice to a young scientist aspiring to pursue a career in chemistry is to build a strong educational foundation by focusing on gaining a solid understanding of chemistry fundamentals during the academic years. This foundational knowledge will prove to be crucial throughout the career. Additionally, it is essential to develop a genuine passion for research within the field of chemistry. Find a specific area that truly interests you, and strive to become an expert in that particular domain. This passion for your research will serve as a driving force, motivating you to explore and innovate.

Another crucial aspect is seeking mentorship. Collaborating with experienced researchers who can provide guidance, support, and valuable insights is invaluable. Mentorship can significantly accelerate your career development and help you avoid common pitfalls.

Moreover, embrace continuous learning because chemistry is an ever-evolving field with new discoveries and technologies emerging regularly. Stay updated with the latest advancements by attending conferences, participating in workshops, and engaging in lifelong learning.

Building a strong network is equally important. Establish connections with your peers, mentors, and colleagues in the field of chemistry. Networking can open doors to collaborative opportunities, potential funding sources, and overall career growth.

Effective communication skills are also essential. Being able to articulate your research findings, both in writing and orally, is crucial. Clear communication enhances your ability to share knowledge, collaborate with others, and disseminate your research effectively.

Persistence and resilience are traits that will serve you well. Scientific research often involves setbacks, challenges, and failures. Don't be discouraged by these obstacles. Instead, view them as opportunities to learn and grow. Consider interdisciplinary collaborations, as many groundbreaking discoveries occur at the intersection of different scientific disciplines.

Pursuing funding opportunities is a practical step. Seek grants, scholarships, and funding sources to support your research projects. Funding is essential for conducting experiments, acquiring specialized equipment, and advancing your work.

Lastly, it is imperative to stay ethical and responsible in your research. Adhering to the highest ethical standards is vital for the credibility and integrity of scientific work.

In summary, a career in chemistry can be incredibly rewarding, contributing to solving real-world problems and advancing our understanding of the natural world. To excel in this field, maintain your curiosity, dedication, and passion for your work. With these qualities and the advice mentioned, you'll be well on your way to making meaningful contributions to the world of chemistry.

BrJAC: For what would you like to be remembered?
Dr. Martinez: For my enduring zest for life, as it is through life's journey that I continually encounter the presence of God.

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POINT OF VIEW

Teaching Experimental Instrumental Analytical Chemistry

Are we forming professionals, training operators or illuding students (and ourselves)?

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The term “science” refers to “any system of knowledge that is concerned with the physical world and its phenomena and that entails unbiased observations and systematic experimentation”.[1] Therefore, laboratory courses are almost universally considered an integral and mandatory part of instruction on science and technology,[2] whatever the field or area of specialization. Consequently, undergraduate chemistry courses (and correlated specialties such as pharmaceutical sciences, biochemistry and some branches of engineering) always include practical disciplines, one of which is instrumental analytical chemistry. Despite being essential for these courses and part of the everyday routine of the faculty affiliated with analytical chemistry departments or programs, several aspects are far from being consensus among lecturers, students and the other people involved. Topics related to course syllabus, general approaches on the implementation and teaching of specific analytical techniques and experiments, their integration with other disciplines, and much more, are a matter of constant discussion and debate.

One aspect to be considered is the considerable change in the profile of students that has occurred in recent years. The teaching tools and didactic approaches that university professors and instructors aged > 40 years experienced in their own training are generally not suitable for the present-day undergraduate audience. With former generations, the usual sources of information were printed books and similar materials available in libraries, whereas students today have a greater affinity for consulting online sources,[3] which are not always reliable and often return a huge number of search results that require careful evaluation to select which information is relevant and/or reliable. Of course, the facility with which online sources of information are manipulated by our present pupils also has some important advantages: in particular, the near-instantaneous speed of information collection and the virtual accessibility to databases from anywhere on the planet. In addition, until the turn of the millennium, university students were more used to long lectures and experimental classes with relatively complex procedures that often took hours of careful manipulation and numerous laboratory operations (many of them repetitive and tedious). Specifically in the case of practical classes, many of today’s students are relatively unaccustomed to experiments that require more than moderate manual skills (at least when compared to their colleagues of previous generations), which may also imply difficulty in organizing their time in the laboratory. Perhaps one of the biggest mistakes we make with our current students is that we consider them ill-prepared or less capable than our generation and attribute their difficulties to this simplistic, crude verdict – when perhaps the biggest problem is that, as educators, we are failing to cope with the rapid evolution in the characteristics and profiles of these young people. The type of change we need so that we can offer practical courses on instrumental analytical chemistry that are more appropriate and better suited for our students is not

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easy because, in addition to the demand that we change the vision of teaching and paradigms that we have considered as absolute since we entered our academic career, we are also confronted with practical reasons that make this type of change difficult. Laboratory classes demand a huge endeavor from technical and instructional staff and teaching assistants; furthermore, they are comparatively expensive, requiring space, fragile glassware, acquisition and maintenance of instruments, proper disposal of consumables and waste, as well as expenses on faculty salaries.[4] The revision of curricula and experimental procedures would impose additional pressure on the workload of the personnel involved and also on the reduced budget availability typical of most public and private universities and colleges (whether in Brazil or anywhere else on the planet).

Although there is no easy, direct and universal solution to the problems mentioned above, some alternatives can be tested and adopted with relative ease in most higher education courses on instrumental analytical chemistry. The workload of the experiments can be reduced, supplementing them or eventually even replacing some with practices carried out in virtual environments, which had an unexpected but necessary boost during the recent COVID-19 pandemic.[5] Several paid or free-to-use tools have been described for this purpose. For instance, Shallice *et al*.[6] describe a downloadable high-performance liquid chromatography simulator that emulates a basic automated liquid chromatography system capable of binary gradient operation. Similar software emulating other instrumental techniques exist, such as UV-Vis's spectrometry, electroanalytical techniques, etc., as stand-alone applications, full online resources or even as Excel spreadsheets. In addition to simulated experiments using virtual analytical instruments, other resources that can be used as a supplement to practical instrumental analysis classes are online video libraries, which have also proliferated after the recent period of restriction on face-to-face activities. A well-known example is the collection of the Royal Chemical Society,[7] which offers videos demonstrating basic principles and practical aspects of various instrumental techniques (from gas and liquid chromatography to nuclear magnetic resonance spectroscopy).

In addition to possible inadequacies due to changes in the profile of typical students currently entering higher education courses in chemistry and related sciences, the evolution of analytical instrumentation, its scope of application and the practical demands imposed on analytical methods that future professionals will apply also pressure us to make changes in the programs of experimental disciplines of instrumental analytical chemistry and in the way we teach the associated techniques. Until the turn of the millennium, the typical program of experimental disciplines of instrumental analytical chemistry in most of the curricula in Brazil and many other countries comprised sequences of isolated experiments using analytical techniques such as gas or liquid chromatography, UV-Vis absorption spectrometry, atomic emission/emission spectrophotometry, etc., with samples and procedures that required relatively simple and quick preparation (usually only sample dissolution, decomposition or extraction). However, due to the natural evolution of analytical instrumentation, the equipment that future professionals will typically find in academic and industrial research laboratories after their impending graduation is very different from that previously employed in the teaching laboratories where their instructors learned analytical chemistry (typically, much simpler equipment that required careful attention and reasonable practical skills from users and operators). The contemporary analytical instruments are much less transparent to the user in their operation; however, as a rule, they incorporate extensive automation and/or mechanization resources, complete control of operation by software and greater operational robustness. From a didactic point of view, modern equipment certainly does not provide students with the same understanding and insight of the basic operational principles of the associated techniques as the instruments of previous generations. However, considering the typical profile of today's students, it possibly would not be advantageous to use those simpler analytical platforms exhaustively merely for didactic purposes. For example, until the turn of the millennium, one of the main demands for students in gas chromatography didactic experiments was to improve and master the manual injection of samples using micro syringes. Today, this didactic approach seems to be preposterous, considering that chromatographs without automatic sample injection are becoming increasingly rare in industrial and even in academic environments. The result is that students are

sometimes subjected to tedious laboratory sessions and often completely lose their focus on what would be fundamental in that practical class. In addition, the present demand is increasingly for professionals to be trained to interpret data and propose solutions based on the information gained and not to act as mere operators of laboratory equipment. Thus, it seems to us that we should increasingly design experiments for undergraduate students that incorporate all stages of the analytical process, with particular attention to sampling and sample preparation (taking advantage of features of modern instruments that allow processing more samples in less time and whose operation is less dependent of user ability and manual dexterity) as well as the interpretation and understanding of the analytical data produced.

Regarding the above-mentioned focus on the interpretation and understanding of analytical data, it is interesting to note that in 2004, when the Analytical Chemistry Division of the Federation of European Chemical Societies (FECS) established the curricular guidelines to be observed in the analytical chemistry disciplines taught in universities of the European Community (the so-called "Eurocurriculum II"),[8] four basic pillars of education in analytical chemistry were defined: Spectroscopy; Chromatography; Chemical Sensors; and Chemometrics and Computer-Based Analytical Chemistry. Thus, it was recognized that in modern analytical chemistry the processing and interpretation of data, as well as its transformation into useful information about the chemical systems studied, is on a par with the three traditional subdivisions of analytical chemistry (spectroscopic and spectrometric techniques, chromatographic techniques and electroanalytical techniques). Although it is important to emphasize that these considerations apply to courses in the European Community, they can be contemplated in the training of chemists anywhere else on the world. Also, they reinforce the indication that the education of analytical chemists should place an increasing emphasis on aspects related to information generation/manipulation and interpretation and not merely on the direct operational aspects of analytical methods and protocols already established.

The discussion so far does not pretend to cover all the relevant points whose evaluation would perhaps be necessary for a comprehensive and exhaustive debate of the current status of college/University courses on instrumental analytical chemistry, as well as the possible alternatives and paths to follow. In addition, this text of course echoes the author's personal opinions and idiosyncrasies, without any pretense at being an absolute expression of the truth (if indeed one exists on this matter). In fact, any discussion of these topics is, by their nature, highly controversial and heavily influenced by the background and area of expertise of those involved. However, we have the firm conviction that the community needs to continuously discuss the education and professional development in analytical chemistry, with emphasis on areas where changes can occur very quickly, always keeping an open mind and a willingness to review supposedly untouchable concepts (and, of course, remembering that its focus should always be on the students).

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LETTER

Electrochemical Biosensors for the Detection of Viruses: Must-Have Products or Just Science for Publication?

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The SARS-CoV-2 pandemic has brought significant light to the urgent need for rapid, precise, and low-cost diagnosis tools. The scientific community has responded as quickly, overflowing the literature with papers describing interesting biosensors for aiding in the diagnosis of COVID-19.[1,2] However, almost none of them, mainly the electrochemical ones have reached the market or never will, with only a few traditional formats used in the daily combat of the virus, including ELISA (enzyme-linked immunosorbent assay), lateral flow assays, and, mainly, PCR (polymerase chain reaction).

The drawbacks of PCR and the need for improved analytical tools

Although PCR-based methods are currently the gold standard for detecting viruses worldwide, these still present various drawbacks. Usually, the commercial detection of viruses (such as SARS-CoV-2) uses the combination of standard PCR (or RT-PCR) and gel electrophoresis due to its sensitivity, reliability, and low price (if compared to other PCR-based methods such as real-time PCR). This approach relies, mainly, on the use of a standard thermal cycler and an electrophoresis tank by a specialized worker. While electrophoresis tanks can be quite affordable, with some of them costing a few hundred dollars,[3] even simple thermal cyclers cost around 5,000 USD[4] – significantly enhancing the investment required for testing. Furthermore, the complete analysis of a sample is slow and can take up to six hours to complete, which prevents an effective sanitary barrier at borders and crowded events, for example. The samples need to be transported to the lab, as no reliable portable PCR and gel electrophoresis equipment are available. The results commonly take from two to five days to be generated – an extremely long delay when considering that these can seriously influence the health of a patient and the spread of the virus. Last, standard PCR does not provide quantitative information – which is vital in some cases to aid in diagnosing the severity of an infection.

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Techniques derived from PCR (such as qPCR, for example), on the other hand, can provide quantitative and more rapid results, but are also more expensive and still require sample transportation. Equipment for performing qPCR ranges from 15,000 USD to 90,000 USD[4] and the use of specific reaction kits containing fluorescent markers also corresponds to a significant increase in analysis costs.

Other commercially available methods for the detection of viruses, ELISA and lateral flow assays, also present significant drawbacks. While ELISA is time demanding (6 h) and requires specialized professionals and equipment to be adequately performed, some lateral flow assays present results with low precision,[5,6] being useful for massive triages in the case of COVID-19, for example.

Although presenting such limitations, PCR-based techniques are still the gold standard for the detection of viruses. This is probably due to its sensitive and well-established features, being widespread along with many medical and research centers around the globe. Furthermore, the development of PCR-based diagnosis kits in urgent scenarios, such as the one imposed by SARS-CoV-2, is straightforward and allows rapid responses from health organizations and governments. The technique can also provide low limits of detection (LOD), with a gold standard RT-PCR assay for COVID-19 presenting a LOD of ~100 copies of viral RNA per mL of transport media, for example. It is important to mention, however, that the LOD of currently approved assays for COVID-19 varies over 10,000-fold, which will generate immense false-negative rates.[7]

How can biosensors improve the diagnosis of viral diseases?

Biosensors present interesting properties to overcome some of the drawbacks presented by PCR. Although thousands of papers have been published in the last years based on the detection of several diseases, almost all the material published has focused on the formation of human resources and not on the market (Table I). There are few discussions in the electrochemical meetings and a tremendous demand to produce new selling and profitable devices for the environment, food, medical, and forensic analyses. In this context, portable potentiostats are commonly available on the market at prices that range from a few thousand dollars (2,000 – 3,000 USD) for full desktop equipment[8] to a few hundred dollars for equipment devoted to a single analysis. There is also significant research interest in the development of portable, miniaturized, and low-cost potentiostat, as highlighted by some articles published in recent years.[9–11] Colorimetric biosensors, in turn, can rely on responses readable with the naked eye or using widespread smartphones. The use of smartphones can also contribute to compiling results and acquiring additional information such as patient location and data. Therefore, if compared to PCR-based techniques, instrumentation costs are decreased while its portability allows point-of-care analysis, significantly increasing the accessibility to tests in remote areas. Analysis time is also greatly diminished as results can be obtained in only a few minutes. Both of these features are of extreme importance when considering healthcare applications that commonly require quick or real-time responses. Furthermore, immunosensors do not require previous sample preparation even when using complex biological fluids, decreasing analysis costs and making it even more rapid. Last, biosensors can be easy to use, usually requiring lower previous preparation from the operator if compared to traditional techniques (Figure 1).

Table I. Examples of recent (2021-2024) publications of electrochemical biosensors for the detection of viruses and their characteristics

Virus	Description	Detection range	Validation	Reference
Herpes simplex virus type 2 (HSV-2)	Electrodes were modified with human cellular receptor nectin-1 and electrochemical impedance spectroscopy (EIS) was applied for the determinations	1 to 10^5 HSV-2 (PFU/mL)	Tested in biological matrix	14

(continues on the next page)

Table I. Examples of recent (2021-2024) publications of electrochemical biosensors for the detection of viruses and their characteristics (continuation)

Virus	Description	Detection range	Validation	Reference
SARS-CoV-2	Gold electrodes were modified with nanochannels based on polystyrene (PS) containing bioreceptors. The blockage of the nanochannels with viruses hampers the diffusion of a redox probe.	1 to 10^8 particles/mL	Tested in biological matrix	15
Hepatitis C vírus (HCV)	Electrodes were modified with fragments of the cell receptor CD81 to determine HCV E2 envelope protein	0.1 to 5 µg/mL of hepatitis C virus-mimetic particles	Tested in synthetic plasma	16
Enterovirus 71 (EV71)	Determination based on the aggregation of AgNPs promoted by the incorporation of EV71	10^{-4} to 10 EV71 (PFU/mL)	Tested in biological matrix	17
SARS-CoV-2	Inkjet-printed nanostructured gold electrodes promote the multiplexed detection of SARS-CoV-2 ORF1ab and N genes with the use of an also inkjet-printed battery-free near-field communication (NFC) potentiostat	10^{-10} to 10^{-5} mol/L of ORF1ab and N genes	Tested in buffer	18

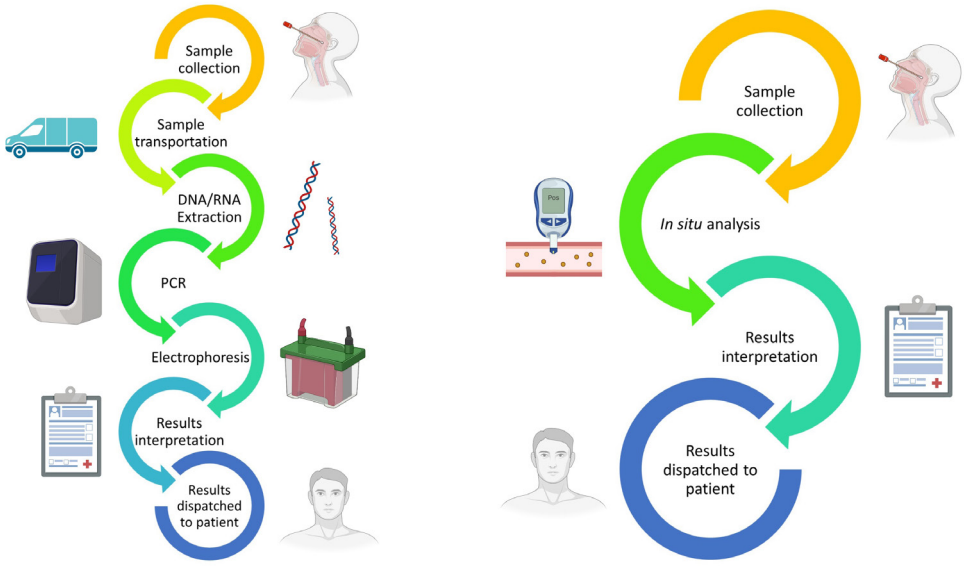

Figure 1. Steps typically involved in a molecular method (e.g., PCR) (left) and the simplicity of a biosensor (right) analysis of biological samples. Created with BioRender.com.

Biosensors can also be readily developed in urgent scenarios, as proven with COVID-19. Numerous examples of electrochemical, colorimetric, and mass-sensitive devices for aiding in the diagnosis of the disease were described in the literature only a few months after the start of the pandemic event.[1,12,13] Devices are commonly validated in biological samples, providing precise results in a rapid, cheap, and simple manner. So, a relevant question is, why are most of these devices still out of the consumers' reach?

Why are biosensors still out of the market?

Despite their advantages, electrochemical biosensors are rarely seen in the market except for particular examples such as the glucometer and a few lateral flow assays. In our opinion, diverse aspects contribute to the existing barrier between publication and commercialization.

1) The biorecognition layer, commonly composed of biomolecules such as antibodies, genetic material, and enzymes, may present stability issues regarding storage, temperature, and chemical conditions. The organization and structural integrity of such elements are essential for the adequate functioning of the devices, which is still a challenge to the field. This aspect mainly influences the shelflife of biosensors, hampering its commerciality.
2) To improve the analytical performance of devices, many of them use complex constructs or high-cost materials, such as nanoparticles, rare elements, or liquid crystals. While the complex constructs might bring a significant challenge for batch manufacturing, increased prices might favor the use of traditional techniques such as immunoassays.
3) For industries to be interested in the fabrication of biosensors, different barriers to market entrance must be transcended and the final product must be profitable. For example, a clear market demand must exist and regulatory agencies must approve the use of the device. Furthermore, the manufacturing must be adequate for low-cost batch production and the adaptation of the machinery or new processes should present cost-efficacy and availability.
4) The validation studies and the development of prototypes should be more discussed for the scientific community and should be a link between the industries and the academy around the world. Among other parameters, the accuracy of the developed tests, for example, needs to be carefully assessed in different scenarios, being compared to well-established, validated techniques to ensure that customers will get precise results.
5) Last, although biosensors present an adequate performance under controlled environments, they commonly present limitations when applied to raw biological samples. The reasons for that are diverse, including the presence of interfering species, biofouling, the formation of complexes, or the nature of the analyte itself. Therefore, the direct application of samples is still a problem.[12]

Recent advancements, however, present great potential to address these challenges. The use of 3-D printed electrodes, for example, might decrease the cost of electrode production while increasing the accessibility of devices, especially in low-resource settings.[19] Using new assembles and labels, in turn, presents the potential to increase the stability, sensitivity, and reliability of biosensors. To improve the biorecognition layer stability, the use of innovative receptors such as biomimetic enzymes, molecularly imprinted membranes, and DNA origami can be of great value,[20-22] while the development of flexible devices can improve the range of their application – including wearables, for example.[23] Last, the combination of artificial intelligence for data analysis and the Internet of Things is crucial for the automation of the procedure and can improve the analytical techniques beyond human potential.[24,25]

It must be clear for analytical chemists that, for achieving these new grounds, innovation and entrepreneurship are essential, stimulating the creation of startups, spin-offs and collaborations with existing companies.[26] Therefore, biosensors are beyond the publication hype and are an inspiration for the future, moving constantly closer to being accessible to the population, being, undoubtedly, not meant to be limited to journal pages.[12]

Conflicts of interest

The authors declare no conflict of interest.

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REVIEW

The Importance of Teaching Metrology for Chemistry Undergraduate Courses

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PITFALLS THAT SHOULD BE ADDRESSED IN CHEMISTRY METROLOGY COURSE SYLLABUS

Are the variances always homogeneous?

Should every analytical calibration curve (ACC) have 1st degree polynomial (straight-line) adjustment?

Where is the most precise region of an analytical calibration curve (ACC)?

Must the measurement uncertainty arising from sampling in chemical analyses be neglected?

Is the laboratory precision the measurement uncertainty of the analytical result?

The Chemistry undergraduate courses, in general, aim to train professionals for research, teaching and activities in industry. In all these areas, the statistical treatment of data supported by metrology disciplines has been a decisive boost to consolidate conclusions in Analytical Chemistry. The main objective of this work was to stimulate the teaching of the metrology in the chemistry undergraduate courses in Brazil and Portugal. In this work we present some concepts and practical cases of metrology and statistics, which together guarantee the suitability of analytical measurement results for specific purposes. Knowledge of these concepts is essential for the chemist to act effectively and responsibly, avoiding some pitfalls when processing data in Analytical Chemistry. A supplementary material with Excel spreadsheets is available, containing the statistical treatment of some examples and complementing the discussions in this work. A survey is presented among the most important universities in Brazil and Portugal, from which we can see the lack of teaching

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these subjects in undergraduate chemistry courses. For this reason, this work recommends a syllabus for creating chemical metrology disciplines in undergraduate chemistry courses. To subsidize this syllabus, an extensive, but not exhaustive, bibliographical references is indicated.

Keywords: homogeneity of variances, analytical calibration curve fit, sampling uncertainty, metrology course syllabus, compliance assessment, chemical metrology

INTRODUCTION

"Metrology", the science of measurement and its applications,[1] develops the knowledge to guarantee that the results of a measurement enable decisions that are as assertive as possible in relation to its intended use; this is what is called the "fitness for purpose" or "fitness to intended use"[1-4] of the "measurement results".[1] In the recent past, analytical chemists were aware of mainly the "measurement trueness"[1] related to the "systematic error"[1] and its "bias"[1] estimation to enable the analytical result "correction".[1] More recently, the awareness with the "measurement precision"[1] as "repeatability"[1] or "reproducibility"[1] or "intermediate precision"[1] and especially with the "measurement uncertainty"[1] as well as with the "metrological traceability",[1] "metrological comparability"[1] and "metrological compatibility"[1] entered within the horizon chemist's interest. All these metrological concepts combined with a solid knowledge of statistics are extremely important for the chemist's professional training and can be a differentiator in employability. In this work, we will show that some statistical concepts are necessary to evaluate some of the above metrological concepts and propose some contents that should be taught in undergraduate chemistry courses and related courses. Also, we will review the availability of Brazilian publications and of the undergraduate and graduate disciplines on metrology. Finally, to help the persons interested in these subjects' relations of bibliographic references and internet sites are presented.

Based on the authors' academic and industrial experience, several times, analytical chemists think that the results from chemical analyses always belong to an ideal universe; that is, they are homoscedastic, the analytical curve is rectilinear (linear on the analyte concentration, the measurand, as in the example of the analytical calibration curve for Cd determination discussed below),[5] and there is no significant influence of the sampling step (not considering the uncertainty due to sampling).[6] All these issues have contributed to the measurement uncertainty. The violation of these assumptions can cause problems or risks in decision-making using the analytical chemistry results.

Often, analytical instrumental responses variability does not have constant variance; that is, it does not present homoscedastic behavior. Thus, the parameters of the analytical calibration curves (ACC) obtained using an unweighted, simple linear regression by ordinary least-squares (OLS),[7-9] when all points have the same influence on the fitting procedure, are not the most adequate, leading to biased fitted parameters. Due to this data heteroscedasticity, these curves are conceptually correctly fitted by a simple linear regression by weighted least-squares (WLS);[10-17] that is, the coefficients of the analytical curve are estimated to give higher weights to the points where the variability (uncertainty) is lower.

Unfortunately, analytical calibration curves (ACC) do not always behave as a straight line (polynomial of degree one) over the entire working range. It is not uncommon that polynomials of a higher degree or non-linear on the fitted parameters fits (e. g., $y = ax^b$, exponential functions $y = ae^{bx}$, $y = e^{a+bx}$, etc) are better suited in instrumental analysis. Thus, analytical chemists must know how to check correctly and decide whether to stick with the rectilinear adjustment or fit the analytical calibration curve (ACC) with a polynomial of higher order on the analyte concentration or a function non-linear on the fitted parameters.

Due to the degree of heterogeneity (i.e., inhomogeneity) on sampling targets, the uncertainty arising from sampling, besides the analytical uncertainty, must be evaluated. Ratifying this statement, item 7.6.1 of ISO/IEC 17025:2017 says:[18-19] "… all contributions that are of significance, including those arising from sampling, shall be taken into account using appropriate methods of analysis." If sampling uncertainty is neglected and is not statistically negligible, the uncertainty information used in the compliance assessment may lead to erroneous conclusions regarding the sampling target and relates only to the laboratory sample.

Misunderstanding related to these very important issues can occur because there is a gap between the analytical chemistry and metrology concepts.[20] Thus, this review aims to shed light on the importance and lack of connection between metrology in teaching and research in Analytical Chemistry in Brazil and Portugal and, finally, propose a syllabus for the metrology course in Chemistry and some related undergraduate courses.

PROBLEMS OR RISKS IN DECISION-MAKING WHEN METROLOGICAL AND STATISTICAL CONCEPTS ARE NOT CORRECTLY APPLIED

As some authors are or were professors in undergraduate and postgraduate Chemistry courses, unfortunately, it was noticed that the disciplines of Analytical Chemistry and Instrumental Analysis do not closely connect with metrological concepts. In these situations, analytical chemists should remember that one of the final steps of the analytical process is the data treatment. Any mistake in this important stage can compromise the results and conclusions.

Are the variances always homogeneous? The case of sulfur determination in mineral oil by X-ray fluorescence

Table I presents the results of concentrations and instrumental responses (IR) for an ACC of the sulfur mass fraction of certified reference materials in the mineral oil matrix.[21] The experiment was carried out on the XOS SINDIE GEN 3 equipment, based on the ASTM D7039-15a (2020) standard method,[22] whose analytical technique is monochromatic wavelength dispersive x-ray fluorescence.

Table I. Analytical calibration curve (ACC) from 0 to 50 mg kg^{-1} for sulfur in mineral oil determination by X-ray fluorescence

Mass fraction, mg kg^{-1}	IR = Counts					
0.00	177	182	172	182	164	199
5.04	922	1085	927	987	991	919
10.29	1842	1841	1791	1962	1843	1897
15.16	2562	2606	2566	2650	2720	2687
25.18	4023	4246	4387	4168	4016	4206
50.48	**8100**	8408	8404	8419	8400	**8306**

Data extracted from the Technical Note[21] titled “Avaliação Metrológica da Curva Analítica para Determinação do Teor de Enxofre em Baixíssimas Concentrações Em Óleo Diesel S-10”. By de Farias, S. I.; da Costa, L. G.; Calili, R. F.; Rios, R. M.; de Oliveira, E. C. *Quim. Nova* **2015**, *38*, (6), 852-858. http://dx.doi.org/10.5935/0100-4042.20150071 with permission granted by Quím. Nova, PubliSBQ, on March 14, 2024.

Although the values in bold were considered outliers according to the Grubbs’ pair test and the residues show independent behavior, according to the Durbin-Watson test, these values weren’t excluded from the ACC statistical treatment since no technical event was reported by the analyst during the analytical work. Also, the standard deviations of the IR smoothly increase with the analyte calibrator concentration, and by removing these two values, the standard deviation of the last calibrated level becomes even lower than that of the first calibrated level.

The ordinary (unweighted) regression ANOVA indicates that the straight-line OLS fit is adequate; however, the Cochran test assesses that the behaviour is heteroscedastic; therefore, WLS linear regression is more suitable.

For this study, the prediction "combined standard measurement uncertainty",[1,23,24] $u(c_{pred})$, were calculated by Equation 1, considering the regression as unweighted (OLS) and weighted (WLS) for two CRMs of concentrations of 10.29 mg kg^{-1} and 25.18 mg kg^{-1} with IR of 1860.67 and 4174.33, respectively, around the centroid (17.7, 3026.58) of the ACC, and for a hypothetical sample solution with IR 9000 using the values available in Table II. Equation 1 (Equation E3.3 of section E.4.3 at reference 24, Equation 6 without the first term at reference 25) is obtained by applying the law of propagation of uncertainty (LPU) on the "measurement function"[1] for the analyte concentration predicted on the ACC as a straight-line,[25] which can be used for all the OLS, WLS and bivariate regressions. Note that the widely used Equation E3.5 at reference 24 is a particular case of Equation 1 and can be deduced from it, but it can be used only when an OLS regression is performed on a homoscedastic ACC.

$$u(c_{\mathrm{pred}}) = u(x^*) = u(x_o) =$$

$$\sqrt{\left\{u^2(y_o)/K + \begin{bmatrix}1 & x_o\end{bmatrix}\begin{bmatrix}u^2(a) & \mathrm{cov}(b,a)\\ \mathrm{cov}(a,b) & u^2(b)\end{bmatrix}\begin{bmatrix}1\\ x_o\end{bmatrix}\right\}b^{-2}} = \qquad \text{Equation 1}$$

$$\sqrt{\frac{s^2(y_o)/K + \left\{s^2(a) + (c_{\mathrm{pred}})^2 s^2(b) + 2c_{\mathrm{pred}}\,\mathrm{cov}(a,b)\right\}}{b^2}}$$

Table II. Parameter fitted by OLS and WLS and their standard-deviations and their covariances; predicted concentrations (c_{pred}) on the fitted ACC, $c_{pred} = (y - a)/b$, for sulfur determination in mineral oil and their standard uncertainties ($u(c_{pred})$). The measured concentrations for two CRMs and a sample solution with their prediction standard uncertainties.

Fitted Parameter	OLS	WLS	c_{CRM} / mg kg^{-1}	$c_{pred} \pm u(c_{pred})$ by OLS / mg kg^{-1}	$c_{pred} \pm u(c_{pred})$ by WLS / mg kg^{-1}
Intercept *a*	171.33 ± 21.65	179.26 ± 4.70	10.29	10.48 ± 0.56	(10.43 ± 0.39)
Slope *b* / kg mg^{-1}	161.39 ± 0.89	161.46 ± 0.79	25.18	24.80 ± 0.56	(24.74 ± 0.58)
cov(*a*,*b*) / kg mg^{-1}	–14.0393	–9440	IR = 9000	54.70 ± 1.21	54.63 ± 2.03

It is observed that the confidence intervals for one standard uncertainty with confidence probability of nearly 68%, for both the OLS and WLS regressions, contain the CRMs values and that the prediction standard uncertainty for the unweighted OLS regression increases symmetrically but very slightly around the centroid, at the concentration 17.6917 mg kg^{-1}, being practically constant (Figures 1(A) and 1(C)) throughout the calibrated range, while for weighted WLS regression the confidence interval increases with concentration (Figures 1(B) and 1(D)).

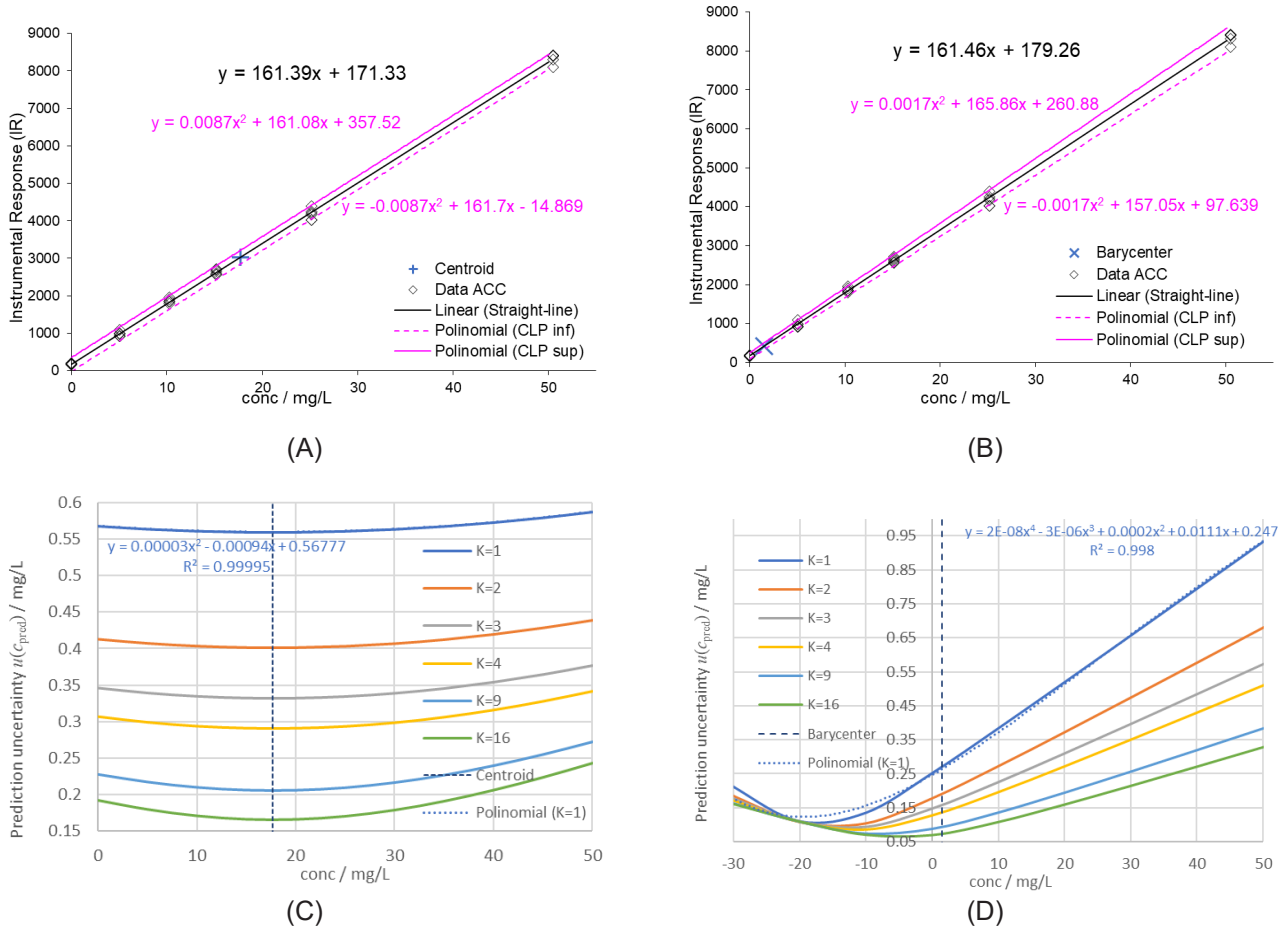


Figure 1. The sulfur analytical calibration curve (ACC) from Table I.

The "calibration diagram"[1] shows the calibration function and the prediction confidence band around the calibration function for a 95% confidence level for (A) OLS and (B) WLS fit, with the upper (CLP sup) and lower (CLP inf) prediction confidence limit curves.

The curves for the variation of the prediction uncertainty, according to Equation 1, against the predicted concentration and the number K of replications of measurement of the IR for the sample solution for (C) OLS and (D) WLS fits. These curves, as well as the CLP sup and CLP inf curves, are hyperbolas, but can be approximated by a polynomial of even degree, as represented for K = 1.

Should every analytical calibration curve (ACC) have 1st degree polynomial (straight-line) adjustment? The case of cadmium determination in water by AAS

The data for the ACC for the determination of Cd in water by atomic absorption spectroscopy (AAS) adapted from Example A5 from QUAM,[24] Table III, must be conceptually fitted by the weighted least squares (WLS) method, as the variances of the calibrated levels IR are heteroscedastic as depicted in the last column in Table III and in Figure 2(A), where a rectilinear line well fit the increasing IR repeatability standard deviations. This heteroscedastic IR of the analytical instruments, based on electromagnetic, but also ionization, radiation detection is naturally expected when consider the intrinsic behavior of this detector noise (see the section "Effect of the instrumental noise on the spectrometric analysis" at reference 26). For teaching purposes, aiming to emphasize the effect of different regression models and techniques on the measurement result (value and uncertainty), this study considers the first (straight-line) and second (parabola, quadratic) degree polynomials models, first using the OLS fitting and after the WLS fitting. Figure 2(B) shows the not conceptually correct use of the OLS to fit a straight-line (dashed line) on the ACC, as realized at reference 24, and to fit a parabola (continuous line), which presents a best coefficient of

multiple determination R^2 = 0.9991. The fitted OLS values for the intercept a and slope b and their standard uncertainty were obtained using the command “Regression” on Excel, a = 0.0097, $u(a)$ = 0.002847 and b = 0.23766667, $u(b)$ = 0.004956. These same values are obtained by the OLS fit (model 1b) using the CCC Software[27] and by the spreadsheet in the tab “Cd Expl A5 QUAM OLS” in the supplementary material. The CCC Software and the spreadsheet present the value of the covariances between these fitted parameters, $\text{cov}(a,b) = -1.228205\times10^{-05}$, not presented by the Excel command “Regression” at "Data", "Data Analysis", or by the function PROJ.LIN. Note that these fitted parameters are very near, within one standard deviation of the values of the uncorrected data fitted at the original publication.[24]

Table III. Data of the analytical calibration curve (ACC) for Cd determination in water by atomic absorption spectroscopy (AAS) (adapted from Example A5 from reference 25)

Concentration (mg L^{-1})	Absorbance (A)			Standard-deviation s_A
0.1	0.028	0.029	0.029	0.000577
0.3	0.084	0.083	0.081	0.001528
0.5	0.135	0.131	0.133	0.002000
0.7	0.180	0.181	0.183	0.001528
0.9	0.215	0.220*	0.216	0.002646

* This value replaces 0.230 in the original publication.[24] Without replacing (correction) the repeatability standard-deviation s_A of the last level becomes 0.008386, larger than threefold the expected from the linear behavior of the first four levels, Figure 2(A). This pathologic behavior is probably due to a typo.

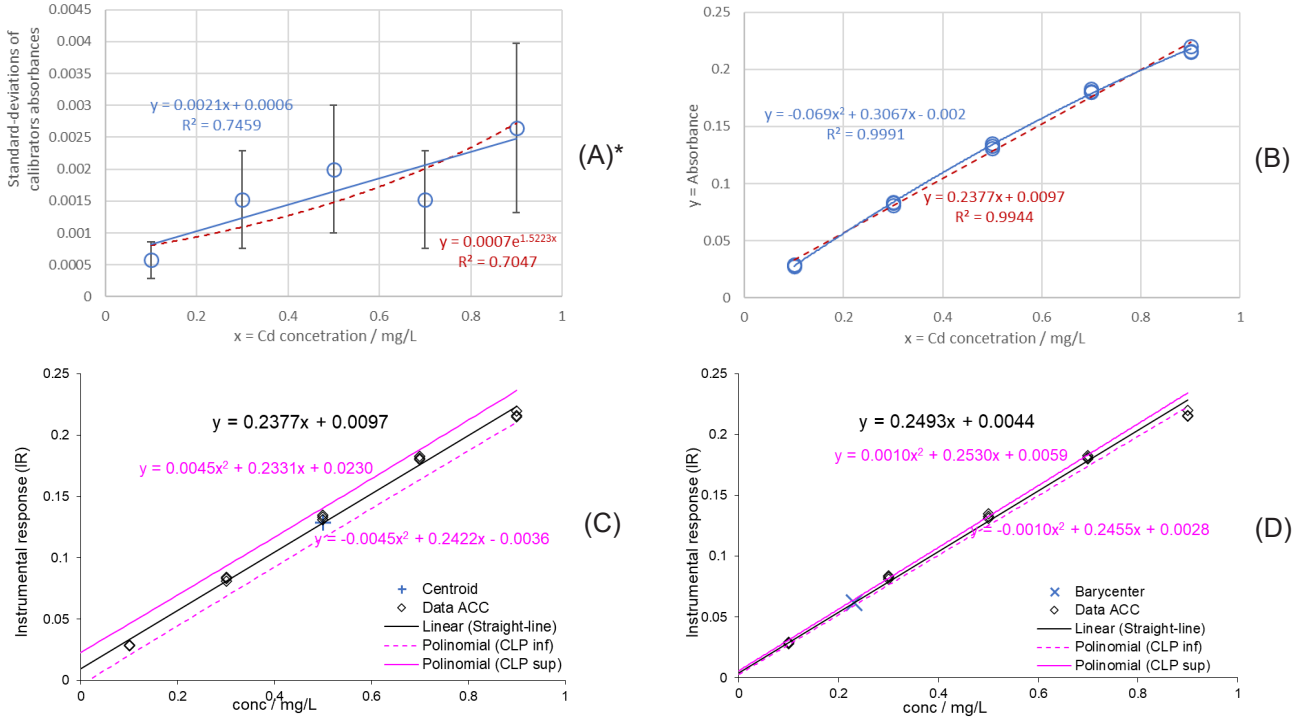
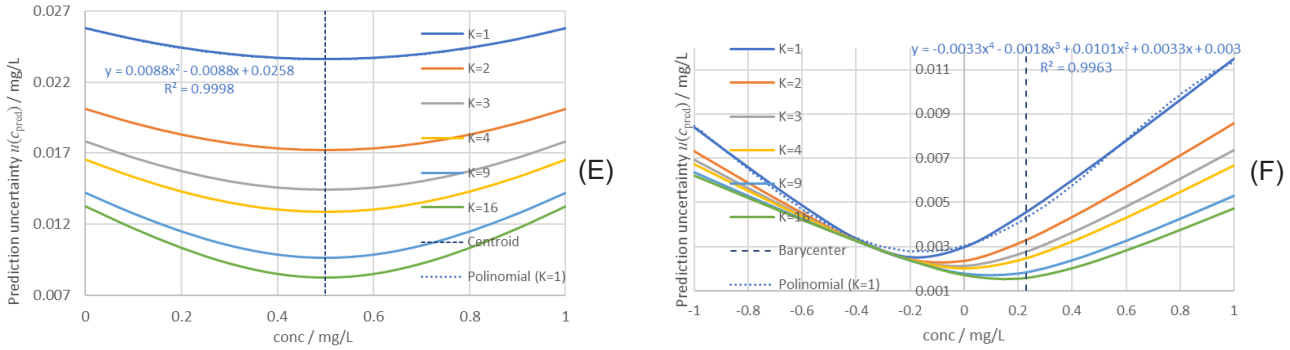


Figure 2. The Cd analytical calibration curve (ACC) from Table III. (continues on the next page)



* The first four levels are well fitted by the straight-line s_A = 0.0017x + 0.0007 with R^2 = 0.5161. Without the replacement of the original 0.23 value by 0.22, the five points of s_A vs. concentration are best fitted by an exponential function: $S_A = 0.0005^{2.6759x}$ with R^2 = 0.8082. The error bars are 50%, as nearly expected by equation E.7 in section E.4.3 of the GUM[23] for three IR replications for each calibration level.

Figure 2. The Cd analytical calibration curve (ACC) from Table III. (continuation)

(A) The OLS fits of the Standard deviations of the instrumental responses (IR) at each calibration level. (B) The Cd ACC is fitted by the OLS as a straight-line and a parabola. The "calibration diagrams"[1] shows the calibration functions, and the prediction confidence bands around the calibration functions for a 95% confidence level for (C) OLS and (D) WLS fits, with the upper (CLP sup) and lower (CLP inf) prediction confidence limit curves.
The curves for the variation of the prediction uncertainty, according to Equation 1, against the predicted concentration and the number K of replications of measurement of the IR for the sample solution for (E) OLS and (F) WLS fits. These curves, and CLP sup and CLP inf, are hyperboles but can well be approximated by a polynomial of even degree, as depicted for *K*=1.

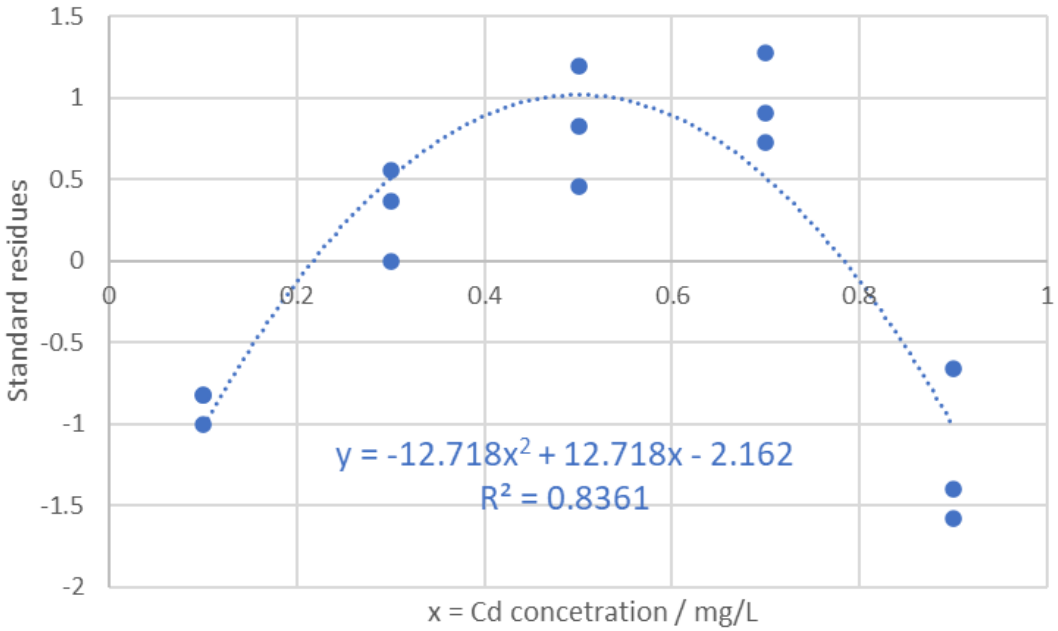


Figure 3. Standard residues, the residues divided by the residual standard deviation, of the OLS rectilinear fit of the analytical calibration curve (ACC) for cadmium determination (Table III).

The rectilinear (first-degree polynomial) OLS fit results show a coefficient of multiple determination $R^2 = 0.9944$ or $r = 0.9972$ (for the rectilinear fit $R^2 = r^2(x,y)$, the square of the coefficient of linear correlation or Pearson correlation coefficient between x and y, see sections 8.3 and 10.3.1.2 at reference 28), with a residual standard-deviation of $s_{res} = 0.005429$. Note that this residual standard-deviation is even larger than the largest IR standard-deviation at the last calibration level! Visually, the rectilinear fit looks like an

excellent model (rejection of the null hypothesis $b = 0$), but it lacks fit (rejection of the null hypothesis $MS_{LOF} = MS_{EE}$), as indicated by Figure 3, and confirmed by the unweighted ANOVA, Table IV. The concave pattern of the plot of the standard residues in Figure 3 also indicates that this ACC is not adequately fitted by a straight-line. This pattern is yet more pronounced from the rectilinear WLS fit of this ACC (see the Figure "Plot of the standard residues" in the tab "CCC WLS rectilinear" in the supplementary material) because the OLS residual standard-deviation is inflated due to the lack of fit.

Table IV. Ordinary (unweighted) Analysis of Variance for first-degree polynomial (straight-line) OLS fit of the Cd analytical calibration curve (ACC) at Table III and Figure 2(B)

Y = 0.0097	**+**	**(0.237666667) X**
Correlation Coefficient	**=**	**0.99719**
% Explained Variation	**=**	**99.44**

SOURCE OF VARIATION	SUM OF SQUARES		DEGREES OF FREEDOM	MEAN SQUARE (MS)
Regression (Reg)	0.067782533		1	0.067782533
Residual (Res)	0.0003832		13	2.94769E-05
Lack of fit (LOF)	0.0004		3	0.000117067
Experimental Error (EE)	0.0000		10	3.2E-06
Total	0.068165733		14	
MS_{Reg}/MS_{Res}	2299.512	>	$F_{CRITICAL}$	4.667
		The model is suitable		
MS_{LOF}/MS_{EE}	36.583	>	$F_{CRITICAL}$	3.708
		The model fit is not suitable		

These results show that even on heteroscedastic data, the OLS fit can be used for an initial exploratory study of the ACC, but not for its definitive fit. The OLS fit can be used to model the plot of the standard-deviations of the IR of the calibration levels against the analyte concentration of the calibration standard solutions. This is because in routine analysis, the number of replications of the measurement of these calibrators is, in general very low, and the corresponding standard-deviations are very uncertain, Figure 2(A).

To try to eliminate the lack of fit, a quadratic term was added, and the OLS fitting of a second-degree polynomial (parabola) was realized, Figure 2(B), resulting in a coefficient of multiple determination $R^2 = 0.9991$ and a residual standard deviation of $s_{res} = 0.002288$, which is half of this one for the rectilinear fit and around the values of s_A in Table III.

Table V. Ordinary (unweighted) Analysis of Variance for second-degree (quadratic) polynomial OLS fit of the Cd analytical calibration curve (ACC) at Table III and Figure 2(B)

$Y = \beta_0 + \beta_1 X + \beta_2 X^2$		
β_0	=	-0.00204
β_1	=	0.30671
β_2	=	-0.06905
r	=	0.99954
% Explained Variation	=	99.91

SOURCE OF VARIATION	SUM OF SQUARES		DEGREES OF FREEDOM	MEAN SQUARE (MS)
Regression (Reg)	0.068102914		2	0.034051457
Residual (Res)	6.2819E-05		12	5.23492E-06
Lack of fit (LOF)	0.0000		2	1.54095E-05
Experimental Error (EE)	0.0000		10	3.2E-06
Total	0.068165733		14	
MS_{Reg}/MS_{Res}	6504.675	>	$F_{CRITICAL}$	3.885
		The model is suitable		
MS_{LOF}/MS_{EE}	4.815	>	$F_{CRITICAL}$	4.103
		The model fit is not suitable		

The predicted standard uncertainty, $u(c_{pred}) = u(x^*)$, for the linear and quadratic OLS models varies from 0.0235 mg L^{-1} to 0.0250 mg L^{-1} (see the Figure 10 in the tab “Cd Expl A5 QUAM OLS” in the supplementary material) and from 0.019 mg L^{-1} to 0.031 mg L^{-1} (see the Figure 12 in the tab “Cd Expl A5 QUAM OLS” in the supplementary material), respectively, what can justify the quadratic fit, leading lower prediction uncertainties at low concentrations. Also, the quadratic OLS fit standard residues are now randomly distributed around the zero, Figure 4, as expected from a good fit, although the lack of fit indicated by the unweighted ANOVA in Table V; however, the heteroscedasticity of the ACC requires the use of the WLS fit.

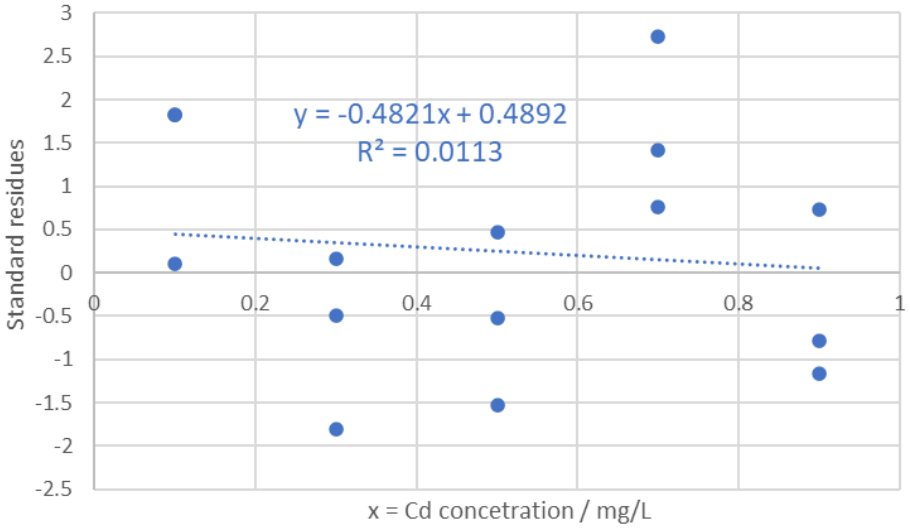


Figure 4. Standard residues of the OLS parabola (quadratic or second-degree polynomial) fit of the analytical calibration curve (ACC) for Cd determination.

The above treatment of the ACC demonstrates that its correct fit should be realized using the WLS. The quadratic WLS fit was performed using the CCC Software[27] using the option "model 2b" which estimates the IR variances (squared standard-deviation) from the replications of each calibrated level. The fitted values, their standard uncertainties, and the covariance matrix among the three fitted parameters are presented in Table VI and the plot of the fitted parabola is in Figure 5(A). The standard residues of this fit are depicted in Figure 5(B) (see also Figure 11 in the tab "Cd Expl A5 QUAM WLS" in the supplementary material). The prediction standard uncertainty ($u(c_{pred})$) calculation for the fit of a parabola is a little cumbersome and can be realized using a generalized matrixial form of the Equation 1. Using the covariance matrix associated with fitted (calculated) values y (y_{fit}) provided by the CCC Software, we calculated the prediction standard uncertainty, only for K = 1, at the five calibrated levels. It was used the LPU as in Equation 2 (see cells U412:U440 in the tab "Cd Expl A5 QUAM WLS" in the supplementary material), where $u^2(y_{fit})$ are the diagonal elements of the y_{fit} covariance matrix from the CCC Software and $s^2(y_o)$ was assumed to be equal the repeatability standard deviation of a calibrator with the same concentration of c_{pred}. This last hypothesis is especially valid for ACC prepared from a blank sample or matrix-matched ACC.

$$u(c_{pred}) = u(x^*) = u(x_o) = \sqrt{\frac{s^2(y_o)/K + u^2(y_{fit})}{\left(\partial y / \partial x\right)^2}} \qquad \text{Equation 2}$$

The behavior of this prediction uncertainty with the analyte concentration is complex, as depicted by the continuous line in Figure 6, but grossly increases within the calibrated region (see Figure 12 in the tab "Cd Expl A5 QUAM WLS" in the supplementary material), as also happen for the case of the WLS straight-line fit, and the lowest prediction uncertainty is below the calibrated range (the minimum of the dashed parabola in Figure 6 is at -4.5 mg/L = -8.0138E-03 / 2 / 8.9039E-04). The pathological local minima at the continuous line, Figure 6, is due to the low IR repeatability standard deviation at a concentration of 0.7 mg/L, below the expected value for this calibration level, as inferred from the straight-line in Figure 2(A). The dashed line parabola in Figure 6 shows the prediction uncertainty $u(c_{pred})$ if the IR repeatability standard deviations of the calibrators and sample test solutions $s^2(y_o)$ obey the straight-line in Figure 2(A).

Table VI. Results of the quadratic (parabola) WLS fit $y = a + bx + cx^2$ carried out by the CCC Software[27] on the analytical calibration curve (ACC) data for Cd determination by AAS in Table III

	Fitted value	Standard uncertainty	Covariance matrix		
a	-0.000918166	0.000689964	+4.760506e-07	-3.472725e-06	+3.767259e-06
b	0.30110662	0.00567432	-3.472725e-06	+3.219796e-05	-3.691866e-05
c	-0.06224945	0.00665795	+3.767259e-06	-3.691866e-05	+4.43283e-05

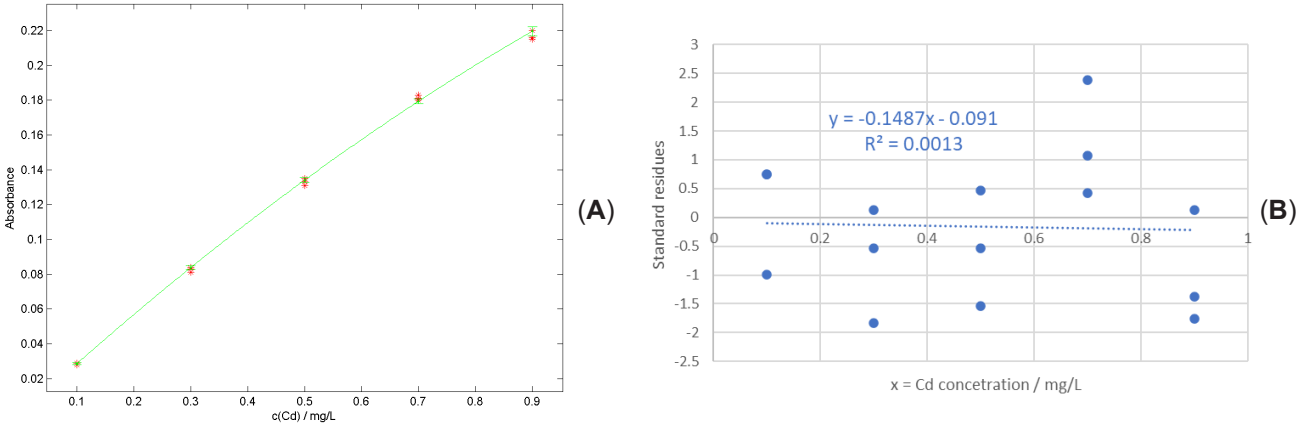


Figure 5. The analytical calibration curve (ACC) for cadmium determination (Table III) and the WLS fitted parabola (quadratic fit). (A) The "calibration diagram"[1] showing the calibration function and the prediction confidence band, as error (uncertainty) bars, around the calibration function for a 95% confidence level as depicted by the CCC Software.[27] The prediction error bars are shown on the fitted curve at the five calibrated levels. (B) Standard residues of the WLS parabola (quadratic) fit. Note that, as expected for a good fit, all the estimated parameters for the straight-line in this figure are nearest from zero than those for the OLS parabola fit in Figure 4.

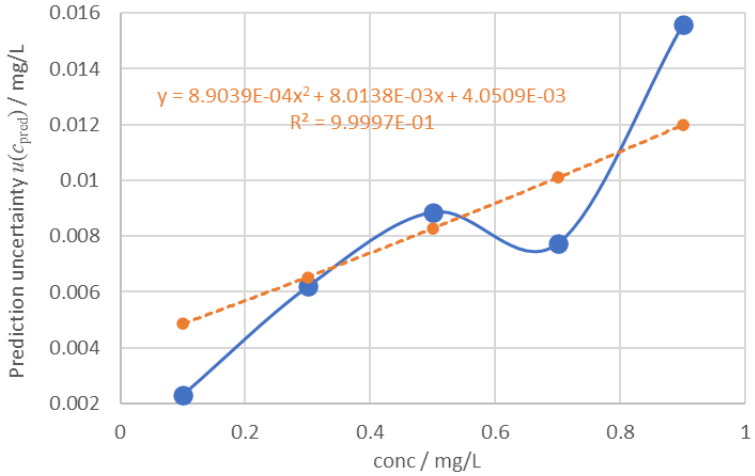


Figure 6. The curve for the variation of the prediction uncertainty, $u(c_{pred})$, according to Equation 2, against the predicted concentration for the WLS parabola fitted on the cadmium analytical calibration curve (ACC) for one unique sample test solution measurement, *K*=1. The dashed line parabola well fits the expected behavior of $u(c_{pred})$ if the IR repeatability standard deviations of the sample test are given by the straight-line in Figure 2(A).

Where is the most precise region of an analytical calibration curve (ACC)?

We learned in our first courses in instrumental analytical chemistry that an ACC should be designed to have the sample test solution in the middle of the calibrated range, which is the more precise region of the ACC. Note that the middle of the ACC is not necessarily equal to its centroid (see the symbol + in Figure 1(A) and Figure 2(C)), the mean values of the calibrators concentrations and their IR. However, this is true only for a very particular and rare case: a homoscedastic ACC with statistically the same IR standard deviation in all calibrated levels, calibrated levels equally spaced, and finally, with the same number of replications of the IR in all calibration levels. This is **not** the case for ACCs in Table I and Figure 1 for sulfur determination, neither in Table III nor Figure 2 for Cd determination.

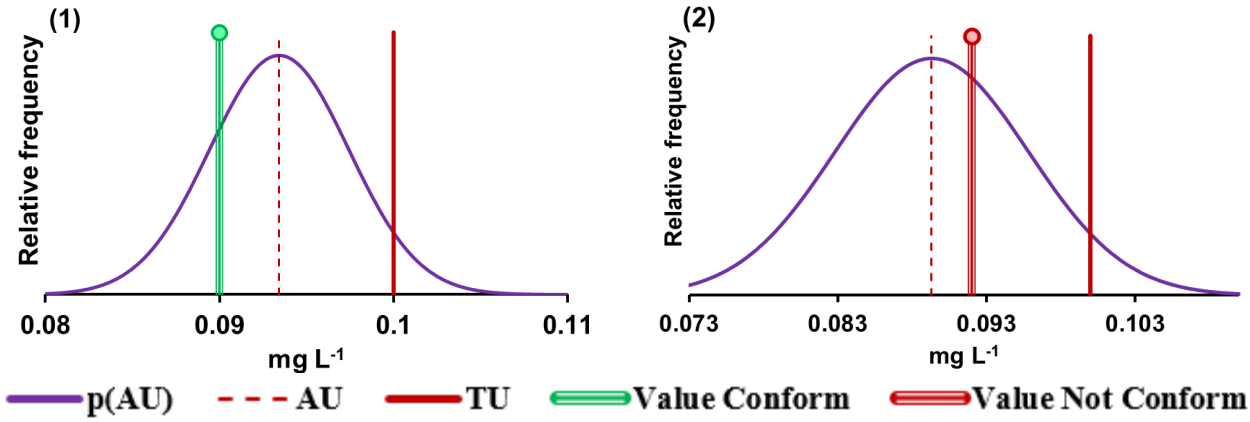
The highly precise region for the OLS regression is at the centroid, at a concentration 17.6917 mg kg^{-1} (Figures 1(A) and 1(C)) for sulfur determination, and at 0.5 mg L^{-1} (Figures 2(C) and 2(E)) for cadmium determination, however none of these ACCs are homoscedastic, but heteroscedastic, the IR standard deviations for each calibration level are different among them. Also, in the case of the sulfur determination, the calibrator's concentrations design of the ACC is not equally spaced, making the centroid not the middle of the calibrated range. Both the heteroscedasticity and the not equally spaced calibrators design dislocate the more precise region of the ACC from its middle point towards the region with a higher density of measured calibrators and with lower IR repeatability uncertainties (standard deviations), which appears to be obvious.

The highest precise region for the WLS regression is below the barycenter, which is generally near the lowest concentrations' levels, at concentration 1.5239 mg kg^{-1} (Figures 1(B) and 1(D)) for sulfur determination and at 0.23 mg L^{-1} (Figures 2(D) and 2(F)) for cadmium determination, when the IR are increasingly heteroscedastic. This behavior becomes clearer when looking for the dependence of the prediction standard uncertainty, $u(c_{\text{pred}})$, against the predicted concentration for both the OLS (Figures 1(C) and 2(E)) and WLS (Figures 1(D) and 2(F)) regression.

To design an ACC owing to a fit for purpose prediction uncertainty, it is necessary to consider not only the expected analyte concentration in the sample test but also prior information concerning the precision (uncertainties) of the IR, $u(\text{IR}_{\text{std}i})$ at the different calibrator concentrations. Finally, if the calibrator concentrations, $u(c_{\text{std}i})$, uncertainties are not negligible, at least $3b$ x $u(c_{\text{std}i}) \leq u(\text{IR}_{\text{std}i})$ for all the calibration levels for a rectilinear ACC (see the equation 23 at the bibliographical reference: Magalhães, W. F. "Cálculo de Incerteza de Grandezas Obtidas por Regressão pelos Métodos de Mínimos Quadrados de Modelos Físico-Químicos Linearizados, uma Abordagem Estatística e Metrológica". *Revista Virtual de Química* **2020**, *12* (5) 1507. Available at: https://rvq.sbq.org.br/), the univariate OLS or WLS treated here cannot be used, but a bivariate least squares (BLS) regression, as performed by the CCC Software.[27] In this case, also the behavior of the calibrator's concentrations uncertainties will contribute to determining the highest precise region for the bivariate regression of the ACC.

Must the measurement uncertainty arising from sampling in chemical analyses be neglected?

Let us think about the determination of the manganese molar concentration in freshwater. Based on an elemental analysis technique ICP-MS, a test result of 0.090 mg L^{-1} complies with the Brazilian specification limit of 0.100 mg L^{-1}?[29] Considering two scenarios. (1): the analytical expanded uncertainty with a coverage factor k = 2 for nearly 95% of coverage probability is 0.008 mg L^{-1}, and (2) the analytical expanded uncertainty, including the sampling uncertainty, is 0.013 mg L^{-1}. Figure 7 shows the upper acceptance limits for the two cases.



p(AU) – probaility density function centered at the upper acceptance limit; AU – Upper acceptance limit; TU – Upper tolerance limit.

(1) Based only on the analytical uncertainty: AU = 0.1 – 0.008/2 x 1.64 = 0.09344 > 0.090 the test result (conform).

(2) Based on the analytical uncertainty plus the sampling uncertainty: AU = 0.1 − 0.013/2 x 1.64 = 0.08934 <0.090 the test result (non-conform).

Where 1.64 is the one-tail normal z-score for a "specific consumer's risk"[30] or a level of significance of 5%.

Figure 7. Risk assessment in manganese molar concentration in freshwaters.

Here, the uncertainty information in compliance assessment was used, considering the guard band concept to be a "specific consumer's risk" of 5%.[30] This measurand was considered compliant based on scenario (1), but not compliant based on scenario (2).

Is the laboratory precision the measurement uncertainty of the analytical result?

A requisite of the laboratory quality systems, as ISO/IEC 17025:2017[18] is the determination during the analytical procedure validation of the "intermediate precision",[1] often called "laboratory reproducibility", "internal reproducibility", "in-house reproducibility", etc. Chemists generally consider this validation performance characteristic as the analytical result measurement uncertainty. Although it is true that the intermediate precision is one of the highest uncertainty sources, it competes with the sampling uncertainty and the recovering uncertainty for this position. Finally, to guarantee metrological traceability, it is important to combine these three previous sources of uncertainties with those ones due to mass, volume measurements and that one due to analytical instrument calibration. This uncertainty combination can be performed using the LPU according to the well-known BIPM[23] or Eurachem/CITAC[24] guides or using a numerical approach like the Monte-Carlo[31] or Kragten[24] methods.

BRAZILIAN JOURNALS

A search in the SCOPUS database was carried out using the terms "metrology", and more specifically "uncertainty", in the article title, abstract and keywords during the years 2001 to 2022, considering the Brazilian journals: Brazilian Journal of Analytical Chemistry (BrJAC), Eclética Química (Ecletica), Journal of the Brazilian Chemical Society (JBCS), Química Nova (QN) and Revista Virtual de Química (RVQ). Few works were found, Figure 8. The authors supposed that this could indicate that the Brazilian chemists are not very familiarized with these knowledges, what justifies its teaching in the university's courses related with chemical analysis.

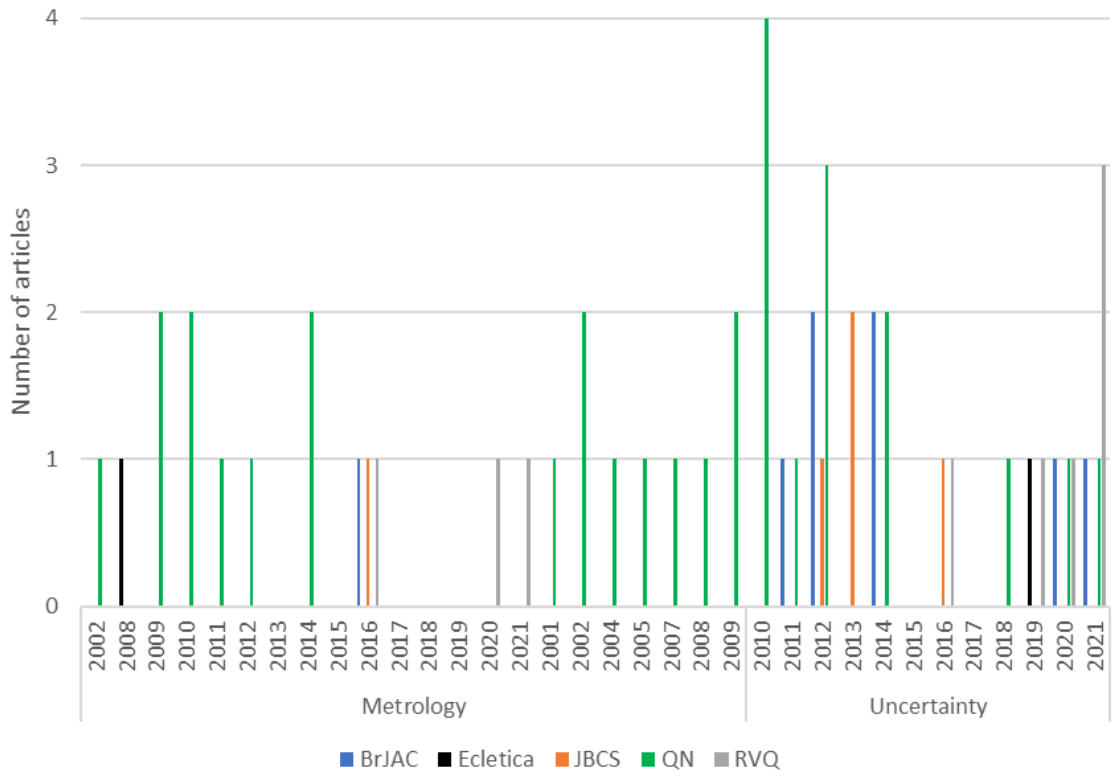


Figure 8. Profile of metrological academic production in Brazilian chemistry journals.

UNDERGRADUATE COURSES

Brazil

Looking at the syllabus design of the first hundred Brazilian Chemistry undergraduate courses according to the "Folha de São Paulo" University Ranking of 2019,[32] only nineteen courses offered in the last years a discipline where some aspects of chemical metrology, as measurement uncertainty, analytical measurement procedure validation, quality control, quality guaranty, quality management or ISO 17025 are treated in their syllabuses our courses programs or bibliographies, as disponible in their course's sites (Table VII). There are only two pos-graduation programs on metrology in Brazil: (i) from the National Metrology Institute (INMETRO),[33] and (ii) from the Pontifical University Catholic of Rio de Janeiro – PUC-Rio,[34] both at the Rio de Janeiro city, RJ, Brazil.

Table VII. Brazilian chemistry undergraduate courses that offered disciplines concerning metrology concepts in the last years

i	RUF Rank	University	University URL	Further information
1	1	USP	www.usp.br	https://uspdigital.usp.br/jupiterweb/obterDisciplina?sgldis=7500077&codcur=75014&codhab=200 https://uspdigital.usp.br/jupiterweb/obterDisciplina?sgldis=7500062&codcur=75014&codhab=200 https://uspdigital.usp.br/jupiterweb/obterDisciplina?sgldis=7500078&codcur=75014&codhab=500
2	2	Unicamp	www.unicamp.br	https://www.iqm.unicamp.br/arquivos/QA851%20-%20Valida%C3%A7%C3%A3o%20de%20M%C3%A9todos%20Anal%C3%ADticos.pdf

(continues on the next page)

Table VII. Brazilian chemistry undergraduate courses that offered disciplines concerning metrology concepts in the last years (continuation)

i	RUF Rank	University	University URL	Further information
3	3	UFRJ	www.ufrj.br	https://siga.ufrj.br/sira/repositorio-curriculo/ListaCursos.html?_ga=2.221801368.1809578526.1658765001-55904377.1658765001&_gl=1*wx8kso*_ga*NTU5MDQzNzcuMTY1ODc2NTAwMQ..*_ga_S9CWPVF04S*MTY1ODc2NTAwMC4xLjEuMTY1ODc2NTA3Ni4w
4	4	UFMG	www.ufmg.br	https://www2.ufmg.br/quimica https://www2.ufmg.br/quimica/quimica/Home/Cursos/Bacharelado https://www2.ufmg.br/quimica/quimica/Home/Cursos/Bacharelado
5	6	UFRGS	www.ufrgs.br	http://www.ufrgs.br/ufrgs/ensino/graduacao/cursos/exibeCurso?cod_curso=343
6	7	UNESP	www.unesp.br	https://www.iq.unesp.br/#!/graduacao1260/cursos/bacharelado-em-quimica/ https://www.iq.unesp.br/#!/graduacao1260/programas-de-ensino/ https://www.fc.unesp.br/Home/Departamentos/quimica201/gradescurriculares/bacharelado-em-quimica-tecnologica.pdf https://www.ibilce.unesp.br/Home/Graduacao450/quimica/quibqa2019.pdf
7	10	UFSC	www.ufsc.br	https://quimica.paginas.ufsc.br/files/2014/03/PPC_QMC_Bacharelado_2021.1-1.pdf https://quimica.paginas.ufsc.br/files/2014/03/PPC_QMC_Tecnol%C3%B3gica_2021.1-1.pdf
8	11	UFF	www.uff.br	https://www.uff.br/?q=curso/quimica/312700/bacharelado/niteroi https://app.uff.br/iduff/consultaMatrizCurricular.uff
9	12	UFPE	www.ufpe.br	gradquimica@ufpe.br 55 81 2126.844
10	14	UFSM	www.ufsm.br	https://www.ufsm.br/cursos/graduacao/santa-maria/quimica/
11	25	UNEB	www.uneb.br	https://dcv1.uneb.br/wp-content/uploads/2021/06/ESTRUTURA-CURRICULAR-farmacia.pdf
12	33	UFPB	www.ufpb.br	http://www.ufpb.br/graduacao/quimicacca
13	37	UNIFESP	www.unifesp.br	
14	39	UFES	www.ufes.br	https://quimica.vitoria.ufes.br/pt-br/grade-curricular
15	45	IFRJ	http://www.ifrj.edu.br/	https://portal.ifrj.edu.br/cursos-graduacao/licenciatura-quimica-nilopolis https://portal.ifrj.edu.br/cursos-graduacao/licenciatura-quimica-duque-caxias
16	50	UFAM	http://www.ufam.edu.br/	
17	52	PUC-Campinas	http://www.puc-campinas.edu.br/	https://www.puc-campinas.edu.br/graduacao/quimica/
18	53	UEPB	http://www.uepb.edu.br/	

(continues on the next page)

Table VII. Brazilian chemistry undergraduate courses that offered disciplines concerning metrology concepts in the last years (continuation)

i	RUF Rank	University	University URL	Further information
19	82	IFMA	http://www.ifma.edu.br/ https://portal.ifma.edu.br/inicio/	https://acailandia.ifma.edu.br/cursosofertados/ https://caxias.ifma.edu.br/cursosofertados/quimica/ https://montecastelo.ifma.edu.br/licenciatura-em-quimica/ https://zedoca.ifma.edu.br/cursosoferecidos/licenciatura-em-quimica/

Portugal

The research was based on websites of the Portuguese society of chemistry, physics, biology, for possible courses in metrology, at universities, and found only five universities that offer metrology in undergraduate courses related to the technological area, Table VIII.

Table VIII. Undergraduate courses in Portugal with metrology disciplines

University	Course	Curricular unit	Six-monthly workload
Universidade Nova de Lisboa	Engineering and Industrial Management	Metrology and Measurement System	38 hours
Universidade de Aveiro	Engineering Sciences	Metrology	60 hours
Universidade de Coimbra	Applied Physics	Metrology	Not available
Universidade da Beira Interior	Engineering and Industrial Management	Instrumentation, Automation and Control	30 hours
Universidade de Évora	Instrumentation Engineering and Metrology		

PROPOSED SYLLABUS FOR A METROLOGY COURSE

Motivation: To propose a knowledge base to implement a metrology discipline, including uncertainty calculation in the curriculum of undergraduate courses in which analytical chemistry is applied;

Name suggestions: Chemical Metrology, Metrology applied to Analytical Chemistry;

Target audience: Undergraduate students in the areas of chemistry, chemical engineering, food engineering, pharmacy, and related areas;

Half-year working hours: 60;

Main objectives: To know the basic concepts of metrology, probability and statistics. Know the main references of the JCGM, International Metrology Vocabulary (VIM) and International Guide to the Expression of Measurement Uncertainty (GUM). Build measurement models. Estimate the result of a measurand with measurement uncertainty. Apply uncertainty as a criterion in decision-making. Use and choose certified reference materials (CRM). Validate analytical measurement procedures. Critically evaluate all previous steps.

Complete Program

1. Objectives of Metrology and Basic Industrial Technology: Standardization and Conformity Assessment, intellectual and industrial property, and management techniques. Purpose of the TIB. TIB as a tool for competitiveness and innovation. Risk management in commercial relationships. Intellectual property (trademarks and patents).
2. International Vocabulary of Metrology – Basic and. General Concepts and Associated Terms (VIM) concepts and definitions: Quantities and their properties. Quantity. Unit of measurement. Unit system. International System of Units. Value of quantity. Measurement. Metrology. Measuring. Measuring principle. Measurement method. Measurement procedure. Measurement result. Metrological traceability. True value of quantity. Measurement accuracy. Measurement accuracy. Measuring accuracy. Measurement error. Systematic error. Measurement bias. Random error. Measurement repeatability condition. Intermediate precision condition. Reproducibility condition. Measurement uncertainty. Type A assessment of measurement uncertainty. Type B assessment of measurement uncertainty. Standard uncertainty. Combined standard uncertainty. Relative standard uncertainty. Expanded measurement uncertainty. Coverage probability. Scope factor. Scope range. Calibration. Validation. Measurement model. Measuring function. Input quantity in a measurement model. Output quantity in a measurement model. Correction.
3. Random variable: Descriptive measures and graphical representations. Continuous random variable. Position and dispersion parameters. Probability distributions. Interval estimation.
4. Least Squares Linear Regression: Assumptions of the Least Squares Method. Cochran test. Estimation of regression parameters. Analysis of the quality of fit. Confidence curves and forecast limits.
5. Linear regression for a second-degree polynomial: Estimation of parameters and matrix of variances and covariances. Estimation of the predicted value and its uncertainty.
6. Special Topics: Analytical calibration curve (ACC) with significant uncertainty in standards. Law of propagation in matrix form. Evaluation of interlaboratory programs. Monte Carlo simulation and Bayesian approach.
7. Measurement model: Measurement function. Expectation and variance properties of a function of random variables. First-order Taylor series approximation of the variance of a function of random variables (law of propagation of uncertainties – LPU).
8. Input quantities: Estimation of position and dispersion of an input quantity. Treatment of outliers (Dixon, Grubbs and Chauvenet tests). Estimation of the standard uncertainty of an input quantity (Type A and Type B). Degree of freedom of an input quantity.
9. Sensitivity coefficients and uncertainty contributions: partial derivatives of input quantities. Uncertainty contributions to variance. Covariance uncertainty contributions.
10. Estimation of the combined standard uncertainty: Application of the Law of Propagation of Uncertainties to the measurement model. Estimation of input quantities. Estimation of the uncertainty of the output quantity by both strategies “Bottom-up” and “Top-down” uncertainty evaluations.
11. Estimate of expanded uncertainty: Scope factor. Coverage probability. t-Student distribution. Equation of the effective degree of freedom. Statement of result with expanded uncertainty.
12. Calibration and Metrological Traceability: Concepts of calibration and metrological traceability of the measurement result. Calibration measurement templates. Applying calibration correction and recovery correction to the measurement result.
13. Reference material and analytical validation: Reference material and certified reference material. Homogeneity and stability tests. Purpose of analytical validation. Concepts of accuracy and veracity in analytical validation. Analytical quality control.

14. Analytical validation parameters: Concepts and applications of accuracy, veracity, precision, selectivity, specificity, working range and quantification limits, decision, and detection limits. Validation planning. Declaration of results.
15. Decision Limit and Decision Rule: Normative limit and reference values. Calculation of the Decision Limit under the assumption of normality. Construction of the Decision Rule. Detection limit.
16. Interlaboratory Trials or Programs: Proficiency Trials (PT) and Collaborative Trials.

Resource Suggestion

Hours distributed in theoretical classes and practical classes in a computer lab with programming in spreadsheet and R software.

Internet sites: (links between <>)

- Agência Nacional de Vigilância Sanitária - Anvisa: <https://www.gov.br/anvisa/pt-br>
- Anvisa, Validação, RDC Nº 166, DE 24 DE JULHO DE 2017 validação de métodos analíticos: <http://antigo.anvisa.gov.br/documents/10181/2721567/RDC_166_2017_COMP.pdf/d5fb92b3-6c6b-4130-8670-4e3263763401>
- Bureau Internacional de Pesos e Medidas – BIPM: <https://www.bipm.org/en/home>
- Carta de nuclídeos, isótopos, isótonos, radionuclídeos, decaimentos radiotativos, etc: <https://www.nndc.bnl.gov/nudat3/>
- Constants, Units and Uncertainty: <https://physics.nist.gov/cuu/Constants/index.html>
- Documentos para acreditação no INMETRO: <http://www.inmetro.gov.br/credenciamento/organismos/doc_organismos.asp?tOrganismo=CalibEnsaios>
- Eurachem: <https://eurachem.org/>
- Euramet: <https://euramet.org/>
- Fundamental Physical Constants: <https://www.nist.gov/pml/fundamental-physical-constants>
- Guias da Eurachem sobre validação, incerteza, qualidade, acreditação, teste de proficiência, rastreabilidade e materiasi de referência: <https://eurachem.org/index.php/publications/guides>
- INMETRO: <https://www.gov.br/inmetro/pt-br>
- IUPAC: International Union of Pure And Applied Chemistry <https://iupac.org/>.
- JCGM Publications: Guides in Metrology: <https://www.bipm.org/en/committees/jc/jcgm/publications>
- Ministério da Agricultura, Pecuária e Abastecimento – MAPA: <https://www.gov.br/agricultura/pt-br/>
- NIST Livro de Química na Web: <https://webbook.nist.gov/chemistry/>
- National Institute of Standards and Technology – NIST: <https://www.nist.gov/>
- Physical Reference Data: <https://www.nist.gov/pml/productsservices/physical-reference-data>
- Redes metrológicas no Brasil: São Paulo, REMESP: <https://www.remesp.org.br/>
  Minas Gerais, RMMG: <https://www.rmmg.com.br/>
  Rio grande do Sul, <https://redemetrologica.com.br/>
- Sociedade Brasileira de Metrologia – SBM: <https://metrologia.org.br/wpsite/>

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CONCLUSIONS

As demonstrated through this work, the knowledge of metrological and statistical concepts, as well as their correct use, is paramount to leads for analytical results that could enable risk-based decision-making. It is shown that the incorrect use of the regression techniques has little effect on the analyte sample test predicted concentration; however, its measurement uncertainty can be completely different according to the used regression techniques. Contrarily, the common sense of the presented examples of analytical calibration curves shows that most of the analytical instruments based on electromagnetic and ionization radiation detection lead to heteroscedastic analytical instruments responses, and its calibration curve data should be fitted using the weighted least squares regression or the bivariate regression, as performed by the free CCC Software. To increase students' interest in statistics subjects, it is strongly recommended that examples of applications from routine chemical work be used. As future work, a critical assessment of the financial impact on the economy resulting from the correct use of data processing in Analytical Chemistry vis-à-vis the pitfalls discussed here is recommended.

Supplementary Material

The tabs "Data", "Var_x" and Var_y" in the Excel file are to be used to perform regressions using the CCC Software as explained by its "User manual (for Release 1.3)" available at the INRIM site.[27] The tabs "Cd Expl A5 QUAM OLS" and "Cd Expl A5 QUAM WLS" are spreadsheets with the OLS and WLS regressions to fit a straight line on the calibration curve data for Cd determination. These spreadsheets can be used to fit straight lines on other calibration curves. The following four tabs with names stating "CCC" have the results from CCC Software V. 1.3 for OLS or WLS rectilinear or parabole fit and some calculations in grey cells for the adapted Example A5 QUAM for Cd determination. The final four tabs have data to be pasted on the first three tabs of the file to use the CCC Software to fit the calibration curves of Cd or sulfur determinations.

This supplementary material can be accessed at the following doi: 10.30744/brjac.2179-3425.RV-98-2023-supp-material.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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ARTICLE

Design of Experiments (DoE) Application in Two Cases of Study in Pharmaceutical Industries

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This study illustrates the practical application of Design of Experiments (DoE) in two real-life scenarios within the pharmaceutical industry. The first case involved optimizing a chromatographic method to determine multiple analytes and their degradation products. The primary variable of interest was gradient time, and the most favorable outcomes were achieved at a pH value of 2. In the second case, we conducted a shelf-life study for a veterinary product, revealing that the vial filling variable exerted a statistically significant impact (p-value < 0.05). The incorporation of DoE in both cases played an important role in ensuring the attainment of dependable and statistically validated results.

Keywords: factorial design, shelf-life, optimization, quality by design, method development

INTRODUCTION

Design of Experiments (DoE)[1] is an important tool for variables characterization, analytical methods optimization,[2] quality assurance in industry, and a variety of important applications in many industrial and scientific fields.[3,4] The use of DoE techniques in pharmaceutical area (by companies or researchers) is,[5] in several cases, an obligation to save time, economic resources, improve the speed for results analysis and acquisition, and mainly to fulfill requirements described by international regulatory agencies,[6] complying with the Quality by Design (QbD),[7,8] and Analytical QbD (AQbD) directives.[9,10]

In a review presented by Patel and Kothari in 2018, the authors presented many aspects related to the implementation of multivariate approaches to degradation study and impurities detection in pharmaceutical companies. The authors emphasized the guidelines presented by the International Council for Harmonisation (ICH).[6]

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The optimization of chromatographic methods[11-13] takes special advantage of DoE[14] and several applications are found in the scientific literature. Peng *et al.*, for instance, presented a review focused in DoE tools for chromatographic conditions optimization.[15] Another field that currently employ DoE is product shelf-life and stability studies in pharmaceutical or food applications. Table I shows some selected papers from the period of 2015 to 2023 that employed DoE to shad light in several pharmaceutical problems. Most of the studies are related to method optimization employing QbD concepts combined with DoE.[16,17]

Table I. Scientific articles related to the use of DoE, chromatographic methods optimization, and shelf-life studies

Goal	Remarks
Formulation optimization[18]	KI tablet formulation optimization using a direct compression method. The authors employed mixture design.
Formulation optimization[19]	Investigation of 3 variables in the preparation of diclofenac sodium.
Stability prediction[20]	The authors proposed phenomenological models to predict the stability of vaccines.
Method optimization[21]	Simultaneous determination of Quinabut and its impurities using HPLC method.
Method optimization[22]	The authors used a central composite design (CCD) to optimize a method for Torsemide and Eplerenone determination.
Dissolution stability[23]	Investigation of modified-release drug product stability.
Method optimization[24]	Chromatographic method optimization to study the stability of ubidecarenone.
Method development[25]	The authors optimized a liquid chromatographic method for determination of glycopyrrolate in formulations.
Increase shelf-life of liposomes[26]	Study of 5 variables (cholesterol concentration, freezing conditions, among others) contribution in physico-chemical properties.
Determination of bioactive cannabinoids[27]	The authors combined DoE and exploratory analysis with (principal component analysis) PCA for data interpretation
Quantification of two analytes: Lamivudine and Zidovudine[28]	The authors used desirability function to simultaneously optimize method conditions for both analytes.

The main goal of this study was to report two frequent pharmaceutical problems that were analyzed using DoE. The first case is related to an analytical method developing using chromatography and the second, to shelf-life (stability) veterinary product investigation. Both studies were performed at pharmaceutical company laboratories by former students from the Chemistry Professional Master Graduation Program (https://www.ppgpq.ufscar.br).

MATERIAL AND METHODS

Material

As described in the previous sections, the experimental part of this study was divided in two sets: (1) chromatographic method development for separation of three actives pharmaceutical ingredients (API) and its impurities, developed at Libbs pharmaceutical company (Embu, São Paulo State, Brazil), and (2) shelf-life study of an API veterinary product. Some details about the experimental part are described in the next sections.

Chromatographic method development (case 1)

The method development was performed using HPLC and UPLC instruments from Agilent (model 1290) and Waters (model Acquity H-Class), respectively. The instruments were equipped with diode array detector (DAD) for chromatographic purity check. The chromatographic columns were Xterra RP8 and RP18 (both Waters), Eclipse XDB C18 and Zorbax SB C18 (both Agilent). Reference analytical standards were used as described by RDC 166/2017.[29] The organic solvents employed (methanol and acetonitrile) were from Merck and a Milli-Q system (Millipore) was used for ultrapure water.

Gradient time (variable 1) and mobile phase pH (variable 2) were investigated using a central composite design (CCD)[30] with centered face and all experiments were made in two authentic replicates. These variables were tested in three different levels (-1, 0 and 1) and varied from 30 min to 50 min in the case of variable 1 and from 2.0 to 4.0 for variable 2. A total of 21 experiments were performed: a 2^2 full factorial design (4 duplicated experiments), axial points with centered face (4 duplicated experiments) and 5 experiments in the center point (variables coded as 0). Figure 1 shows the CCD configuration with all experiments performed.

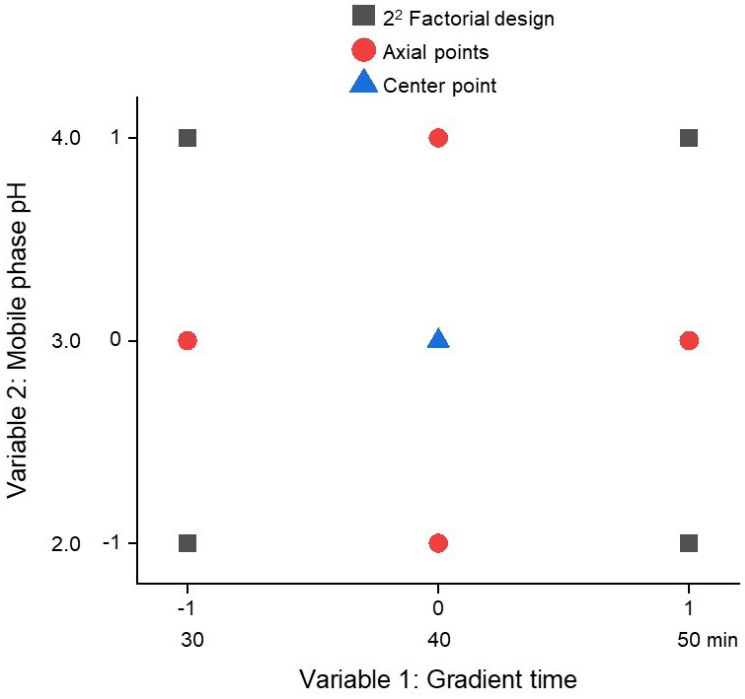

Figure 1. CCD performed in the case 1: 2^2 factorial design (black squares), axial points (red circles) and center point (blue triangle). Both normalized (between -1 and 1) and real conditions for variables 1 and 2 are presented.

Table II shows the experiments performed, and four responses were evaluated: (1) number of obtained peaks (y_1), (2) number of obtained peaks with resolution (R) higher than 1.5 (y_2), (3) global desirability (D) combining both y_1 and y_2,[31,32] and (4) resolution for a critical pair (R_{cp}).

Table II. CCD for case 1 (chromatographic method development) / The resolution values are related to the critical pair (R_{cp})

Experiment	Variables in normalized scale		Monitored responses			
	Time	pH	y_1	y_2	D	Resolution for the critical pair (R_{cp})
1	-1	-1	11	10	0.53	3.1
2	-1	-1	11	9	0.46	3.1
3	1	-1	10	9	0.33	3.0
4	1	-1	10	9	0.33	3.0
5	-1	1	10	9	0.33	2.8
6	-1	1	11	10	0.53	2.8
7	1	1	11	8	0.38	2.5
8	1	1	11	7	0.27	2.5
9	-1	0	15	7	0.46	2.9
10	-1	0	15	7	0.46	2.9
11	1	0	16	7	0.50	2.7
12	1	0	16	8	0.71	2.7
13	0	1	9	6	0.00	2.5
14	0	1	9	6	0.00	2.5
15	0	-1	11	8	0.38	2.9
16	0	-1	10	7	0.19	2.9
17	0	0	10	7	0.19	2.8
18	0	0	10	7	0.19	2.8
19	0	0	10	7	0.19	2.8
20	0	0	10	7	0.19	2.8
21	0	0	10	7	0.19	2.8

Responses y_1 and y_2 were combining after normalizing each one between 0 (not desired response, lowest resolution) and 1 (target response, highest resolution). In this case, each response (y_i) was transformed in individual desirability (d_i) as described in Equation 1.

$$d_i = \left(\frac{y_i - L}{T - L}\right)^s \qquad \text{Equation 1}$$

Where T and L are the target (highest value) and the lowest value, respectively. The index s is a weight, and in this specific case is 1.

The results for experiment 1 (see Table II, y_1 = 11 and y_2 = 10), for instance, can be normalized using the following mathematical expressions:

$$d_1 = \left(\frac{y_1 - L_1}{T_1 - L_1}\right)^S = \left(\frac{11 - 9}{16 - 9}\right)^1 = \frac{2}{7} = 0.29$$

$$d_2 = \left(\frac{y_2 - L_1}{T_2 - L_2}\right)^S = \left(\frac{10 - 6}{10 - 6}\right)^1 = \frac{4}{4} = 1$$

Both values can be combined using geometric mean, to obtain the Global desirability (D) for the first experiment:

$$D = \sqrt[n]{d_1 \times d_2 \times \cdots \times d_n} = \sqrt[2]{0.29 \times 1} = \sqrt{0.29} = 0.53$$

These calculations were repeated for all experiments and can be seen at Table II. More details will be presented in the Results and Discussion section.

All regression models obtained were calculated using Octave version 7.2.0 and the data organization and visualization were performed using Microsoft Excel®. Homemade mathematical routines[30,33] were prepared to perform analysis of variance (Anova) and statistical evaluation of the obtained regression models. In all cases for model performance evaluation, the confidence level was 95%.

Shelf-life study (case 2)

In this part of the study, HPLC was used to monitor the concentration of an API used in veterinary pharmaceutical product. The monitoring method employed a stationary phase Gemini C18 (Sigma-Aldrich), and the mobile phase was composed of methanol:acid solution (55:45% v v^{-1}) at gradient mode. The analytical signal was monitored at 330 nm.

Fifteen experiments were performed using a Doehlert design.[34] In this type of design, the variables can be verified in different number of levels. Then, three variables were tested: (1) time (days) in seven different levels (1, 21, 41, 61, 80, 100, and 120), (2) temperature (°C) in five levels (-20, -5, 10, 25 and 40), and (3) vial filling (%) in three levels (25, 62.5 and 100).

The normalized values for the time were from -0.866 (1 day) up to 0.866 (120 days). In the case of variable (temperature), the coded values varied from -1 (-20 ºC) up to 1 (40 ºC). Variable 3 (vial filling) coded values goes from -0.817 (25%) up to 0.817 (100%). As the time is a very important aspect of shelf-life study, more importance was given to this variable that was monitored in 7 levels (from 1 day up to 120 days). The monitored response was the API concentration in % m m^{-1}. The variable 3 (vial filling) was intended to understand how the air inside de vial and its interaction with time and temperature can contribute to the API stability. The level 25% means that 75% of the vial was empty. Table III shows the performed experiments and three replicates were prepared at the central point (variables coded as 0). Like the previous section, the data and regression models obtained were also handled using Microsoft Excel® and Octave, respectively. The confidence level for model evaluation was also 95%. Figure 2 shows the configuration of the 3 variables in a 3D visualization.

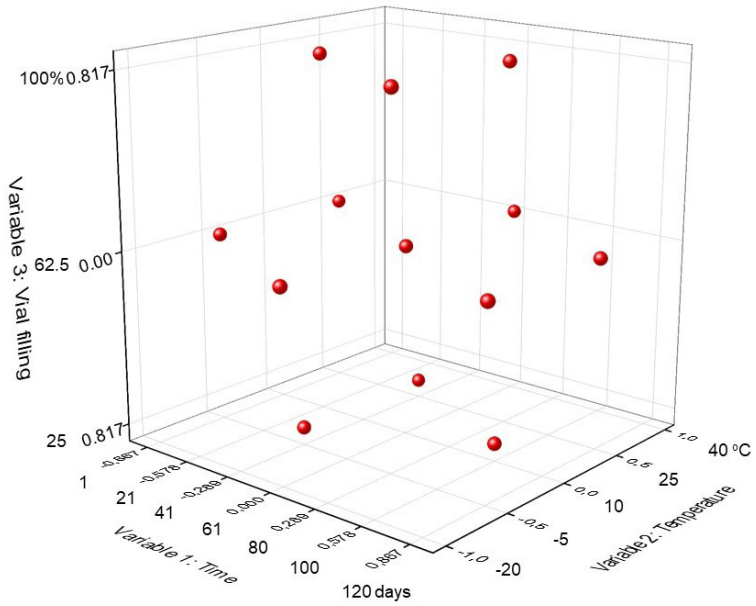

Figure 2. Doehlert design performed in the case 2. Both normalized and real conditions for the three variables (time, temperature and vial filling) are presented.

Table III. Doehlert design for case 2 (shelf-life study for a veterinary API)

Experiment	Variables in normalized scale			Monitored response
	Time	Temperature	Vial filling	[Analyte] % m m^{-1}
1	0	1	0	60.48
2	0.866	0.5	0	60.54
3	0.289	0.5	0.817	60.94
4	0	-1	0	61.01
5	-0.866	-0.5	0	60.21
6	-0.289	-0.5	-0.817	59.84
7	-0.866	0.5	0	60.29
8	-0.289	0.5	-0.817	60.06
9	0.866	-0.5	0	59.43
10	0.577	0	-0.817	60.41
11	0.289	-0.5	0.817	61.66
12	-0.577	0	0.817	61.35
13	0	0	0	61.24
14	0	0	0	60.41
15	0	0	0	60.93

RESULTS AND DISCUSSION

Chromatographic method development (case 1)

Figure 3 shows a pictorial description of a typical obtained chromatogram. The two red peaks are the critical pair that was also monitored, and the resolution (R_{cp}) between them was considered as response.

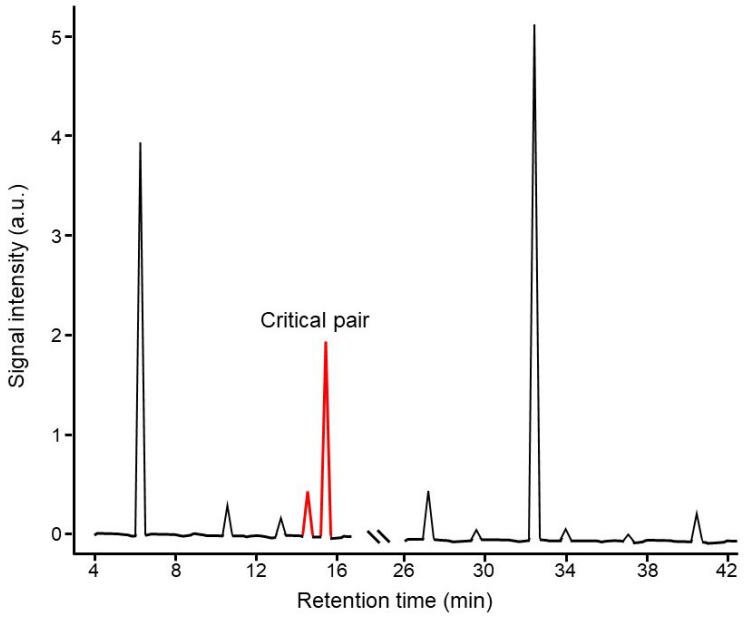

Figure 3. Pictorial description of chromatogram for case 1 (HPLC method optimization). The peaks illustrated in red are related to the critical pair.

Table II shows the four responses obtained: y_1, y_2, D (Global desirability combining y_1 and y_2), and resolution of the critical pair. The goal of this first study of case was to maximize all monitored responses. The individual models for y_1 and y_2 presented lack-of-fit and the *p*-values[35] obtained when the mean square of regression (MSR) and mean square of residue (MSr) were 0.002 and 0.003, respectively. A global model was calculated combining both y_1 and y_2 (see details in section *Chromatographic method development (case 1)*), and global desirability (D) was taken into consideration.[2] Six coefficients(b_i) were calculated using least squares[36] as describe in Equation 2.

$$b = (X^T X)^{-1} \times X^T y \qquad \text{Equation 2}$$

Where X is a matrix with n rows (number of experiments) and m columns (coefficients b that will be calculated). In the specific situation of this case of study, n = 21 (experiments, see Table II) and m = 6 (coefficients). The calculated coefficients are b_0 (constant), b_1 and b_2 (linear coefficients for the variables, time and pH), b_1^2 and b_2^2 (quadratic coefficients for the variables), and b_{12} (interaction between the two variables). The general idea behind Equation 2 is to minimize the error between the predicted response $\hat{y}$ (in this case $\hat{D}$) and the experimental value y (*D*, see Table II).

The *p*-value obtained for MSR and MSr was 0.0006 that demonstrate the both MS are statistically different. On the other hand, the proposed model presented LoF, and the *p*-value comparing MSLoF and MS of pure error (MSPE) was 0.01 (lower than 0.05, then significative). As the model presented lack-of-fit, the MSLoF was used to calculate the confidence interval of the coefficients.[30] Figure 4 shows the results obtained. As can be observed only the intercept or constant (b_0) and the quadratic coefficient for variable 1, time (b_1^2) were statistically significative.

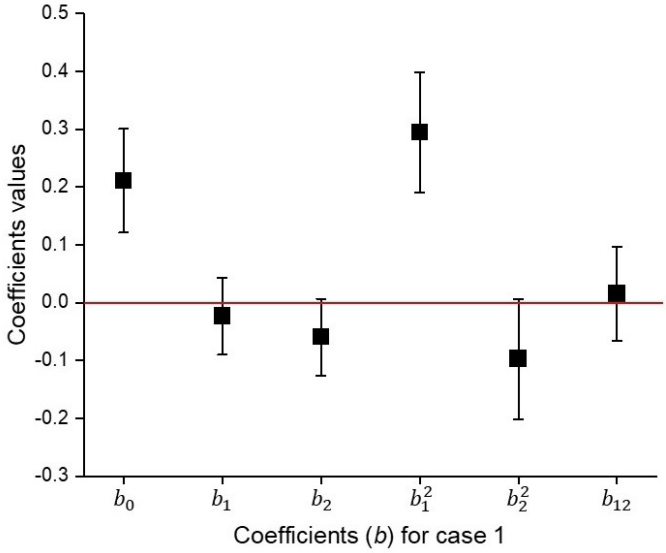

Figure 4. Coefficients (b) and its confidence interval (error bars) for the case 1 considering D as response.

From Figure 4 it is noted that some confidence intervals (C.I.) can assume the 0 value, and their correspondent coefficients are not significant. The C.I. was calculated according to Equation 3.

$$C.I. = \sqrt{MSLoF \times main\ diagonal\ of\ (X^t X)^{-1}} \times t_{(n-1.95\%)} \qquad \text{Equation 3}$$

Where $t_{(n-1.95\%)}$ is the tabulated value of *t* for *n* – 1 degree of freedom of the MSLoF.

The Anova table of the proposed model can been shown in Table IV.

Table IV. Anova table for the proposed model using D as response

Parameters	Sum of Squares (SS)	Degrees of freedom	Mean of Squares (MS)	Calculated F	*p*-value
Regression	0.480	5	0.096	$\frac{MSR}{MSr} = \frac{0.480}{0.173} = 8.34$	0.0006
Residue	0.173	15	0.012		
Total	0.653	20	0.033		
Pure error	0.069	12	0.006	$\frac{MSLoF}{MPE} = \frac{0.104}{0.069} = 6.01$	0.01
Lack-of-fit	0.104	3	0.035		

The non-significant coefficients (b_1, b_2, b_{12} and b_2^2, see Figure 4) were removed, and the Sum of Square of Pure Error was calculated again using the new replicates. The model was also calculated again, and Equation 4 shows the final proposed model (95% of confidence level) when D was monitored.

$$D = 0.17 + 0.27 v_1^2 \qquad \text{Equation 4}$$

As can be noted, only the constant (b_0 = 0.17) and the quadratic coefficient for variable 1 (b_1^2 = 0.27, time) were statistically significant (*p*-value < 0.05). Figure 5 shows the response surface obtained and it is possible to see that both short (30 min) or long (50 min) gradient times can be used. In the case of pH, any value in the range from 2.0 to 4.0 can be used to obtain both high y_1 and y_2 (high D value).

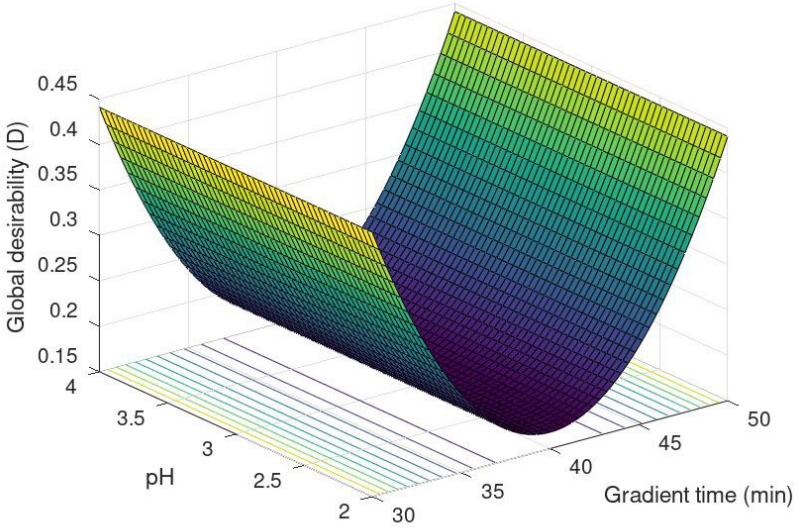

Figure 5. Surface response for case 1 with variables 1 (gradient time, min) and 2 (pH). The monitored response was the global desirability (D) considering responses y_1 and y_2 (see details in Table II).

On the other hand, the D was the only response monitored and the R_{cp} need to be also considered. The regression model for the R_{cp} can be seen at Equation 5.

$$R_{cp} = 2.76 - 0.10v_1 - 0.20v_2 + 0.078v_1^2 - 0.050v_1v_2 \qquad \text{Equation 5}$$

This regression model presented more significative coefficients and Figure 6 shows the surface response obtained. The best results can be obtained using only 30 min for variable 1 (shorter time) and pH = 2.0. Several validation experiments were performed using these instrumental conditions, and the precision values (%) for the APIs monitored varied from 99.9±0.7 up to 101.2±0.2.

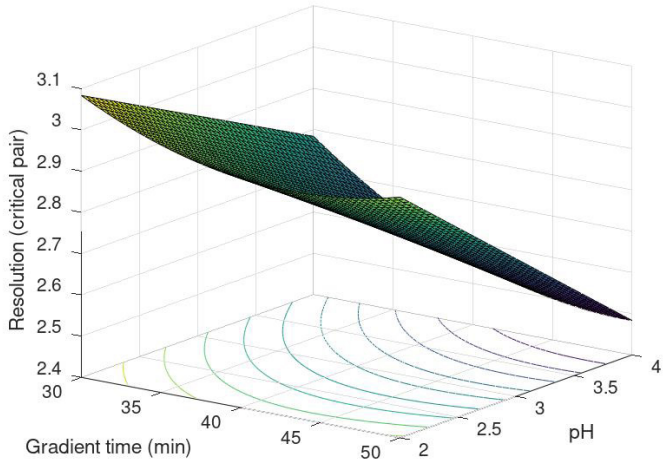

Figure 6. Surface response for case 1 with variables 1 (gradient time, min) and 2 (pH). The monitored response was the R_{cp} (see details in FIGURE 1 and Table II).

Shelf-life study (case 2)

The goal of the second case was to observe if variables related to shelf-life are affecting the concentration of the API in the final product. Figure 7 shows a pictorial description of typical chromatogram, and the concentration of the API is proportional to the signal area sum of the four analytes depicted: 1, 1a, 2a+2b and 2. Table III shows the results obtained for the Doehlert design performed, and as can be observed, the concentration of the API presented a very narrow range: from 59.43% m m^{-1} (experiment 9) up to 61.66 (experiment 11), with average, standard deviation and median of 60.59, 0.60 and 60.48, respectively. The Kurtosis and Skewness[37] of the values were -0.31 and -0.03, respectively, that reflects in a flat distribution of the data and similarity between average and median.

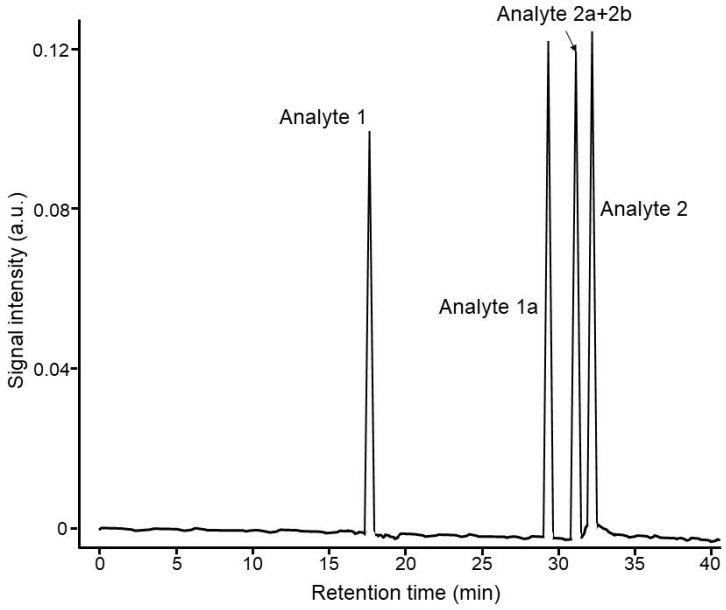

Figure 7. Pictorial description of chromatogram for case 2 (shelf-life study). The peaks identified (1, 1a, 2a+2b and 2) correspond to the veterinary API.

A regression model was calculated and evaluated to observe which variable and in which extension affect the response. But now 10 coefficients were calculated: b_0 (constant), b_1, b_2, b_3 (the linear coefficients for each variable), b_1^2, b_2^2, b_3^2 (quadratic coefficients), b_{12}, b_{13} and b_{23} (interaction coefficients). The three replicates performed at the central point (experiments 13, 14 and 15) were used to calculate the MSPE. Figure 8 shows the coefficients calculated and its interval of confidence (error bars). As can be noted only b_0 and b_3 were significant with 95% of confidence level.

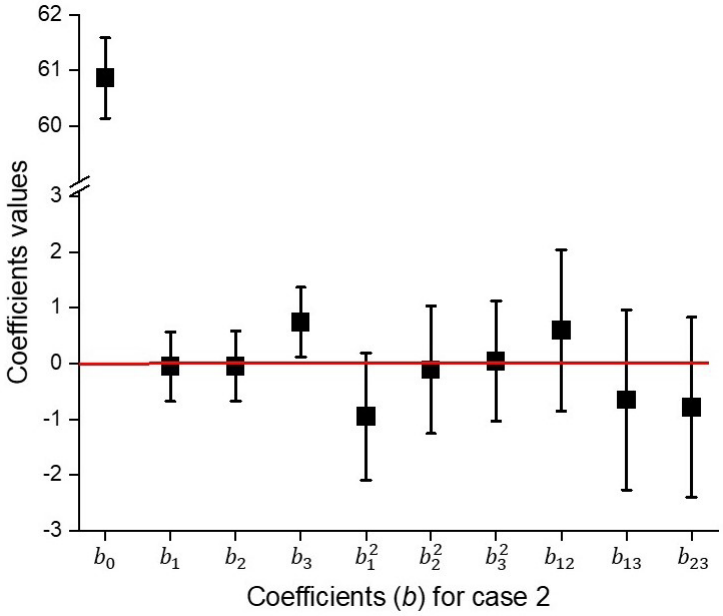

Figure 8. Coefficients (b) and its confidence interval (error bars) for the case 2 considering the concentration of the API as response.

As can be observed using Table III and Figure 8, the regression model calculated presented very poor statistical parameters due to the fact the low variation of the response. The model was recalculated excluding the non-significative coefficients and the obtained one can be seen in Equation 6.

$$[API]\%\, m\, m^{-1} = 60.86 + 0.74 v_3 \qquad \text{Equation 6}$$

Figure 9 shows the distribution of the residues when the experimental and predicted values were compared. As can be noted the residues follow a normal distribution with average, standard deviation and median of -0.00013, 0.45, and -0.047. The Kurtoses and Skewness[37] of the results were 1.87 and -0.96 that reflects in distribution in cume and low asymmetry (similarity between average and median).

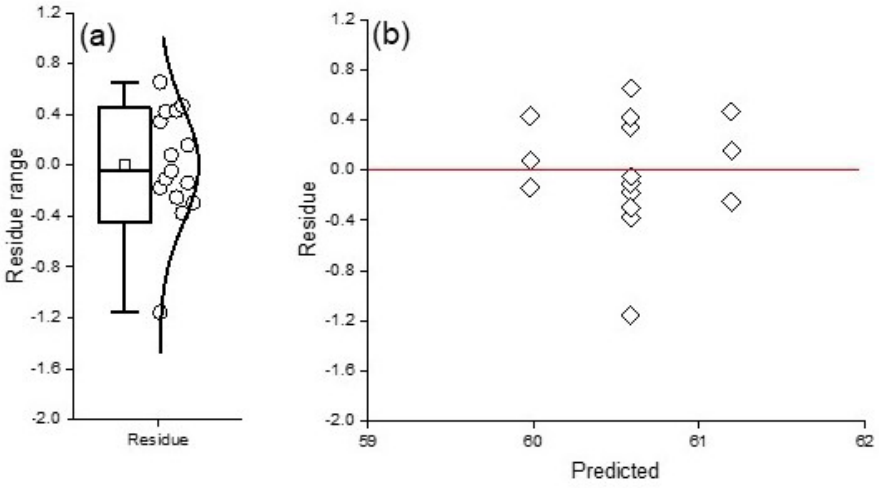

Figure 9. Statistical evaluation of the residues of the proposed model: (a) The box size is proportional to the standard deviation, the small square and the horizontal line inside the box are the average and median, respectively. The bars show the range of the values (minimum and maximum); (b) predicted $\hat{y}$) *versus* residues values for the proposed model.

The residues were evaluated in combination with the predicted values using Shapiro-Wilk[38] test, and it was observed a normal distribution

Figure 10 shows the surface response for the regression model obtained. The vial filling parameter effects and its confidence interval is positive and around 1.5±1.2% m m^{-1}. The other variables contributions were negligible (*p*-value > 0.05) in the studied range, being the API stable for a period of up to 4 months, even when submitted to temperatures from -20 up to 40 ºC. The volume of product inside the vial is a significative variable (variable 3), but its contribution for the final concentration is not critical for the purpose of the veterinary pharmaceutical product.

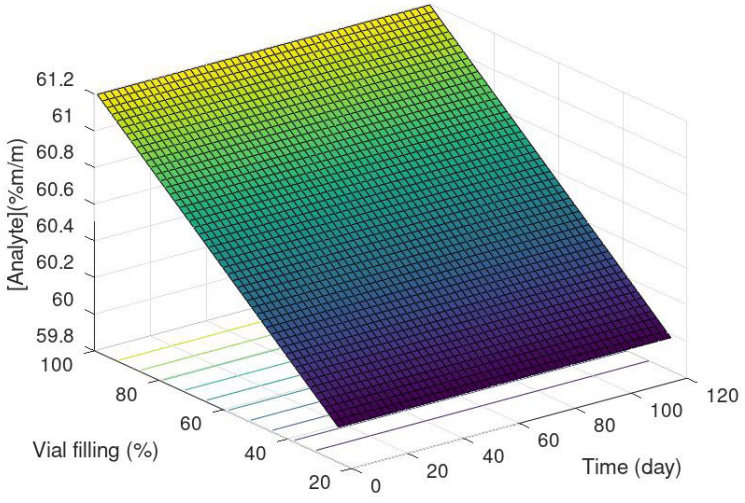

Figure 10. Surface response for case 2 with variables 1 (time, day) and 3 (Vial filling). The monitored response was the concentration of the veterinary API. The variable 2 (Temperature) was fixed in 10 ºC (normalized as 0).

CONCLUSION

Both examples presented in this study yielded reliable results that underwent statistical evaluation. Furthermore, the optimization of methods and assessment of shelf life reflected into significant economic benefits for the companies involved, underscoring the essential role of Design of Experiments (DoE) in achieving diverse goals. In the context of method optimization, retention time emerged as the most important variable, while pH played a critical role in enhancing resolution for the critical pair (see Figure 6). In the stability study (case 2), vial filling was the most critical variable. Notably, the contact between the product and air, although statistically significant, had a relatively minor effect, accounting for less than 2% of the observed variance.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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ARTICLE

Development and Performance Evaluation of Low-Cost Cellophane Paper-Based Biosensors for Polyphenol Detection in Teas: A Cost-Effective Alternative to Teflon® Membranes Biosensors

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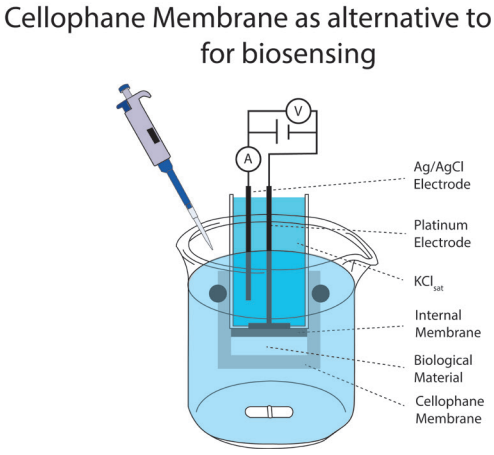

This study introduces biosensors developed using cellophane paper with immobilized polyphenol oxidase (PPO) from dwarf banana and eggplant peels, integrated with glutaraldehyde and a Clark electrode. These biosensors, optimized for enzyme concentration at 75 units for dwarf banana peel and 100 units for eggplant peel, demonstrated high sensitivity to catechol, paracetamol, pyrogallol, and hydroquinone, similar to traditional Teflon® Clark electrodes. Despite the lower measurement capacity of cellophane membranes (20-30) compared to Teflon's (300-400), the cost-effectiveness and accessibility of cellophane offer a strategic advantage. These biosensors effectively quantified polyphenols in teas, with results closely matching those from the standard Folin-Dennis method and Teflon® electrodes, maintaining a 95% confidence level and an error margin of less than 1%. Hence, the cellophane paper-based biosensors present a cost-effective, efficient alternative for polyphenol detection in teas, promising broader application due to their affordability and performance.

Keywords: Polyphenol oxidase, cathecol, eggplant peels, dwarf banana peels, Clark electrode, low-cost phenol sensing

INTRODUCTION

Selectivity is one of the biggest problems in analytical chemistry, and it is usually obtained only through intense control of the experimental conditions. However, in nature, highly selective structures are found in enzymes, antibodies, and others. Enzymes, in particular, have several advantages, such as a joyful combination of selectivity with sensitivity, allowing the use of various transduction technologies.[1] Current scientific publications describe various devices that exploit enzymes (biological material) in combination with electrochemical transducers.[2-6]

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In Brazil, federal legislation establishes a differentiated maximum limit of total phenols concerning the disposal of effluents in bodies of water. This resolution imposes a maximum concentration of 0.5 mg L^{-1} of phenols.[7] Phenolic compounds, abundant secondary metabolites in the plant kingdom, are extensively studied for their diverse roles. These compounds primarily serve as defense mechanisms in fruits and vegetables, imparting astringency, color, flavor, and aroma. In humans, consuming these compound-rich fruits and vegetables is beneficial mainly due to their potent antioxidant properties, which safeguard against oxidative stress and aid in reducing inflammation and preventing cardiovascular diseases, certain cancers, and premature aging. Interestingly, many medicinal drugs are derived from plants rich in phenolic compounds. For instance, *Cannabis sativa* is notable for its high phenolic content, utilized in controlled doses for the treatment of behavioral disorders under professional guidance. However, it is crucial to acknowledge the potential toxicity of these compounds; unregulated consumption can lead to adverse effects such as liver damage. In the pharmaceutical industry, even synthetic drugs like paracetamol, containing a phenolic group, demonstrate the dual nature of these compounds – acting as effective analgesics at prescribed doses while posing risks of severe liver damage when consumed excessively. Similarly, hydroquinone, used in chemical peels, exemplifies the potential for skin damage and high toxicity.

This underscores the critical need for efficient, rapid, and sensitive methods to quantify phenolic compounds, balancing their beneficial uses against potential risks. Thus, growing investment in developing technologies that carry out this monitoring, such as biosensors, is promissory. Biosensors reveal the great potential for monitoring phenol compounds, enabling speed and efficiency.[8] They could be advantageous to be low-cost depending on the enzyme source and immobilization process. In addition, they have unique characteristics, such as selectivity, low cost of construction, storage, and detection limit; ease of automation; and construction of simple and portable equipment for on-site monitoring.[9]

The incorporation of molecules with biological activity has significantly increased the development of these biosensors using the enzyme polyphenol oxidase (PPO) extracted from crude extracts of vegetables and fruits,[2,10-14] for quantification of these analyses in different applications for environmental, pharmaceutical, and food areas and using expensive substrates to immobilize the biologically active molecule. Related to PPO enzyme sources, we can cite works in 2015 by Ribeiro et al.[15] developed a yam biosensor for detecting phenols in wastewater. Vega and collaborators[16] built a nanosensor for detecting phenols, analyzing wastewater in agriculture, and containing polyphenols from fertilizers and pesticides. Sousa and collaborators[17] built a portable voltammetric biosensor using Wi-Fi technology to analyze phenolic compounds in an urban environment using a biosensor composed of three microelectrodes, with the PPO pure enzyme placed on the working microelectrode, and the sample to be analyzed needs to come into contact with the three microelectrodes and the enzyme.

In summary, the literature has shown the development of biosensors using PPO enzyme and Teflon® membrane to immobilize the enzyme with glutaraldehyde (a polymerizing agent)[18-21] is an interesting combination. However, there are still challenges to effectively commercializing biosensors, and sometimes, it is related to the substrate for the immobilization of the enzyme. Hence in the present work, the enzyme PPO from crude extracts was immobilized on a cellophane paper, a low-cost material compared to Teflon®, with glutaraldehyde. The advantage of the proposed developed method is based on reducing the cost and complexity in the construction of the biosensor without loss of stability, response time, selectivity, and detection limit. The method for quantifying and immobilizing is straightforward, involving a single-step process where polymerization and enzymatic immobilization happen concurrently. PPO facilitates the conversion of monophenols and diphenols into quinones, utilizing molecular oxygen in the oxidation process.[22] The quinones formed in this reaction polymerize to form melanins.[22] Thus, the concentration of phenolic compounds will decrease the oxygen concentration in the samples measured with a Clark-type oxygen electrode.

MATERIALS AND METHODS

Equipment

To obtain the raw extracts of eggplant and dwarf banana peels in 0.1 mol L^{-1} phosphate buffer solutions of pH 6.5,[23] a crusher (blender) and an Analyzer model 300 pH meter in addition to a centrifuge from Du Pont Instruments Sorvael, model RC-5B Plus. An Analyzer Orion model VIS 7220 spectrophotometer was used to determine the extracts' enzyme activity and total protein. The Clark-type electrode and the oxygen analyzer used to study biosensors are from DIGIMED, model DM-CO1 (electrode), and model DM-4 V5 (analyzer).

Reagents

Polyclar SB-100 (Indústria ISP do Brasil Ltda), Polyvinylpyrrolidone (PVP), was used to remove excess natural polyphenols in the crude extracts studied through hydrogen bonding. It stabilizes juices, wines, and beer, insoluble in water, presenting easy removal. Analytical grade reagents were used to prepare 0.1 mol L^{-1} phosphate buffer solutions (pH 6.0-7.5), 0.1 mol L^{-1} acetate buffer solutions (pH 3.0-5.5), and catechol and pyrogallol standard solutions (Sigma Chemical Co-St. Louis, Mo, USA), paracetamol, and hydroquinone (Reagen, Laboratory Products, Paraná, Brazil), which was prepared daily. Bovine serum albumin solution (1%) m/V (Sigma Chemical Co) was used as a standard in protein analysis. The glutaraldehyde solution (2.5%) m/V (Reagen, Laboratory Products, Paraná, Brazil) will be used together with a cellophane paper membrane (acquired in the packaging market) as a support for the enzyme immobilization. Samples of some types of filter bag teas, such as mate, black, chamomile, mint, fennel, and green from the Oetker brand, were used to quantify polyphenols by the amperometric biosensors produced by dwarf banana and eggplant peels. All tea samples were obtained from the local market. Some additional information about the composition of the teas used:

- Black tea: high content of flavonoids, polyphenols, tannins, caffeine, theophylline, and B complex vitamins.
- Mate tea: caffeine, alkaloids, proteins, carbohydrates and lipids.
- Chamomile tea: Chamazulene, α-bisabolol and α-bisaboloxides.
- Mint tea: menthol, ascorbic acid, flavonoids and terpenes.
- Green tea: pigments and carbohydrates.
- Fennel tea: coumarin and malic acid.

The tannic acid solution (0.1 mg L^{-1}) and saturated sodium bicarbonate (Reagen) were prepared for spectrophotometric analysis of total polyphenols and the Folin-Denis reagent for the standard curve of the tea samples.

Analytical procedure

Biological Material

Eggplants and dwarf bananas were purchased in the local market (250 g each), approximately two eggplants and 2 dwarf bananas.

Extraction of the enzyme Polyphenol oxidase from eggplant and banana peels

Twenty-five grams of each biological material were peeled into small pieces and homogenized in a blender with 50 mL of 0.1 mol L^{-1} phosphate buffer solution, pH 6.5, containing 2.5 g of Polyclar for the eggplant pee[24] and 7.5 g of Polyclar for dwarf banana peel.[23] Then, they were centrifuged at 14000 rpm for 20 minutes. The supernatant solutions were stored in a freezer as a source of PPO enzyme for activity measurements, total protein, and biosensor construction.[13]

Determination of polyphenol oxidase activity in crude extracts

The activity of soluble PPO, found in biological substances, was determined by measuring the absorbance at a wavelength of 410 nm. This measurement corresponds to the melanin produced from

the polymerization of quinine, which occurs after a reaction. This reaction involves mixing 0.2 mL of the supernatant solution, derived from each extract mentioned previously, with 2.8 mL of catechol in a 0.1 mol L^{-1} phosphate buffer solution at a pH of 6.5 and a temperature of 25 °C.[14] The reaction was monitored for 2 minutes,[25] the time necessary to reach V_{max}. The activity unit is described as the quantity of enzyme necessary to produce a 0.001 increase in absorbance units per minute, following the conditions outlined in Equation 1.

$$a = \frac{\Delta A.\,60.1000}{\Delta t.\,d.\,V_{sample}} \qquad \text{Equation 1}$$

where: a = activity / U mL^{-1}; ΔA = change in absorbance; Δt = time variation / min; d = diameter of the cuvette; V_{sample} = sample volume / mL.

The total supernatant protein solutions of the crude extracts were quantified by the biuret method[25] using bovine serum albumin as a standard.

Biuret solution for dosage of total protein in the studied crude extracts

The solutions were prepared by adding 1.50 g of $CuSO_4.5H_2O$ and 6.0 g of sodium potassium tartrate separately dissolved in 500 mL of distilled water. With constant stirring, 300 mL of 10% NaOH was added. It was diluted to 1 L with distilled water and stored in a refrigerator. This solution is generally stable for over 1 year, but if a red precipitate of CuO appears, it is discarded and prepared again.

Standard solution of bovine serum albumin protein (1%) m/V

A 1% w/v bovine serum albumin solution (Sigma Chemical Co) was used, and it was prepared in 0.1 mol L^{-1} phosphate buffer solution, pH 6.5.

Method of immobilizing polyphenol oxidase (PPO) enzyme with glutaraldehyde bifunctional reagent using cellophane paper and biosensor preparation

A cellophane paper was used to support the immobilization of PPO present in crude extracts of dwarf banana and eggplant peels. The circular areas of the membranes were covered, with different amounts of units of PPO solutions, from the crude extract of dwarf banana peel (25 U; 50 U; 75 U, and 100 U) and the crude extract of eggplant peel (50 U; 75 U; 100 U and 150 U) with glutaraldehyde 2.5% w/v, in the same proportion.[2] The membranes were dried in a desiccator at 25 ºC for 10 hours.

These polymerization step, particularly using glutaraldehyde, is crucial in our biosensor design for several reasons. Primarily, it promotes the covalent bonding of the enzyme protein to the membrane surface, leading to the enzyme's insolubilization. This immobilization is essential as it retains the enzyme's catalytic activity while preventing its dissolution into the solution during the biosensor's operation. Furthermore, the use of glutaraldehyde does not only immobilize the enzyme but also forms a gel-like layer on the membrane's surface. This gel layer is significant for creating a stable microenvironment around the enzyme, which can enhance the overall stability and activity of the biosensor. The gel matrix allows for a controlled diffusion of analytes to the enzyme's active sites, which is crucial for consistent and reproducible sensor responses.

Two minutes before the determinations, they were introduced into a 0.1 mol L^{-1} phosphate buffer solution, pH 6.5, at a temperature of 4 ºC to carry out the amperometric measurements. To perform the amperometric measurements, the membranes were placed at the end of the oxygen electrode and fixed with a rubber ring (Figure 1).

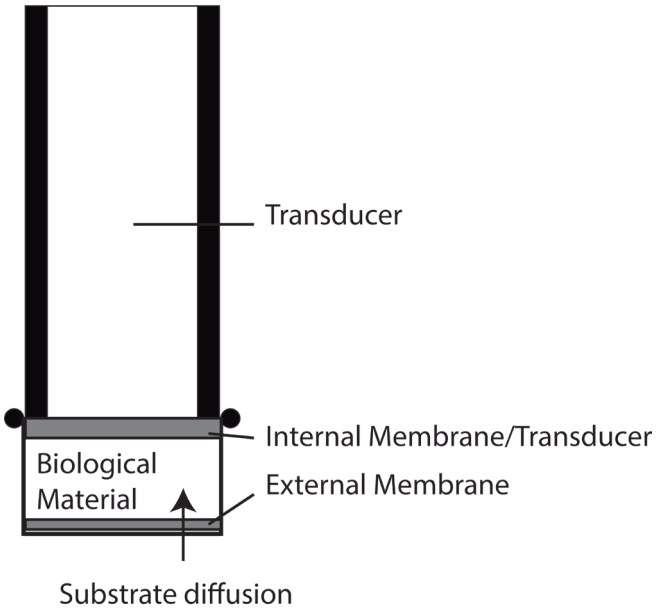

Figure 1. Schematic representation of the experimental set-up.

The biosensor, which consists of an oxygen electrode equipped with each created enzymatic membrane, was positioned in a glass cell. This cell contained 50 mL of phosphate buffer solution with a concentration of 0.1 mol L^{-1} and a pH of 6.5, maintained at a temperature of 25 ºC. After stabilization, 1.0 µL of catechol solution 5.0×10^{-5} mol L^{-1} is added with constant stirring.

The biosensors were used for amperometric measurements to quantify polyphenols in some tea samples. For comparison, Teflon® membranes (Celgard 2400) were fabricated using the same procedure, changing the cellophane paper for Teflon® membranes.

Amperometric quantification of phenolic substrates concentration

The biosensor, which consists of an oxygen electrode equipped with each created enzymatic membrane, was positioned in a glass cell. This cell contained 50 mL of phosphate buffer solution with a concentration of 0.1 mol L^{-1} and a pH of 6.5, maintained at a temperature of 25 ºC. After stabilization, increasing volumes of 10 mmol L^{-1} catechol substrate (10-200 µL) were added at 1-minute intervals between each addition, with constant agitation. The addition of the substrate causes a decrease in the O_2 concentration with the consequent decrease in the reduction current of this species.[1] The sample concentration containing polyphenols was determined by the same procedure, using the analytical curve thus obtained (a curve was plotted for each sample studied).

Effect of immobilized polyphenol oxidase concentration on biosensors

The crude extracts of eggplant and banana peels studied showed the best source of the enzyme PPO; thus, they were selected for this project.[23,24] The effect of the concentration of PPO (units) immobilized on the cellophane paper matrix was studied, as indicated in “Method of immobilizing polyphenol oxidase (PPO) enzyme with glutaraldehyde bifunctional reagent using cellophane paper and biosensor preparation”, concerning the response of these biosensors and/or stability.

Effect of pH on the response of amperometric biosensors

The effect of pH on the biosensor response of eggplant and dwarf banana peels was studied in the pH range ranging from 3.0-7.5; using the catechol substrate at a concentration of 5.0×10^{-5} mol L^{-1}.[1]

Determination of response time for the studied biosensors

The response time for the biosensors of eggplant and dwarf banana skins was determined using catechol 5.0 × 10^{-5} mol L^{-1} as substrate, and the number of determinations performed for each biosensor was also studied.

Relative response of biosensors to different phenolic substrates

The relative response to the biosensors of eggplant and dwarf banana skins was determined using phenolic substrates such as catechol, pyrogallol, paracetamol, and hydroquinone (2.5 ×10^{-3} mol L^{-1}) in 0.1 mol L^{-1} phosphate buffer pH 6.5.[23,26]

Preparation of tea samples

The samples were prepared by infusing 10 g of tea in 15 mL of double-distilled water (previously heated, 80 ºC). The infusion time was 10 minutes, after this infusion period, the temperature of the solution was approximately 40 ºC. Then, this solution containing the tea was placed in a volumetric flask, and the volume was made up to 25 mL with 0.1 mol L^{-1} phosphate buffer solution, pH 6.5.

Determination of total Folin-Denis phenols (Standard Spectrophotometric Method) for polyphenols in tea samples

The Folin-Denis reagent was prepared as described in the literature.[27] The standard curve is prepared using aliquots of 0-10 mL of tannic acid (0.1 mg L^{-1}), 5 mL of Folin-Denis reagent, and 10 mL of sodium bicarbonate (saturated solution) in a 100 mL flask. After 30 minutes, the absorbance at 760 nm is determined.

The blue-green color produced in this method results from a colorimetric reaction involving the reduction of phosphomolybdic-phosphotungstic acid by phenolic compounds. The intensity of the color produced depends on the phenolic compounds amount present in the sample.

RESULTS AND DISCUSSION

Determination of polyphenol oxidase activity and total protein in crude extracts of dwarf banana and eggplant peels

Crude extracts from banana and eggplant peels were studied as biocatalytic materials for the oxidation of the catechol phenolic substrate. Table I shows the activities (U mL^{-1}) found in these extracts, total protein (mg mL^{-1}), and specific activity (U mg^{-1} of total protein). As we can see in this table, the specific activity of PPO in the crude extract of dwarf banana peel was better than in eggplant peel; however, compared to the purified enzyme, both showed higher activity and could be used for biosensor fabrication. The stability times of crude extracts of dwarf banana and eggplant peels about PPO enzyme were studied being stored in a freezer, it was 40 days for the banana peel and 34 days for the eggplant peel, being able to work at temperatures close to 35 ºC for the two extracts, without significant loss of enzyme activity.

Table I. Activity, total protein and specific activity of polyphenol oxidase in crude extracts of dwarf banana and eggplant peels

Material	Activity (U mL^{-1})	Total Protein (mg mL^{-1})	Specific Activity (U mg^{-1} of protein)
Crude extract of dwarf banana peel	30900	24.0	1287.5
Crude extract of eggplant peel	22350	27.5	812.8
Pure Enzyme*	-	-	2400
Purified** Enzyme mushroom	1075	23.0	47

* Sigma The linear regression for the standard curve of bovine serum albumin for the dosage of total protein in the two extracts studied was Absorbance = 0.00548 + 0.03507 (Protein mass/mg); **Ref. No. 28.

Effect of polyphenol oxidase concentration of crude extracts studied on cellophane paper biosensor and comparison with Teflon® membrane

Crude extract of dwarf banana peel

The study of the effect of PPO concentration of crude extracts on cellophane paper biosensors and comparison with Teflon membrane using Clark´s electrode showed that the biosensor of crude extract of dwarf banana peel in cellophane paper behaves similarly to the biosensor built-in Teflon (Figure 1), with respect to the concentration of immobilized enzyme (75 U).

Hence, there is an advantage in using cellophane paper because the cost-benefit in relation to Teflon is much higher (cellophane paper 1 dollar per meter while a 0.5 cm diameter Teflon membrane costs 88 dollars). The biosensor response increases with increasing concentration of the enzyme immobilized on the outer membrane of the oxygen electrode up to 75 U for both cases, decreasing next (Figure 2). This could be attributed to the thickening of the enzymatic layer, which hampers the transport of both the substrate and molecular oxygen, consequently leading to a reduction in oxygen consumption.

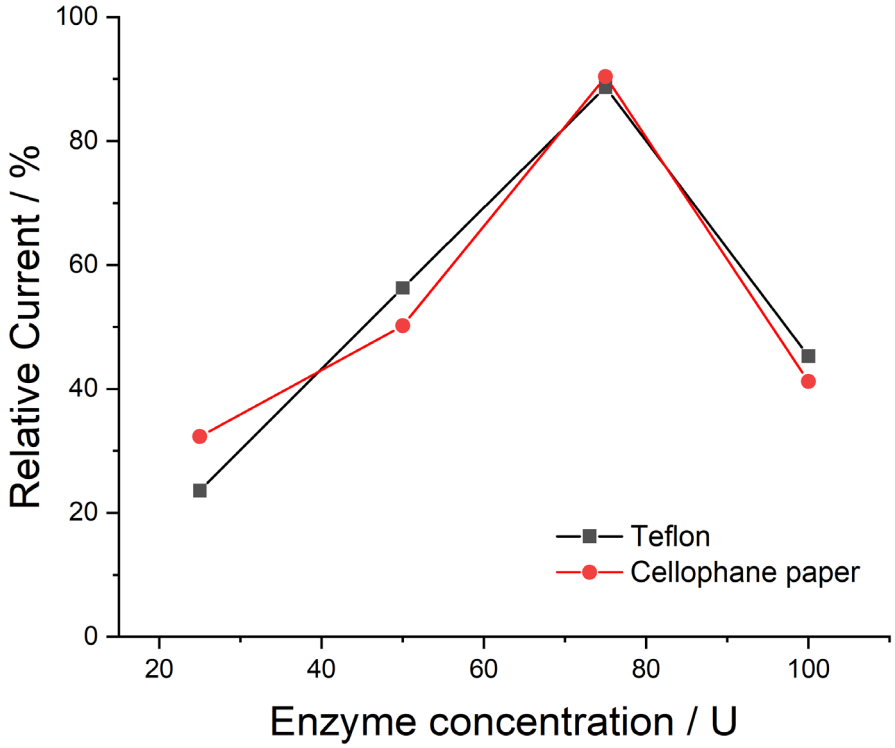

Figure 2. Effect of polyphenol oxidase enzyme concentration (U) on the relative current response [If/Ii × 100] of the biosensor obtained from crude dwarf banana peel extract in phosphate buffer solution, pH 6.5, at 25 ºC. Where If = % final current and Ii = % initial current. Conditions: substrate catechol 5×10^{-5} mol L^{-1}, in Teflon membrane and cellophane paper.

Crude eggplant peel extract

The biosensor built from eggplant skin on cellophane paper behaves better than the biosensor built on Teflon, as the amount of immobilized enzyme, that is, in cellophane paper (100 U) was lower than in Teflon (150 U). The greater permeability of cellophane can explain this compared to Teflon, which may also include the material´s thickness. With this, there is an advantage in using cellophane paper because the cost-benefit of Teflon is much higher. The biosensor response increases with increasing concentration of the enzyme immobilized on the outer membrane of the oxygen electrode up to 100 U for cellophane,

decreasing next (Figure 3). This phenomenon might be linked to the enhanced thickness of the enzymatic layer, which impedes the movement of the substrate and molecular oxygen, resulting in a decreased consumption of oxygen.

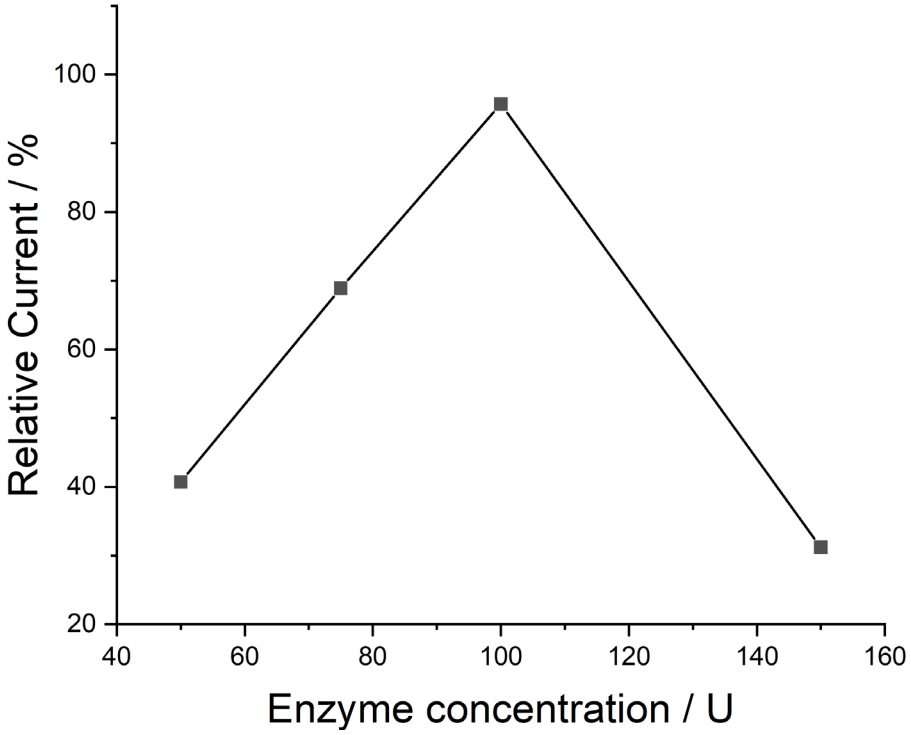

Figure 3. Effect of polyphenol oxidase enzyme concentration (U) on the response [$I_f/I_i \times 100$] of the crude eggplant peel extract biosensor in phosphate buffer solution, pH 6.5, at 25 ºC, for the substrate 5×10^{-5} mol L^{-1} catechol, in cellophane membrane. Where I_f = % final current and I_i = % initial current.

Effect of pH on the response of amperometric biosensors of crude extracts of dwarf banana and eggplant peels in cellophane paper

The study of the effect of pH (3.0-7.5) on the response of biosensors from crude extracts of dwarf banana peel and eggplant peel to the catechol substrate 5.0×10^{-5} mol L^{-1} was determined using a cellophane membrane with 75 Units of Polyphenol oxidase (PPO) for dwarf banana peel and 100 U for eggplant peel, at 25 ºC. It used 0.1 mol L^{-1} acetate buffer solution (pH 3.0-5.5) and 0.1 mol L^{-1} phosphate buffer solution (pH 6.0-7.5).

The best immobilization pH on the responses of biosensors of crude extract of dwarf banana peel and eggplant peel was 6.5 (Figures 4 and 5). It used 0.1 mol L^{-1} phosphate buffer solution (same values found with the enzyme immobilized on the Teflon membrane as in Signori and Fatibello-Filho,[14] evidencing that cellophane works as well as Teflon immobilization support for PPO in the two extracts studied.

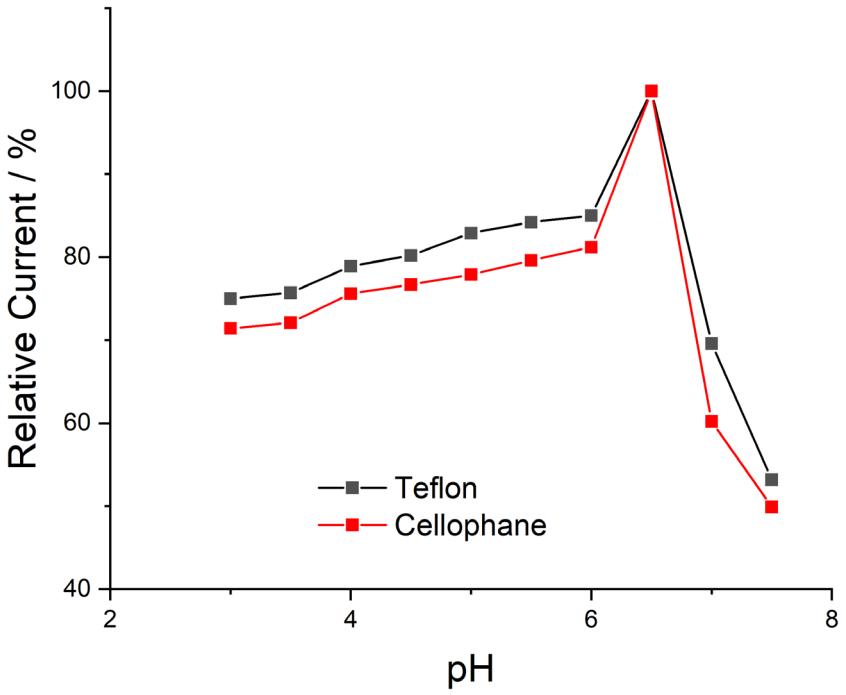

Figure 4. Effect of pH on the response [I_f/I_i x100] of the dwarf banana peel biosensor, at 25 ºC, for the catechol substrate 5×10^{-5} mol L^{-1}, using 0.1 mol L^{-1} acetate buffer solution (pH 3.0-5.5) and 0.1 mol L^{-1} phosphate buffer solution (pH 6.0-7.5). Where I_f = % final current and I_i = % initial current.

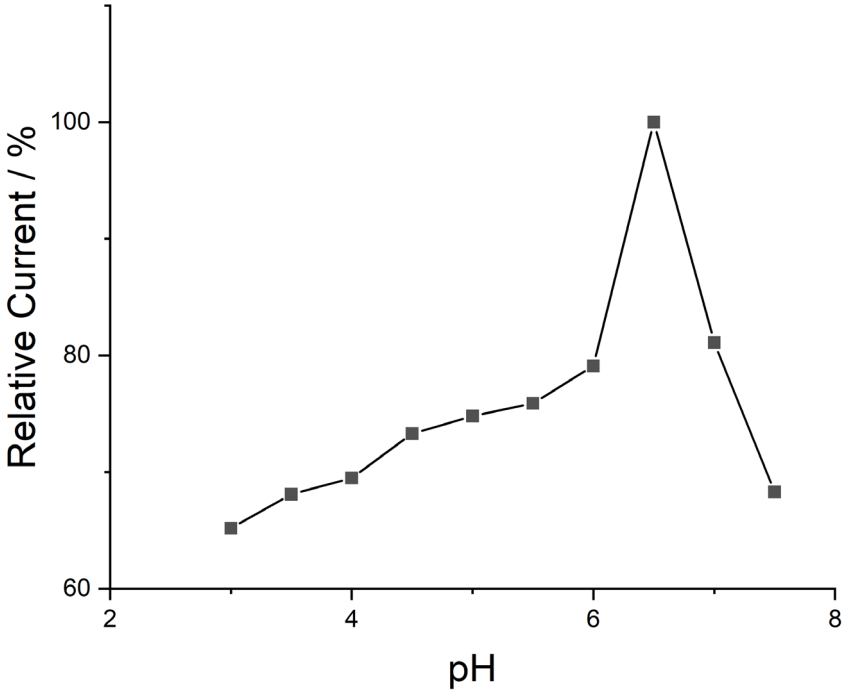

Figure 5. Effect of pH on the response [$I_f/I_i \times 100$] of the eggplant skin biosensor, at 25 ºC, for the catechol substrate 5×10^{-5} mol L^{-1}, using 0.1 mol L^{-1} acetate buffer solution (pH 3,0-5.5) and 0.1 mol L^{-1} phosphate buffer solution (pH 6.0-7.5) on cellophane paper. Where I_f =% final current and I_i =% initial current.

Response time study for the biosensors of crude extracts of dwarf banana and eggplant peels

The response time for the banana peel biosensor was shorter using cellophane paper (40 seconds) as support for immobilization than for Teflon 58 seconds.[14,23] The same can be observed for the eggplant skin biosensor in cellophane paper 45 seconds and in Teflon 64 seconds. This can be explained because cellophane has a thinner membrane thickness than Teflon, facilitating the diffusion of the substrate to the biosensor and consequently decreasing the response time. This study was also performed using two membranes with immobilized enzymes coupled to the amperometric detector instead of just one; the response time was approximately 5 minutes, showing the impossibility of using more than one membrane. Therefore, it can be concluded that with two membranes, the phenolic substrate cannot diffuse to the detector, making the quantification impossible.

Interfering species test: Relative response of biosensors of crude extracts of dwarf banana and eggplant peels in cellophane membrane for different phenolic substrates

Tables II and III show the response of cellophane membrane biosensors to these phenolic substrates: catechol, pyrogallol, paracetamol, and hydroquinone. Biosensors were more sensitive to catechol, followed by paracetamol, pyrogallol, and hydroquinone; evidencing catechol as the main substrate for determining the activity of PPO and the calibration curves of biosensors for determinations of polyphenols. The biosensors studied with cellophane agreed regarding the relative response for different phenolic substrates with the biosensors studied by Signori and Fatibello-Filho,[14] using The Teflon membrane as an immobilization matrix. Thus, it can be used to determine polyphenols instead of the Teflon biosensor; because it has low cost and easy access (national product).

Table II. Relative response of the biosensor of crude extract of dwarf banana peel in cellophane paper, using phenolic substrates (5×10^{-3} mol L^{-1}) in phosphate buffer solution 0.1 mol L^{-1} pH 6.5 at 25 ºC

Phenolic Substrates (5×10^{-3} mol L^{-1})	Relative current (%)
Catechol	*100*
Paracetamol	99.8
Pyrogallol	98.7
Hydroquinone	98.3

Where: [I_f/I_i x 100] = % relative current

Table III. Relative biosensor response of crude extract of eggplant peel in cellophane paper, using phenolic substrates (5×10^{-3} mol L^{-1}) in phosphate buffer solution 0.1 mol L^{-1} pH 6.5 at 25 ºC

Phenolic Substrates (5×10^{-3} molxL^{-1})	Relative current (%)
Catechol	*100*
Paracetamol	99.5
Pyrogallol	97.2
Hydroquinone	97.1

Where: [I_f/I_i x 100] = % relative current

Study of the number of determinations for the biosensors of crude extracts of dwarf banana and eggplant peels in cellophane membrane as immobilization matrix

With each enzyme membrane of the banana peel electrode with polyphenol oxidase immobilized on cellophane paper, it was possible to make an average of approximately 30 determinations, and with the eggplant peel electrode, around 20 determinations, resulting in two days of use for each studied electrode. The membranes with the immobilized enzyme must not be removed from the surface of the oxygen electrode during this determination. They must be kept in 0.1 mol L^{-1} phosphate buffer solution, pH 6.5 at 25 ºC during and after its use. This can be explained due to the low resistance of this membrane in its handling; that is, if it is removed from the surface of the oxygen electrode, it tends to be damaged, making further determinations impossible. Comparing the Teflon membranes (300-400 determinations and approximately 30 to 40 days of use for the electrodes of dwarf banana and eggplant peels, with cellophane membranes, we can verify that the latter is less resistant, having to be replaced more quickly than the Teflon membrane. We can then conclude that we have practically disposable enzymatic electrodes if the matrix is made of cellophane paper. Due to the ease of obtaining this polymer (national product) and its low cost compared to Teflon (imported product), we can build several membranes with cellophane paper and obtain advantages in the determinations before Teflon.

Quantifying polyphenols in tea samples, with biosensors from crude extracts of dwarf banana and eggplant peels using cellophane paper as a support, for the immobilization of Polyphenol oxidase present in these extracts

To evaluate the performance of these biosensors, the content of polyphenols (mg g^{-1} of dry tea) was determined in samples of teas (mate, black, chamomile, mint, fennel, and green). Tables IV and V below compare the polyphenol contents obtained by the proposed amperometric method and the spectrophotometric (standard).[29]

The levels of polyphenols were found to agree with those obtained by the standard method at a confidence level of 95% and within an acceptable error range (< 1%), evidencing the efficiency of the developed method. The limit of quantification (LOQ) for our biosensor method has been determined to be 10^{-5} mol L^{-1}. This was established in a 0.1 mol L^{-1} phosphate buffer solution at pH 6.5 and a temperature of 25 ºC. These details are in Tables II and III, where we present the data supporting our LOQ determination.

The LOD has been calculated to be 10-6 mol L^{-1}, representing the lowest analyte concentration in a sample that can be reliably detected, not merely distinguished from zero. This has been determined through triplicate measurements, with the standard deviation and relative error presented in Tables IV and V.

The biosensors studied presented a good analytical performance with smaller relative errors and more significant results than the biosensors of banana peel and eggplant built with Teflon.[23-25] After 2 days of use in both biosensors, it was observed that there was a significant loss of immobilized material (lyophilization).

Table IV. Determination of polyphenols in tea samples using the amperometric method proposed and the standard spectrophotometric method,[29] with a biosensor of crude extract of polyphenol oxidase from the dwarf banana peel (*Musa acuminata*) on cellophane paper

Samples	Phenol concentration (mg g^{-1} dry tea) Spectrophotometric	Phenol concentration (mg g^{-1} dry tea) Amperometric	Relative Error (%)
Yerba mate tea	2.80 ± 0.02*	2.78 ± 0.01	- 0.71
Black tea	2.91 ± 0.01	2.89 ± 0.03	- 0.69
Chamomile tea	1.95 ± 0.03	1.94 ± 0.04	- 0.51
Mint tea	0.17 ± 0.02	0.17 ± 0.01	0.00
Fennel tea	< LOQ	< LOQ	-
Green tea	< LOQ	< LOQ	-

* standard deviation of the mean with a confidence level of 95%, for n=3.

Table V. Determination of polyphenols in tea samples using the amperometric method proposed and the standard spectrophotometric method,[29] with a biosensor of crude extract of polyphenol oxidase from eggplant peel (*Solanum melongena L.*) on cellophane paper

Samples	Phenol concentration (mg g^{-1} dry tea) Spectrophotometric	Phenol concentration (mg g^{-1} dry tea) Amperometric	Relative Error (%)
Yerba mate tea	2.15 ± 0.01*	2.13 ± 0.03	- 0.93
Black tea	2.71 ± 0.04	2.69 ± 0.02	- 0.74
Chamomile tea	1.85 ± 0.02	1.84 ± 0.03	- 0.55
Mint tea	0.15 ± 0.01	0.15 ± 0.01	0.00
Fennel tea	< LOQ	< LOQ	-
Green tea	< LOQ	< LOQ	-

* standard deviation of the mean with a confidence level of 95%, for n=3.

CONCLUSIONS

In conclusion, developing biosensors using cellophane paper immobilized with polyphenol oxidase enzymes from crude extracts of dwarf banana and eggplant peels, glutaraldehyde, and a Clark electrode proved to be a cost-effective alternative to Teflon® membrane electrodes for polyphenol detection in teas. The biosensors optimized enzyme concentration and pH value, exhibiting greater analytical performances, and the polyphenol levels found agreed with those obtained by the standard method with an acceptable error range (<1%). Despite the lower number of measurements for cellophane membranes compared to Teflon, the easy access and lower cost of cellophane paper allow for constructing several membranes, resulting in cost advantages and functioning as a disposable membrane. Therefore, the proposed method offers a promising approach to the detection of polyphenols in teas and has the potential to be extended to other applications.

Conflicts of interest

Authors declare no conflicts of interest.

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ARTICLE

Development and Validation of an Analytical Method for the Determination of Fipronil and its Degradation Products in 28 Organic and Regular Honey Samples by GC-ECD

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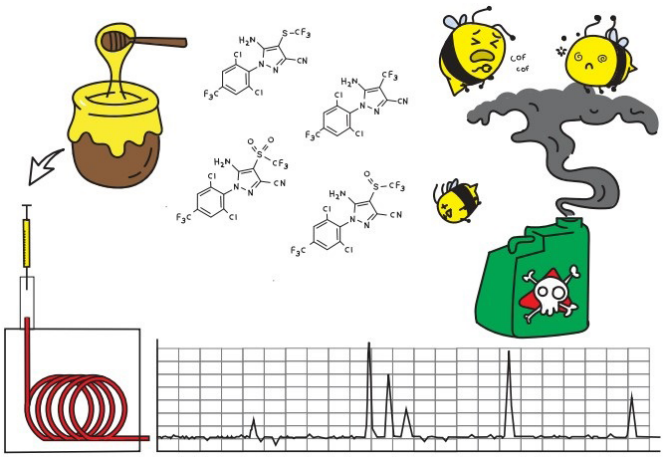

Recently, there has been a worldwide problem of increased bee mortality (Colony Collapse Disorder) and the intensive use of pesticides is suspected as one of the causes. Honey samples are one of many indicators to assess bee exposure to pesticides. In this work, a method for the simultaneous analysis of the pesticide Fipronil and its degradation products in honey samples by gas chromatography with electron capture detector (GC-ECD) is presented and validated. The extraction procedure was investigated using C18-SPE with different solvents and methanol showed the best performance. The analytical quantification was performed by internal standard matrix-matched calibration, which resulted in analytical curves presenting correlation coefficients higher than 0.99. The proposed method was validated with good results, such as recoveries around 70 – 99%, limits of detection and quantification bellow 0.014 and 0.072 μg mL^{-1}, respectively, and relative standard deviations below 7%. The method is simple, effective and was successfully applied to 28 commercial honey samples, regular and organic, from different floral sources. The results showed the presence of fipronil desulfinyl, the main degradation product of fipronil, in some samples, even among the organic ones.

Keywords: honey, fipronil, degradation products, gas chromatography, matrix matched method

INTRODUCTION

Every day, honeybees (*Apis mellifera*) make successive flights, covering a wide area, collecting nectar, water and pollen from flowers. During travel, they come into contact with various particles (e.g. pesticides) and microorganisms in the air, soil or water that may become embedded on the surface of their bodies or be inhaled and adhered to their respiratory system.

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Bees and their products can be considered as bioindicators of environmental contamination.[1] The level of contamination of hives by pesticides is directly related to the proximity of a source of pollution and thus can provide information about a specific polluted environment.[2-4] Although honeybees are not targeted by pesticides, their exposure to these products can cause their death in cases of acute toxicity,[3] or impair their foraging behavior and affect colony health and development in cases of chronic toxicity.[4]

The pesticides, when carried to the hive, contaminate the bee products (honey and pollen), reducing their beneficial properties. These products, if highly contaminated, can pose threats to human health when ingested.[5] In addition to foraging contamination, bees and honey can also be contaminated by direct application of pesticides to treat hives.[6]

The loss of hives, a phenomenon known as colony collapse disorder, observed in several countries in the northern hemisphere,[7,8] has been highly associated with diseases caused by Varroa (an ectoparasite mite), Nosema (a kind of fungus), certain viruses, and exposure to pesticides.[9] Thus, concern for the preservation of bees is increasing worldwide, especially since about 80% of plant species depend on pollination to exist, and bees are important pollinators.[10] Therefore, the loss of bees can cause a collapse in the economy of many countries, both by reducing the productivity of honey derivate and negatively impacting agriculture due to decrease in pollination.[7,8,10]

Fipronil, 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile, is a widely used insecticide that belongs to the chemical family of phenylpyrazole.[11] This compound interferes with Gamma-Amino Butyric Acid (GABA) receptors, interrupting the influx of nerve transmissions. In sufficient doses, it causes excessive neural excitation, severe paralysis, and insect death. The main degradation products of fipronil (Figure 1) also showed strong insecticidal properties.[11,12]

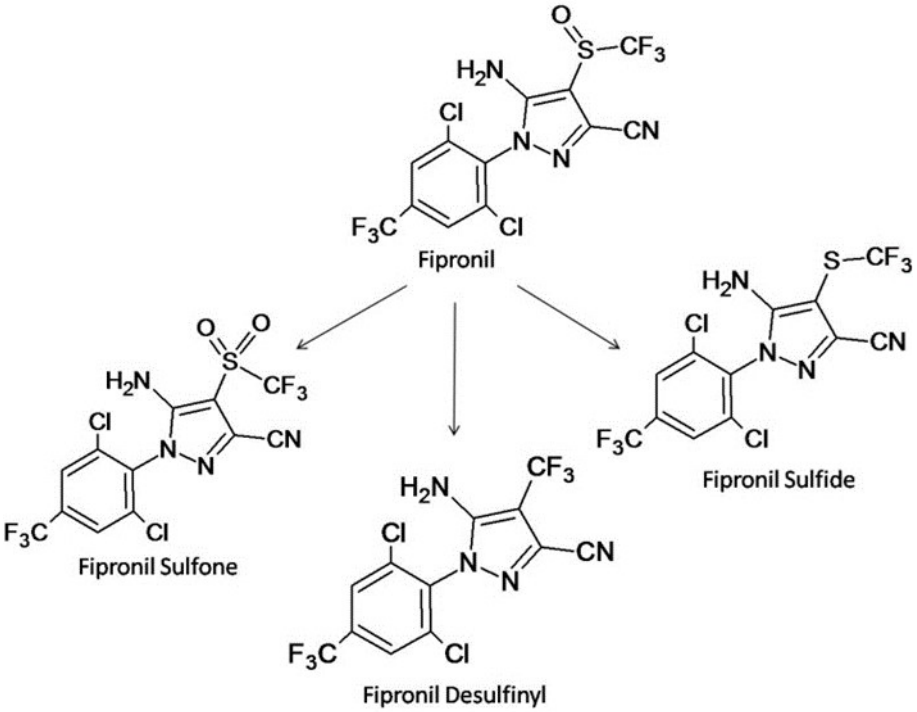

Figure 1. Fipronil and its degradation products: fipronil sulfone, fipronil desulfinyl and fipronil sulfide.

Fipronil has been extensively studied in recent years due to its harmful effects on non-target organisms, such as birds, aquatic organisms and honeybees.[12] For a variety of birds, this compound is highly toxic

through acute and chronic exposure. For aquatic organisms, it can be accumulated in tissues. For honeybees, fipronil has high acute toxicity, and its lethal dose (LD50) ranges from 4 to 6.2 ng/bee.[13,14] Fipronil degradation products, even at low concentrations, have also shown high toxicity to aquatic organisms.[11]

A relevant study confirmed the threat of fipronil to *Apis mellifera* and found that sublethal exposure of fipronil causes motor and behavioral changes in bees, culminating in the collapse of all colonies and the abandonment of hives. These researchers strongly recommended discontinuing the use of this active ingredient.[15]

Several works in the literature describe the determination of fipronil residues in several environmental samples[16] and different honey matrices.[17-21] However, to the best of our knowledge, there is no scientific paper dedicated to optimizing the figures of merit (e.g., low LOQ and high precision) for simultaneous quantification of fipronil (F) and its three most important degradation products, namely fipronil desulfinyl (FD), fipronil sulfone (FSO) and fipronil sulfide (FSE) in commercial honey samples reported in the literature. Therefore, the objective of this study was the development and validation of a fast and accurate method using SPE and GC-ECD for the determination of F and its most frequent degradation products in commercial honey samples.

MATERIALS AND METHODS

Chemicals and materials

Certified individual standards of F (97%), FSO (100%), FD (98%), FSE (99.6%) and the internal standard (IS) decachlorobiphenyl (PCB209) were purchased from AccuStandard (New Haven, USA). Residue analysis-grade methanol, n-hexane, isopropyl alcohol and acetone were purchased from Tedia (Ohio, USA) and ultrapure water was obtained from a Milli-Q® system (Millipore, Milford, MA, USA). The stock and working solutions of each compound were prepared in methanol and stored at 4 °C. Solid phase cartridge, C18, was purchased from Silicycle (Specifications: C18-17%; Bed Weight - 500 mg; Cartridge Volume - 6 mL; Ultrapure Silica Gels). For extraction under vacuum, an SPE manifold (Supelco, Sigma Aldrich, USA) coupled to a vacuum pump (Alfa Mare, Brazil) was used. For method optimization and validation, an uncontaminated honey sample was used as a blank sample for matrix-matched standards.

Sampling

Twenty-eight samples of commercial honey were purchased in local markets in Brasilia (Brazil). These samples come from different floral sources, being twelve predominantly wild, two from orange tree, one from eucalyptus and the other thirteen samples do not present this information. All samples were kept in their original packaging at room temperature in a dark place. Nine of these samples were organic honeys.

Extraction

The extraction method was enhanced by optimizing it, leveraging insights from prior research as a foundation.[18] Initially, the C18 solid phase cartridges (500 mg / 6 mL) were preconditioned by passing 5 mL of methanol followed by 5 mL of purified water (both by gravity) and not allowing complete drying of the sorbent after each wash. An aqueous solution (15 mL) was prepared with 5 g of uncontaminated honey, spiked with 400 µL of F standard and extracted.

Four trials were done to evaluate and select the solvent extraction mixture to be used. They were performed in duplicate with the following solvent mixtures and under vacuum: (1) 3 x 3 mL of methanol, (2) 3 x 3 mL of isopropyl alcohol, (3) 2 x 3 mL of methanol + 3 mL of isopropyl alcohol and (4) 3 x 3 mL of a mixture of n-hexane:isopropyl alcohol (1:1, v/v). Solvent extraction was performed at concentration levels of approximately 0.19 µg g^{-1}. This step is presented in Figure 2A.

Each honey sample (1.0 g) was diluted with water to a volume of 3 mL and a 1 mL aliquot of this solution (previously evaluated to avoid saturation of the cartridge) was passed through the pre-conditioned solid phase cartridge at a flow-rate of about 10 mL min^{-1}. Thereafter, the SPE was washed with 5 mL of water and

the retained compounds were finally eluted by passing 3 x 3 mL of methanol. The eluate was evaporated to dryness using a gentle steam of nitrogen at 40 °C, resolubilized in methanol containing 0.032 μg mL^{-1} of the IS and quantitatively transferred to a 1-mL vial. For analysis, 1 μL of each extract was injected into the GC-ECD system. This step is presented in Figure 2B

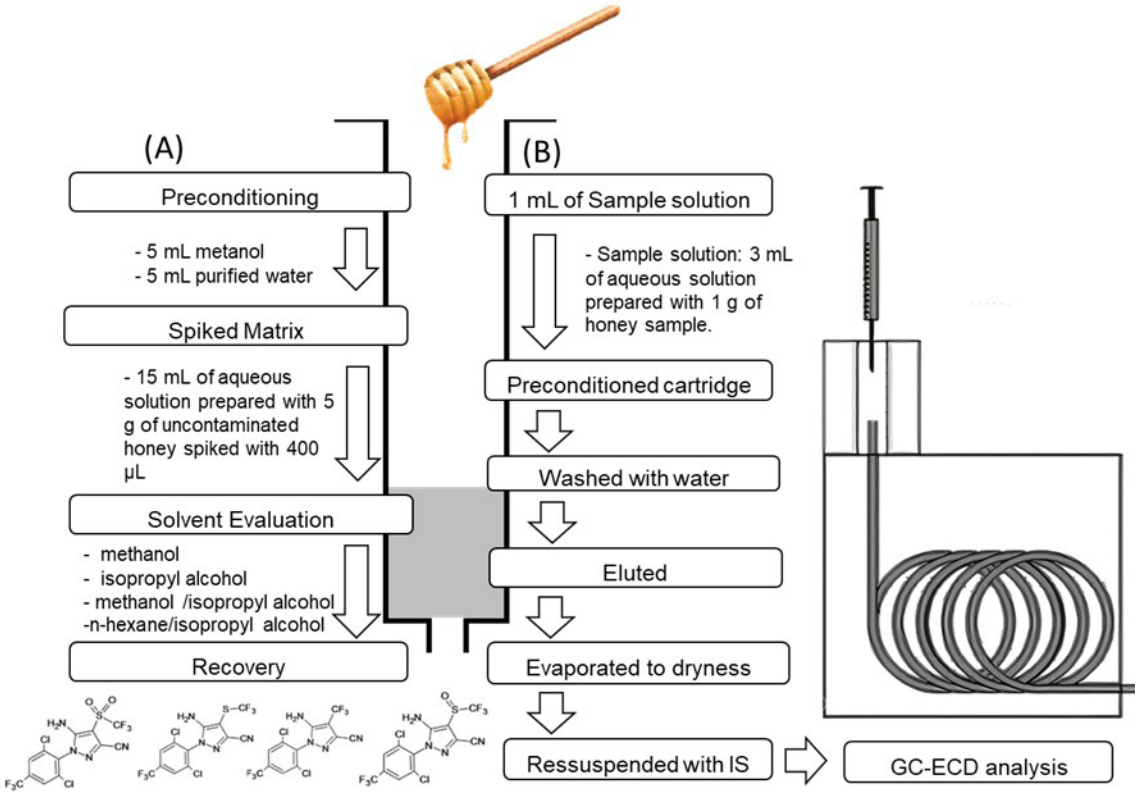

Figure 2. Sample preparation scheme. (A) strategy sequence for solvent selection and (B) sample preparation.

GC-ECD conditions

The chromatographic conditions were initially based on previous work.[22] A Shimadzu GC-2010 gas chromatograph equipped with an electron capture detector (GC-ECD), with a 30 m × 0.25 mm i.d. capillary column coated with a 0.25 μm thick film of 5% phenyl and 95% methylpolysiloxane (Rtx®-5 Restek) was used. Helium was used as carrier gas at a flow-rate of 1 mL min^{-1}. The injection port temperature was 260 °C and 1 μL of standard and samples were injected under splitless mode for 2 min, followed by the split ratio of 1:20. The detector temperature was 305 °C, with nitrogen as make up gas (20 mL min^{-1}). The oven temperature program was: 100 °C (1 min), 15 °C min^{-1} up to 230 °C, 2 °C min^{-1} up to 256 °C (2 min) and 20 °C min^{-1} up to 280 °C (10 min). The total analysis time was 35.87 min.

Analytical Performance and Method Validation

Due to low linearity observed in the analytical curves in methanol, additional curves were prepared in honey extract free of the target compounds (blank honey extract). IS was used in all curves. The comparison of the analytical curves (in methanol and honey extract) for each analyte was based on their angular coefficients. All analytical curves are presented in Table I, each with 6 levels called P1, P2, P3, P4, P5 and P6 in triplicates, and matrix-matched standards were produced according to described previously in the Extraction topic. Pesticides stock and working solutions were prepared in methanol.

Table I. Concentration of F, FD, FSO and FSE in methanol and in honey extracts for each point of the calibration curve

Analyte	Analytical Curve (μg mL^{-1})					
	P1	P2	P3	P4	P5	P6
F	0.0230	0.0322	0.0414	0.0552	0.0690	0.0828
FD	0.0240	0.0336	0.0432	0.0576	0.0720	0.0864
FSO	0.0236	0.0330	0.0425	0.0566	0.0708	0.0850
FSE	0.0230	0.0322	0.0414	0.0552	0.0690	0.0828
F	0.0230	0.0322	0.0414	0.0552	0.0690	0.0828
FD	0.0240	0.0336	0.0432	0.0576	0.0720	0.0864

A validation of the method was carried out with the following parameters: matrix effect, analytical curve and linearity, limits of detection (LOD) and limits of quantification (LOQ), precision (repeatability and intermediate precision) and recovery.

Linearity was evaluated according to the coefficient of determination (r^2) of all analytical curves with a minimum acceptable correlation coefficient equal to 0.99.[23] LOD was established considering the lowest concentration capable of generating a detectable signal, while LOQ was the lowest concentration capable of generating a linear analytical curve (P1 in Table I). For recovery assays with an acceptance criteria between 70 and 120%[24] a honey sample free of the target compounds was fortified at two levels, 100 and 200 ng g^{-1}, for each compound. The precision of the method, in terms of repeatability, was assessed by extracting and analyzing fortified samples at 0.046 μg mL^{-1} in six replicates. To evaluate the intermediate precision of the method, different days were used, also in six replicates. The acceptance criteria was ≤ 20%[24] and precision was calculated according to the Equations 1 and 2, as follows:

$$s = \sqrt{\frac{\sum_{i=1}^{I}\sum_{j=1}^{L}(\hat{x}_{ij}-\bar{x}_i)^2}{I\,(J-1)}} \qquad (1)$$

Where, S is the combined standard deviation, I is the number of samples and J is the number of replicates.

Precision was assessed using the relative standard deviation (RSD) in accordance to equation 2:

$$RSD(\%) = \frac{s}{\bar{x}}\,100\% \qquad (2)$$

RESULTS AND DISCUSSION

Solvent selection

In order to evaluate solvents with a wide range of polarity for elution, different solvents and mixtures were tested. The results of the recovery studies are shown in Table II.

Performing a F-test to compare the recovery results, it can be seen that the recovery for the four solvent combinations did not differ statistically, as any calculated F-value was greater than the unilateral limit of the F-distribution of Fisher-Snedecor with 5% significance level to 3 and 6 degrees of freedom (F_{critic} = 4.191). Thus, pure methanol was chosen as the elution solvent, considering the availability of methanol in the laboratory and the convenience of using the same solvent for extraction and instrumental analysis.

Table II. Recoveries for the tests of the choice of solvent for F, FD, FSO and FSE with the respective calculated values for the F-test

Analyte	Hexane/Isopropyl alcohol (%)		Isopropyl alcohol (%)		Methanol/Isopropyl alcohol (%)		Methanol (%)		F-test
	R1	R2	R1	R2	R1	R2	R1	R2	
FD	85	98	85	100	101	99	97	84	0.55
F	65	70	59	51	73	69	73	60	2.87
FSO	50	54	45	49	55	52	55	50	1.82
FSE	71	73	70	72	74	72	71	72	1.11

Degradation of the compounds

F and its degradation products apparently degraded during storage or during chromatographic analysis. Confirmation of the presence of degradation products was performed by injecting each pesticide standard solution individually. In the FD, F and FSO chromatograms more than one peak was observed. In the chromatogram of the combined standard solution (Figure 3), additional peaks (e.g., F+FSO and two FD peaks) can be seen.

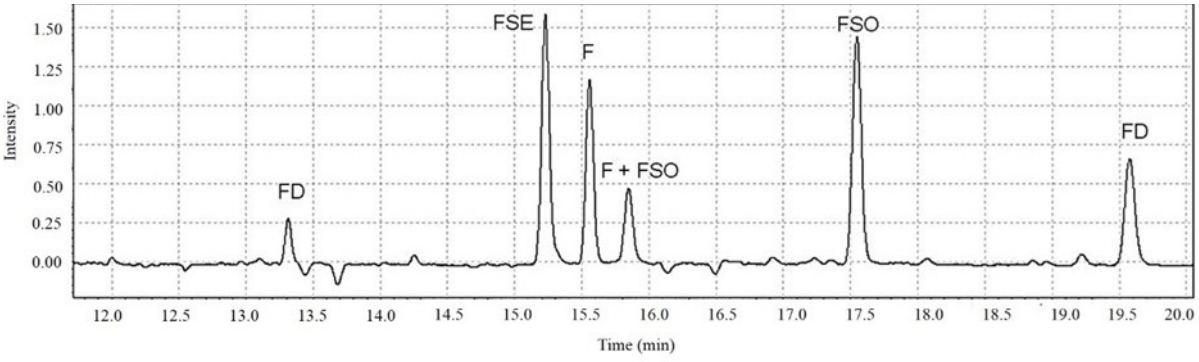

Figure 3. GC-ECD chromatogram for the standard solution containing F, FD, FSE and FSO.

Validation conditions

The analytical curves in methanol did not show good linearity for all analytes in question, with a determination coefficient lower than 0.990 for F, FSO and FD. The first alternative to improve the linearity of the analytical curves was to build a new curve considering the sum of the area of all analyte signals (Σ F, FSO, FD and FSE) *versus* the sum of their concentrations. The new equation showed better linearity (r^2 = 0.999) compared to the individual curves, but lost information about the individual analytes.

Thus, in order to obtain better results for each individual analyte, new standard solutions were prepared in blank honey extracts (matrix-matched). Comparing each analytical curve in methanol with each analytical curve in blank honey extract, a strong matrix effect can be observed, resulting in better sensitivity and linearity for the matrix-matched curves, as can be seen in Figure 4.

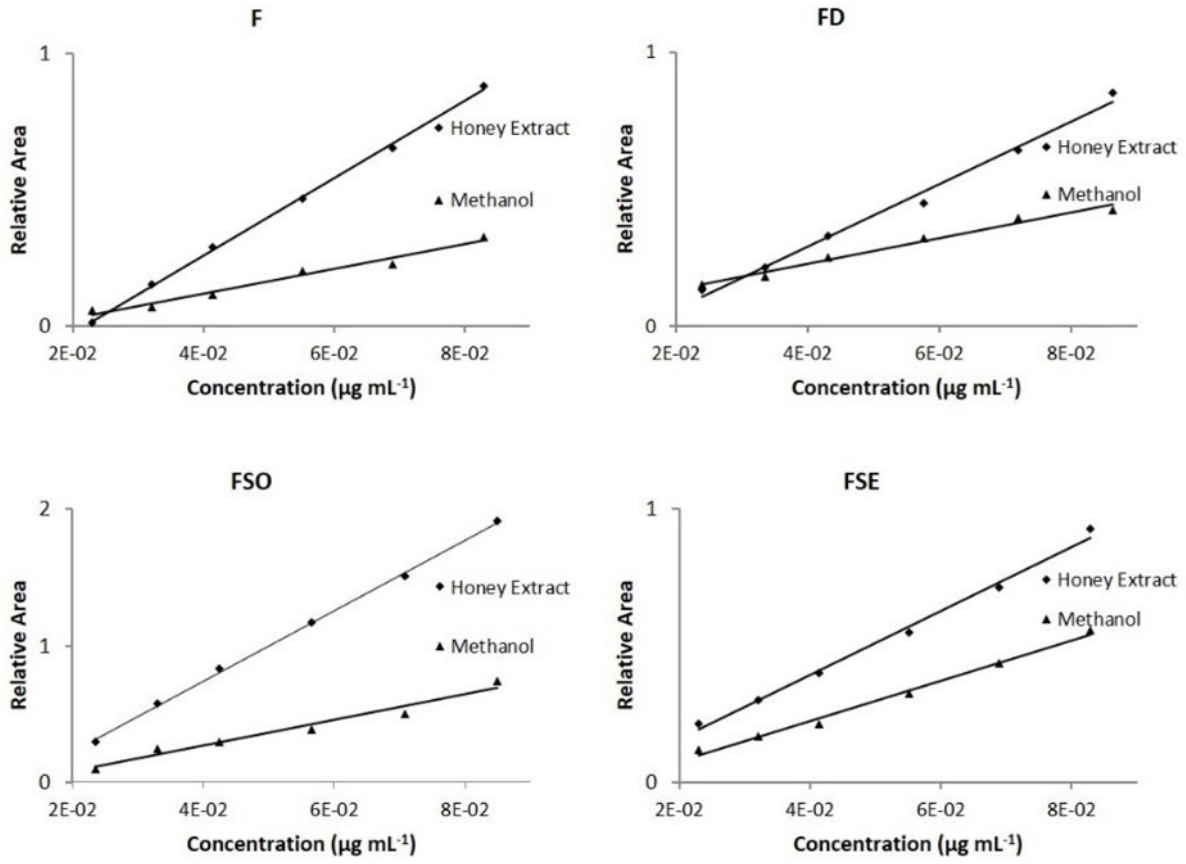

Figure 4. Analytical curves of F, FD, FSO and FSE in methanol and in honey blank extracts with PCB 209 as IS.

This matrix effect may be due to the injector, low analyte concentrations and matrix properties. In relation to the injector, when standards are prepared in pure solvent and analyzed by GC, the active sites of the liner formed by free silanol groups and metal are available for the retention of the analytes, causing a lower transfer of the molecules to the column and consequently to the detector. When standard solutions prepared in blank honey extracts are injected, a competition between the matrix compounds and the analytes for the active sites of the liner occurs, allowing a greater amount of the pesticide to reach the detector.[25]

A stronger matrix effect was also observed at lower analyte concentrations, probably because if present at higher concentrations, the percentage of analyte that is trapped in the active sites of the liner will be much lower and the error associated with each injection will also be less expressive.[25] With regard to matrix properties, the sample and co-extractives can also influence the strength of the matrix effect, depending on the size of molecules, thermal stability, polarity and volatility. Co-extractives such as lipids, pigments and other high molecular weight compounds present in honey can remain in the extract, interfering with chromatographic analyzes and promoting a matrix effect.[25]

For all the above reasons the calibration was performed using matrix-matched standards prepared as described in the experimental section.

The parameters for linear regression (y = ax + b) obtained for a six-level calibration curve, in triplicates, are shown in Table III. Good linearity was obtained for all analytes in the concentration range within the evaluated interval, with determination coefficients greater than 0.990.

Table III. Matrix-matched analytical curve parameters and limits of the method for F and its degradation products

Analyte	Linear regression y = ax + b	r^2	Limits of the method (μg g^{-1} of honey)	
			LOD	LOQ
F	y = 14.17x - 0.108	0.998	0.0138	0.069
FD	y = 11.39x - 0.165	0.990	0.0101	0.072
FSO	y = 25.85x - 0.093	0.998	0.0097	0.069
FSE	y = 11.77x + 0.020	0.993	0.0091	0.071

LODs and LOQs

The LODs were established considering the lowest concentration capable of generating a detectable signal, and the LOQs were established as the lowest concentration capable of composing a linear analytical curve.[23] The LODs and LOQs for F and its degradation products are also presented in Table III, expressed per mass of analyte per mass of honey. These values are similar to those obtained by Flores-Ramirez et al. when working with the same analytes in soil samples.[22]

The LODs reported in the literature for quantification of F and its degradation products in honey through a multicomponent method vary from 0.83 to 1.16 ng g^{-1} of sample using solid phase extraction and LC/MS-MS, while the values found in this work vary between 9.10 and 13.8 ng g^{-1}. Despite achieving lower LODs, techniques such as LC/MS-MS[26] are more sophisticated and expensive compared to GC-ECD, which allows performing analyzes with good sensitivity and linearity. A more recent study compares the determination of F and some of its degradation products in chicken eggs, using LC-MS/MS and GC-ECD techniques. The work concludes that the methods for the analyzed samples are extremely compatible, presenting practically equal results.[27]

Precision and Recovery

Method precision was determined by repeatability and intermediate precision, expressed by relative standard deviations, RSD_R and RSD_{IP}, respectively. These data are shown in Table IV. The method was found to be precise (RSD < 10%) for all the compounds studied at both spiking levels.

Table IV. Precision (n = 6) in terms of repeatability (RSD_R) and intermediate precision (RSD_{IP}) and recovery for F and its degradation products

Analyte	Precision		Recovery (%)	
	RSD_R (%)	RSD_{IP} (%)	Spike level 100 ng g^{-1}	Spike level 200 ng g^{-1}
F	6	10	99 ± 6	74 ± 4
FD	4	8	71 ± 3	72 ± 5
FSO	3	7	84 ± 7	70 ± 6
FSE	5	8	79 ± 4	71 ± 2

The recovery results of the honey samples, at two levels of fortification, were between 70 and 99%, as shown in Table IV. Considering the acceptability criteria for recoveries between 70 and 120%,[24] it can be concluded that the method is in agreement with the required parameters.

Prior research on fipronil and its degradation products in honey

While there are some review papers on the determination of pesticides in honey or other food samples,[28-32] there is none exclusively dedicated to F and its degradation products. Even though there is a growing interest in multi-analyte methods, the determination of F and its derivatives in honey is not yet well-established in the literature. Thus, the quest for creating a robust method remains a challenge, primarily due to the risk that F and its derivatives pose to bees.

Table V presents some analytical strategies previously used to determine F and some of its degradation products in honey samples. As we can see, some more sophisticated methods require a more complex sample preparation, such as the use of extraction and clean-up steps. The addition of more steps, combined with more expensive analytical methods, makes the method even more costly and, consequently, may hinder the analysis of such importance in areas of interest.

Even the more sophisticated methods in the literature, such as LC-MS/MS, have some merit figures similar to those found in this study, such as recovery values, linear range, and LOD, as presented in Table V. It is also worth highlighting that among the methods found, few focused on the determination of its degradation products, which are important sources of information about the contamination of floral sources for honey production.

Table V. Analytical strategies for the determination of F and some of its degradation products in honey samples reported in the literature

Analytes	Sample Preparation		Analitycal Method	Linear range	LOD	Recovery (%)	Ref
	Extraction	Clean-up					
F	LLE[a]	SPE by Florisil Columm	GC-ECD	0.001 – 2 mg kg^{-1}	1 µg kg^{-1}	72.0 – 93.0	17
F	Modified QuEChERS[b]		LC-UV	0.03 – 0.25 mg kg^{-1}	30 µg kg^{-1}	70.7 – 101.1	33
F	OCLLE[c]		LC-MS/MS	2.5 – 10 ng g^{-1}	0.015 µg kg^{-1}	72.0	34
F	Modified QuEChERS		LC-MS/MS	0.005 – 0.05 mg kg^{-1}	0.0004 mg kg^{-1}	68.4 – 83.8	35
F		SPE	GC-MS	10 – 300 ng g^{-1}	1.4 ng g^{-1}	99.0 – 106.0	36
F	LPE[d]	SPE by Florisil	UHPLC-MS/MS	0.1 – 5 µg kg^{-1}	0.03 -1.51 µg kg^{-1}	75.0 -125.0	37
F	QuEChERS		LC-MS/MS		0.01 mg kg^{-1}	87.6 – 111.0	5
F	LPE		UHPLC-MS/MS		0.05 – 10 µg kg^{-1}	81.9 – 98.1	38
F	QuEChERS		LC-MS/MS	0.2 – 10 ng g^{-1}		30.0 – 96.0	39

(continues on the next page)

Table V. Analytical strategies for the determination of F and some of its degradation products in honey samples reported in the literature (continuation)

Analytes	Sample Preparation		Analitycal Method	Linear range	LOD	Recovery (%)	Ref
	Extraction	Clean-up					
F, FSO, FSE, FD, and fipronil carboxamide	LPE	SPE by Florisil Adsorbent	LC-MS/MS	2 – 18 ng mL^{-1}	0.83 – 1.16 ng g^{-1}	89.9 – 98.8	26
F, and FSO	QuEChERS		LC-MS/MS	1 – 100 ng g^{-1}	1.3 and 0.3 ng g^{-1}	82.0 – 97.0	40
F, FD, FSE, and FSO	QuEChERS		LC- MS/MS	0.001 – 0.1 mg kg^{-1}	0.001 mg kg^{-1}	75.0 – 120.0	41
F, FD, FSE, and FSO		SPE	GC-ECD	0.0230 – 0.0864 µg mL^{-1}	0.0091 – 0.0138 µg kg^{-1}	70.0 – 99.0	This study

[a]LLE – Liquid-Liquid Extraction; [b]QuEChERS – Quick, Easy, Cheap, Effective, Rugged and Safe; [c]OCLLE – On-Column Liquid-Liquid Extraction; [d]LPE – Liquid-phase

Analysis of commercial honey samples

The only compound found in the analyzed samples was FD. However, in some of these samples, the generated signal was lower than the LOQ. The results are presented in Table VI. No signals related to the retention time of F, FSO and FSE were observed.

Table VI. Results of analyzes of honey samples for FD

Organic samples	Concentration (µg g^{-1})	Conventional Sample	Concentration (µg g^{-1})	Conventional Sample	Concentration (µg g^{-1})
1	<LOQ	11	<LOQ	21	<LOQ
2	<LOQ	12	<LOQ	22	<LOQ
3	0.13 ± 0.01	13	<LOQ	23	0.081 ± 0.003
4	<LOQ	14	0.16 ± 0.03	24	0.075 ± 0.001
5	<LOQ	15	0.094 ± 0.009	25	<LOQ
6	0.10 ± 0.01	16	<LOQ	26	0.129 ± 0.005
7	<LOQ	17	<LOQ	27	0.076 ± 0.004
8	0.079 ± 0.002	18	0.075 ± 0.003	28	<LOQ
9	<LOQ	19	0.080 ± 0.001		
10	<LOD	20	<LOQ		

FD, the main degradation product of F, is very persistent in the environment and considered bioacumulative.[42,43] This compound proved to be more toxic to rats and mosquitoes than F and presented high toxic potential for human health.[44] Therefore, in addition to being a potentially dangerous contaminant, it can also be considered a marker of fipronil use.

The presence of F and its degradation products does not depend on the production type of honey, whether organic or conventional, probably because bees, when foraging, can go further than expected reaching areas of conventional agriculture. Thus, although the beekeeper does not directly use any type of pesticide in the treatment of hives, bees can be contaminated in crops treated with pesticides. Furthermore, in Brazil the use of F has been suspended due to serious adverse effects on bees.[45]

CONCLUSIONS

The method for determining F and its degradation products in honey samples using IS matrix-matched analytical curves proved to be simple and effective. Extraction with a C18 SPE column and elution with methanol resulted in clean extracts capable of being injected in the GC-ECD system.

Validation parameters were all within the expected range, resulting in recoveries ranging from 70 ± 6 to 99 ± 6%. All determination coefficients for the IS matrix-matched analytical curves were above 0.990, higher than the ones obtained for external standard analytical curves in methanol.

The optimized method was applied to 28 commercial honey samples, including organic and conventional, and 11 samples showed a signal of FD higher than LOQ, regardless of whether the sample was organic or not.

In this study, we cannot infer the initial contamination level of each sample, as these active ingredients are subject to degradation over time. Thus, it is possible that samples with pesticide concentration results lower than the LODs were contaminated at the time of collection, but with the passage of time the analytes were completely degraded.

The analyte found in the samples, FD, is a product of the photodegradation of F and represents a potential risk to human and bee health.

Conflicts of interest

The authors declare that they have no financial conflicts of interest.

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Technical Note

Rapid Prediction of ANFO Based Explosives through ATR-FTIR Analysis – *Use of ATR-FTIR in Explosives*

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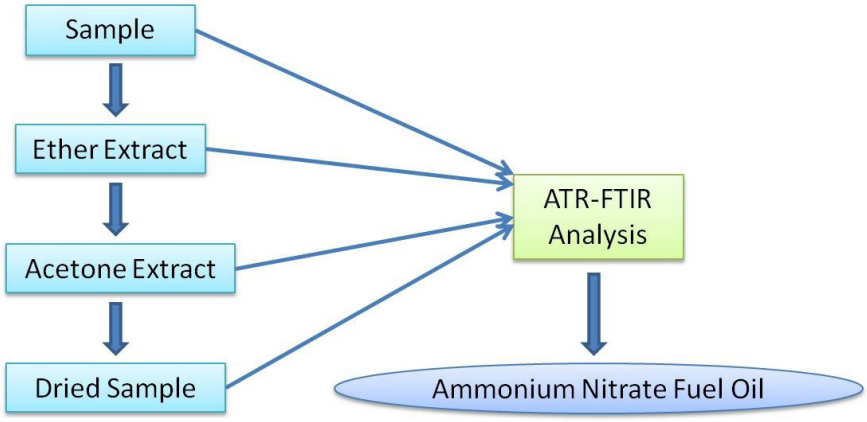

Ammonium Nitrate Fuel Oil (ANFO) is preferred mining explosives in worldwide. It is composed of ammonium nitrate (94-96%) and liquid hydrocarbon as fuel oil (4-6%), which is detonated through an explosive charge. In India, Forensic Science Laboratories received many criminal cases from investigation agencies for chemical analysis of sample as semi-solid materials supposed to be explosive material. In the present study, we developed an Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) procedure for easily analyzing the real crime exhibits related to ANFO based explosives. Firstly, semi-solid material is directly used on the ATR. Further, the sample is extracted using appropriate solvents (diethyl-ether/acetone). Extracts are subsequently analyzed on ATR-FTIR in comparison with standards for ammonium nitrate and diesel. The residue after acetone extract is dried and left solid material directly used on ATR for the detection of water-soluble compounds. The results significantly showed the presence of ammonium nitrate with the residue of diesel in a real crime exhibit. Hence, the proposed modify procedure can be advantageous for the rapid detection of diesel components mixed in ammonium nitrate through ATR-FTIR spectroscopy without the use of other chemical or instrumental analysis in a short period of time and also for easily identifying the presence of organic explosives (if any) among different samples received for the forensic opinion.

Keywords: Ammonium nitrate, ATR-FTIR, explosives, forensic analysis, fuel oil

INTRODUCTION

Ammonium nitrate (AN) is an extensively used chemical compound with numerous important applications. It is a crystalline powder varying in color from almost white to brown.[1] It is made by the reaction of ammonia (NH_3) with nitric acid (HNO_3) in water (H_2O) followed by careful evaporation of the

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water to give up a solid material. It is a popular fertilizer because nitrogen is a key component of the two ingredients of the compound, i.e., ammonium (NH_4^+) and nitrate (NO_3^-). Ammonium nitrate as such does not ignite; but in contact with other flammable materials, it boosts fire risk and also supports fire even in the absence of oxygen. When involved in a fire, will stimulate burning and be classified as an oxidizer.[2] It is one of the main components in several types of mining explosives. It is combined with fuel oil and detonated through an explosive charge, where it is called an Ammonium Nitrate Fuel Oil (ANFO)-based explosive. ANFO is used as an explosive for mining, quarrying and civil construction purposes. As the constituents of ANFO are comparatively trouble-free to find, there is a potential for their improper use in improvised explosive devices (IEDs).[3]

ANFO is composed of around 94 to 96% ammonium nitrate and 4 to 6% fuel oil by weight. Ammonium nitrate (NH_4NO_3) is a strong oxidizing agent; and fuel oils are used for supply of fuel in preparation of ANFO-based compositions.[4] The fuel diesel, gasoline and sometimes kerosene are typically used in ANFO explosives, which can also be used as an indicator for fuel source recognition in explosive cases.[5] As ANFO consist of inorganic and organic ingredients, So the analysis of ingredients from ANFO based material has been classified into the determination of ammonium nitrate (inorganic part) and fuel oils (organic part) constituents. ANFOs are most widely used explosives all over the world.[4] During the last decades, ammonium nitrate has been involved in many industrial blasts, including the Beirut explosion in 2020 that killed 220 people and injured more than 6,500 instantly, Texas fertilizer plant explosion in 2013 that killed 15 people, a North Korean railway blast in 2004 that left 161 dead, and the 1995 Oklahoma City Bombing that left 168 souls lost, including 19 children, with several hundred more injured.[6] The most well-known incidents includes the explosion of an ammonium sulfate nitrate silo in Oppau (Germany) killed 507 and injured 1917 people in 1921.[7] 2000 suffered injuries in Texas serial plast due to ammonium nitrate during 1947,[8] and in 1994, the Asociación Mutual Israelita Argentina (AMIA) Jewish community center in Buenos Aires was bombed with the same chemical, killing 87 people.[9] At present, ANFO-based explosive materials account for most of the explosions that occurred in India, for example, the civil hospital blasts in Ahmedabad in 2008 and Mumbai in 2011.[10-11] In country like India, ANFO explosives are apparently found as ready mixed in a well packed plastic bag in the suspect's house during police patrolling and sometimes found as an undetonated product at the place of occurrence in the majority of criminal cases in the state of Madhya Pradesh. Investigation agencies usually send this suspected semi-solid material in plastic bags or plastic containers stated to be explosive material for the confirmation of chemical components to Forensic Science Laboratories.

Thus, the analysis of ingredients from any suspected explosives material is an important task for forensic chemists to recognize the type of explosive based on ingredients and ultimately support the investigation agency in exposing the connection to their probable root and origin. Many techniques have been usually employed for the identification of ANFO based preparations and their post blast residues. For example, Ion Chromatography (IC) was effectively able to detect ammonia and nitrate ions; presence of heavier hydrocarbons of fuel oil detected through Gas Chromatography–Mass Spectrometry (GC–MS).[12-13] Confocal Raman Spectroscopy was used to analyze the mixtures of ammonium nitrate (75% concentration) that may be found in real crime case samples of explosive threats.[14] Previously, Isotope Ratio Mass Spectrometry (IR-MS) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) were evaluated for the discrimination of various forms of ammonium nitrate and its precursors to generate characteristic isotopic and elemental profiles.[15-16] However, the most fascinating area in the field of analytical chemistry is the use of non-destructive and eco-friendly techniques.[17] Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) has opened the window of opportunity for the identification and detection of bulk chemical components of crime exhibits in many forensic laboratories.[16] The ATR-FTIR could be used as a complementary and fast procedure for the analysis of post-blast residue in consumer fireworks.[18] The main benefits of the ATR method include a nominal or no requirement for sample preparation and its provision for high lateral spatial resolution. Suppajariyawat et al. reported the analysis of synthesized ANFO samples using GC-MS and ATR-FTIR.[19] However, the use of these techniques on real time samples

received by Forensic Science Laboratories has not been published. In the present study, an attempt has been made to develop an appropriate modified procedure for forensic researchers and explore the important role of the non-destructive ATR-FTIR technique in the analysis of real crime samples received in Forensic Science Laboratories for ANFO/Organic explosive ingredients. This study also highlighted the recovery of solid ammonium nitrate from real crime sample of ammonium nitrate fuel oil-based explosives using minimal use of organic solvents, which made this modified procedure as an eco-friendly technique in forensic chemistry.

MATERIALS AND METHODS

Diethyl ether (HPLC & Spectroscopy Grade, Purity 99.5%, CASR 60-29-7) and acetone (GC Grade, Purity 99.90%, CASR 67-64-1) were purchased from Finar Limited, Ahmedabad Gujrat, India. Iso-propyl alcohol (Purity 99%, CAS No. 67-63-0) was purchased from Loba Chemie Pvt. Ltd., Palghar, Maharashtra India. Whatman filter papers (No. 42, Ashless, Diameter 125 mm) was purchased from the authorized local vendor. The deionized water having Resistivity > 10 MΩ.cm was produced using Ion Exchange Lab Q Water System (ION EXCHANGE INDIA LTD. Mumbai, India) and used during chemical analysis.

Samples were seized by the investigating agencies as suspected explosive material. It was deposited for chemical identification of any “Explosive Substance” composition or any residue of explosive material. Figure 1 shows a schematic representation of the procedure for the analysis of a suspected explosive/ ANFO sample.

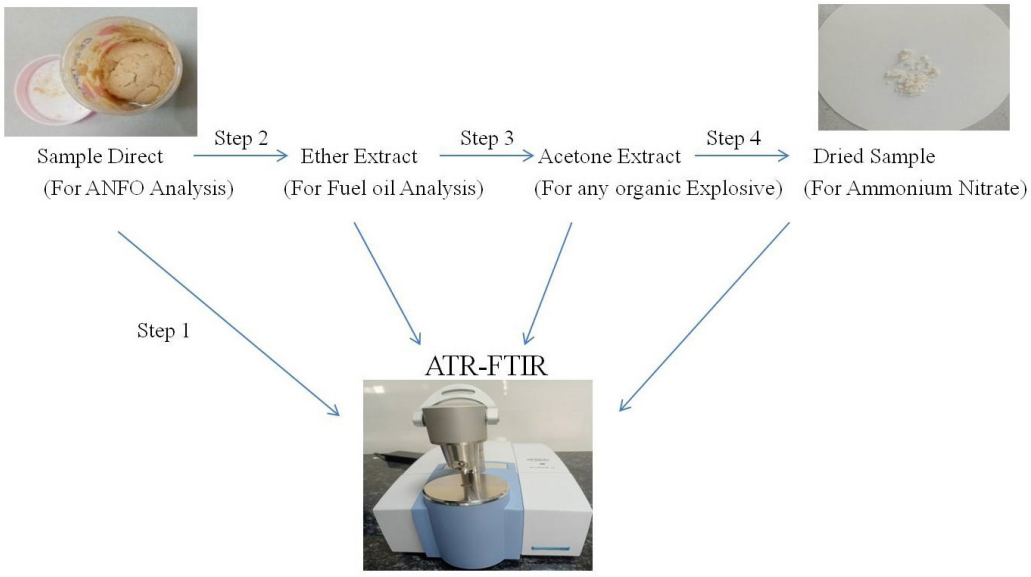

Figure 1. Schematic diagram of the procedure used for sample analysis.

Sample preparation/extraction procedure

Direct sample analysis

Some creamish coloured semi-solid sample suspected to be ANFO is directly applied and analyzed on the ATR-FTIR without any solvent treatment.

Diethyl-ether extraction

Some amount (approximately 1 g) of a creamish coloured semi-solid sample suspected to be ANFO is taken in test tube and extracted with diethyl-ether for fuel oil analysis. Briefly, 1 gram of sample and 2 ml of diethyl-ether are properly mixed using vortex. The diethyl-ether layer is poured from the test tube in a glass vial and concentrated to 20 µl. 10 µl of extract is taken and analyzed using a micropipette on ATR-FTIR for fuel oil analysis.

Acetone extraction

After extraction of diethyl-ether, residue of sample in test tube is further extracted with solvent acetone for the analysis of organic explosives. The acetone layer is poured from the test tube in a glass vial and concentrated to 20 µl. 10 µl of extract is taken and analyzed using a micropipette on ATR-FTIR for organic explosives analysis (if any).

Solid ammonium nitrate recovery procedure

After acetone extract preparation, acetone is evaporated from the residue of sample and the sample is dried at room temperature, resulting in the formation of powdery material. Finally, powdery material is directly analyzed on the ATR diamond for ammonium nitrate recovery.

ATR-FTIR analysis

Infrared (IR) spectra are recorded on a Bruker ALPHA II FT-IR spectrometer (Brucker Optick GmbH, Ettlingen, Germany) equipped with a Single-reflectance diamond ATR accessory exhibiting a Single-reflectance (45°) diamond crystal (Bruker Platinum ATR) with an approximately 0.6 mm × 0.6 mm active area and a deuterated triglycine sulfate (DTGS) detector. IR absorption spectra are recorded from 4000 to 400 cm^{-1} using a spectral resolution of 4. Before each measurement, the crystal is carefully cleaned with isopropanol, and a background spectrum is recorded. For each real sample analysis, samples are dropped over the dry surface of the ATR prism. Five replicated spectra are recorded to assess precision and ensure the reproducibility of each sample. For every measurement, the system performed twenty-three scans, which are automatically averaged using the Opus 8.7.31 software in order to obtain spectra with a good signal-to-noise ratio. The ATR prism is cleaned and tested regularly between the measurements, and an absorption spectrum is recorded without the sample to ensure that it has no peaks above the regular environmental noise level. The laboratory conditions of relative humidity and temperature during the analysis are kept around 39 ± 8.0% and 23.7 ± 2.0 °C, respectively.

RESULTS AND DISCUSSION

Functional groups in the ingredients of chemical substances could be easily examined by the characteristic absorption band pattern of each group of atoms in the FT-IR spectra.[11] ATR-FTIR spectra of ammonium nitrate standard crystals are presented in Figure 2.

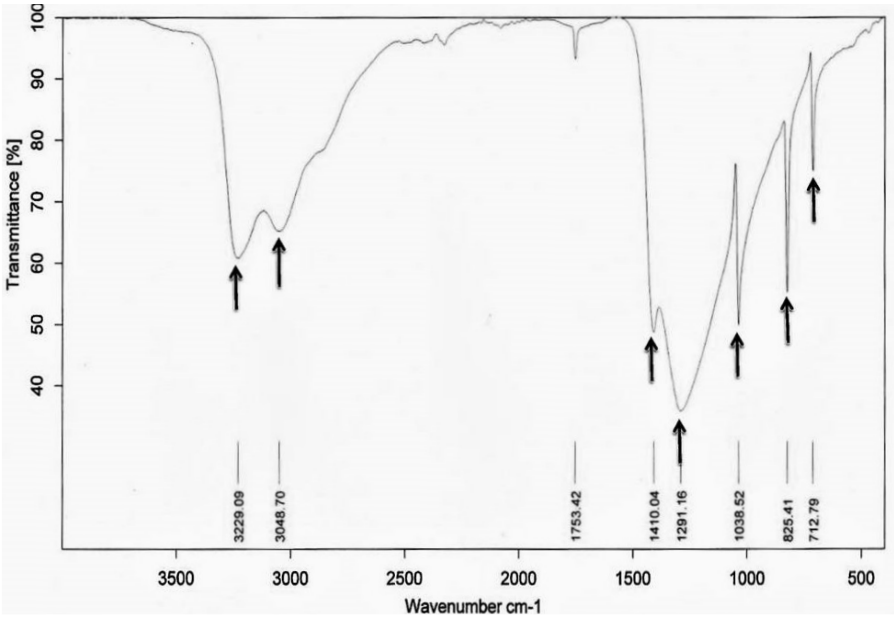

Figure 2. FTIR spectra of ammonium nitrate.

In general, ammonium nitrate spectra show two main characteristic signals of NH_4^+ ions and NO_3^- ions. The NH_4^+ ions have consistently 2 characteristic bands; i.e., a very strong broad band at 3020 to 3330 cm^{-1} and a strong absorption region at 1325 to 1480 cm^{-1}. It has no bands developing below 714 cm^{-1}. The NO_3^- ions have a very strong broad absorption band at 1280–1520 cm^{-1}, three weak bands in the region of 700–740 cm^{-1}, 1020–1070 cm^{-1} and 1720–1800 cm^{-1}, and a sharp, less intense band also observed at 840–800 cm^{-1}. These signals are the major characteristic of the ammonium nitrate compound in ATR-FTIR, which confirmed the vibrational mode of both ions.[13] The spectrum show two peaks labelled at 3048 cm^{-1} and 3225 cm^{-1}, could be related to the vibrational frequencies of NH_4^+ ions. The strong vibrational frequencies of NO_3^- ions peaks have labelled at 1291 cm^{-1} and 1409 cm^{-1}. The bands appearing at 713 cm^{-1}, 826 cm^{-1}, 1040 cm^{-1} and 1753 cm^{-1} have resulted from weak vibrational frequencies of NO_3^- ions.

The diesel sample from the local area is taken for analysis, and a spectrum is shown in Figure 3. As aliphatic hydrocarbons (paraffin) are the main components of diesel fuel, they mostly express the vibrational features of an aliphatic C-H stretching in the region at 2800–3200 cm^{-1} and the C-H deformation at around 1350–1470 cm^{-1}.[14] IR spectra of the diesel sample have confirmed the presence of three characteristic peaks of diesel, labelled at 2853 cm^{-1}, 2921 cm^{-1} and 2954 cm^{-1} which are related to the vibrational aliphatic C-H group stretching. The peaks observed and labelled at 1377 cm^{-1} and 1459 cm^{-1} resulted from the absorption band of C-H group deformation.

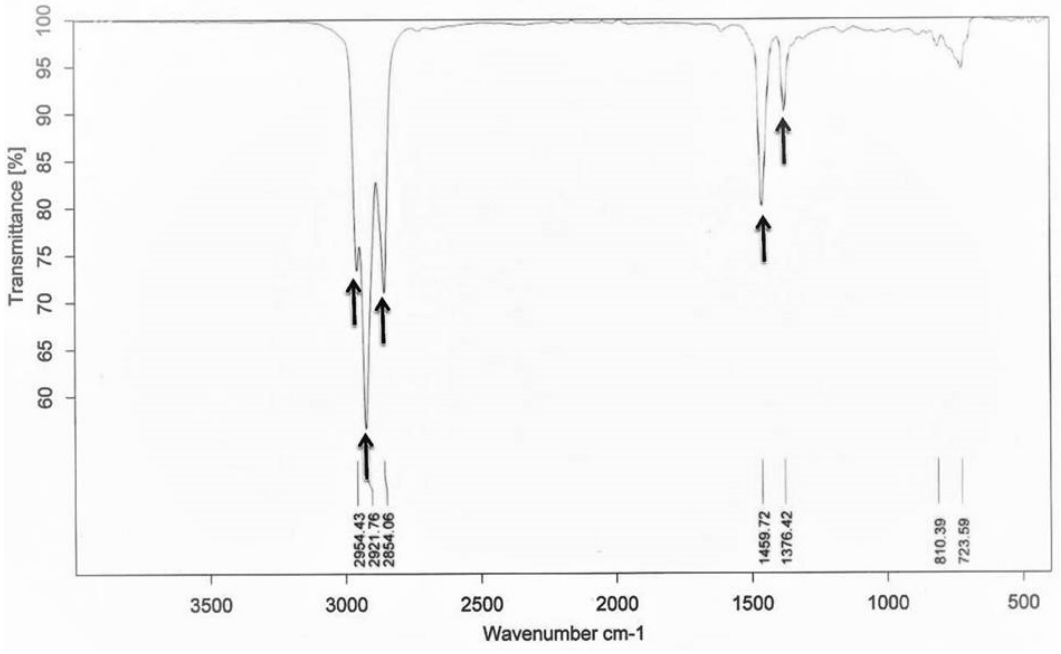

Figure 3. FTIR spectra of diesel.

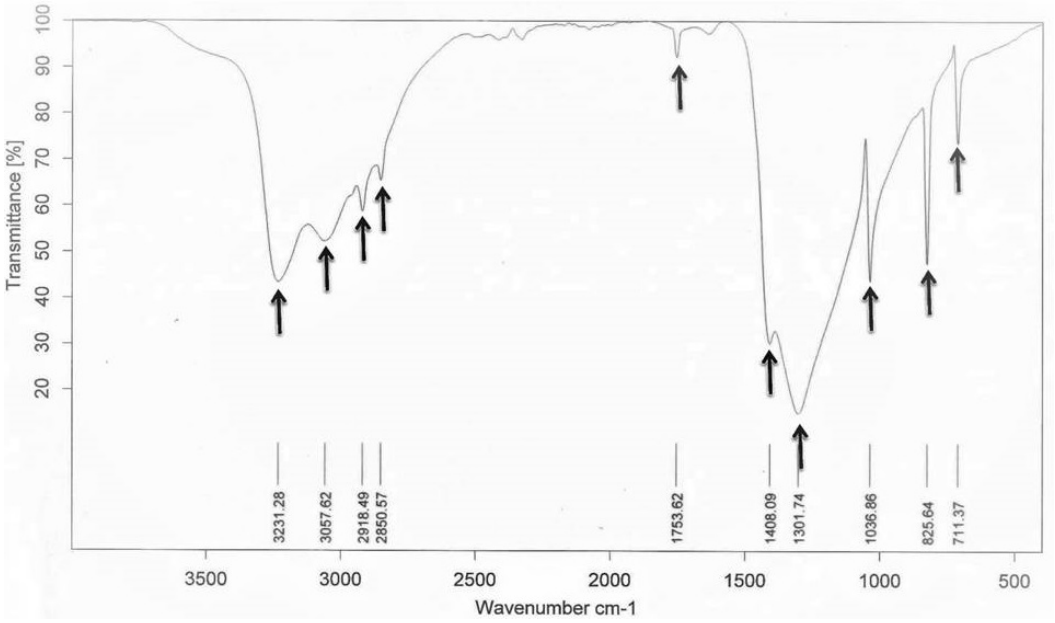

Figure 4. FTIR spectra of semi-solid sample.

Firstly, the semi-solid sample stated to be explosive was directly analyzed on ATR-FTIR and presented in Figure 4. The sample has shown spectra that are slight similar to those of ammonium nitrate spectra along with characteristic peaks of N–H stretching vibration in the region 3020 to 3330 cm^{-1} and N-O stretching and NO group deformation in the regions of 700–770 cm^{-1}, 800–860 cm^{-1}, 1015–1070 cm^{-1}, 1280–1520 cm^{-1} and 1700–1800 cm^{-1}. The FTIR spectrum of ammonium nitrate produced strong signals resulting from NO_2 group asymmetric stretches in the range 1325–1425 cm^{-1} and NO group deformations in the region 860–800 cm^{-1}. These signals from a semi-solid sample in IR could be the main aspect of the ammonium nitrate compound. But the FTIR spectra of the tested sample did not visually display any significant differences when compared to the standard spectrum of ammonium nitrate. Also, the main FTIR signals of diesel standard, including C-H deformation and C-H stretches, are completely hidden under the ammonium nitrate spectrum of the semi-solid sample. This is expected because FTIR analysis does not usually present enough sensitivity to a low percentage of unknown substances, which could be buried in the suspected sample as explosive material.[19] As FTIR did not evidently distinguish different peaks specifying the components of ANFO, it is difficult to say that a suspected explosive compound is ANFO or not; because it is uncertain whether ammonium nitrate is present in the form of a pure substance or blended with any fuel oil. It is necessary to extract the fuel oil component from the matrix. So, the sample is extracted using a suitable solvent, i.e., diethyl-ether to extract out the fuel oil part of the sample. The FTIR spectrum of concentrated organic extract is characterized by ATR-FTIR and is presented in Figure 5.

The maximum intensive bands are caused by C–H groups stretching in the region between 3000–2800 cm^{-1} and angular deformations at 1461 cm^{-1} and 1383 cm^{-1}; which confirmed the presence of characteristic peaks of diesel residue in semi-solid sample, as previously shown in Figure 3. Other peaks could corroborate the presence of aromatic compounds along with components of biodiesel. Aromatic compounds showed characteristic bands of low intensity in the region 900–675 cm^{-1} from the C–H out of line angular deformation. Nespeca et al reported that biodiesel in diethyl-ether extract is observed by the carbonyl absorption band near 1750–1735 cm^{-1}.[20] As a good practice, it is essential to screen the sample for high explosive residues during forensic examination. So, after the fuel oil analysis, acetone extract is prepared for screening of high explosive residues. However, it is also of greater importance in handling the ANFO-like material in the laboratory during analysis. Scientists should ensure that such type of compund is not exposed to strong shock waves from other explosives and also avoid contamination with combustible materials or organic substances and inorganic materials that may contribute to its sensitivity to explosion.[21]

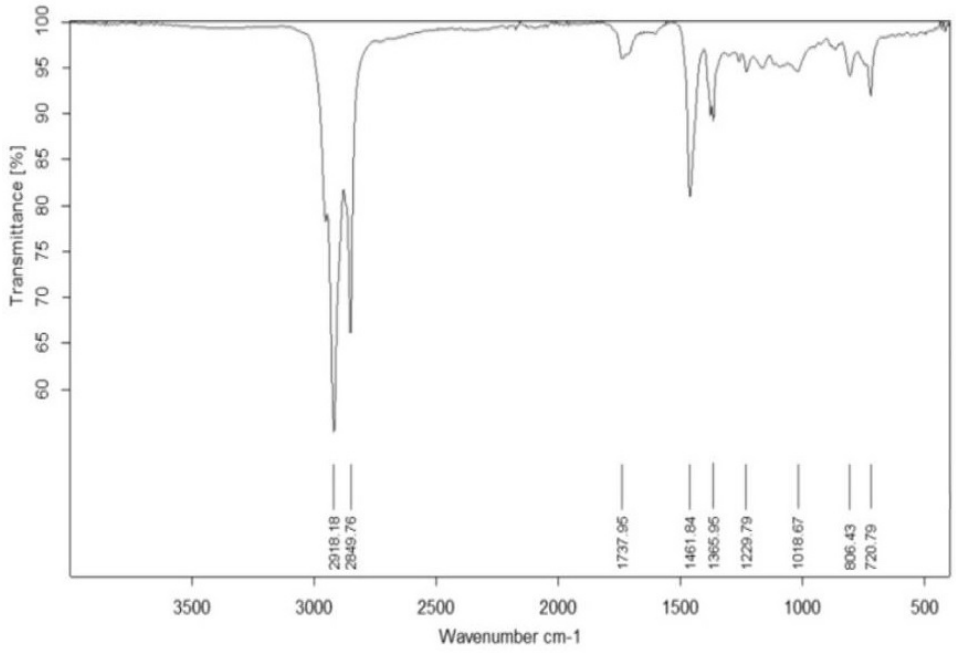

Figure 5. FTIR spectra of diethyl-ether extract from sample.

Acetone extract is dried on aluminium foil and then analyzed on an ATR diamond. No high explosive residue was detected in the acetone extract of the sample (data not presented). Finally, a powdery material is prepared after the evaporation of acetone from the sample. Powdery material is directly analyzed on the ATR-FTIR and spectra are presented in Figure 6.

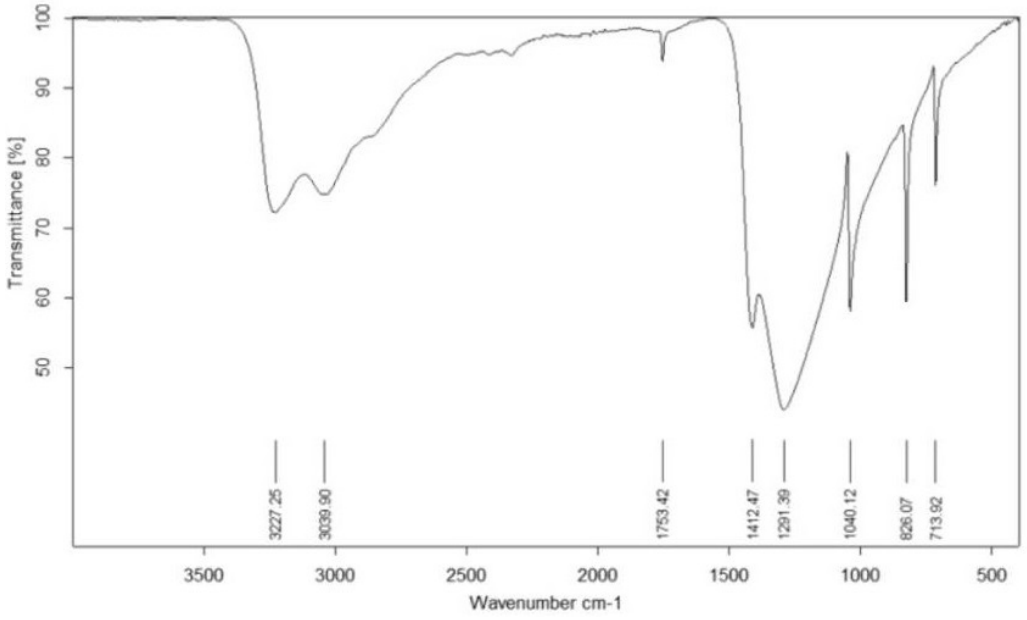

Figure 6. FTIR spectra of powdery material obtained after extract.

Two characteristic bands (as mentioned previously in Figure 2) of ammonium nitrate can be easily identified in the powdery sample, which confirmed the presence of characteristic peaks of ammonium nitrate in the extract from the sample. The spectra of semi-solid material do not give a clear conclusion

about the presence of ammonium nitrate and fuel oil components in the sample, as ammonium nitrate dominates in the spectra. But after following the procedure as mentioned in Figure.1, spectra of ether extract closely match with the diesel standard. Similarly, the spectra of powdery material after acetone extract are matched with the ammonium nitrate standard. ANFO is cheaper than other high explosives such as Trinitrotoluene (TNT), Cyclonite (RDX), Pentaerythritol tetranitrate (PETN), Nitroglycerin (NG) etc. Its easy availability and easy preparation as improvised explosive devices which made it more popular with blasting agencies, anti-social elements and terrorists.[4] So, analysis of this type of sample should be carried out by the forensic laboratories in India. Hence, the proposed procedure can be advantageous in forensic investigation for easy and fast detection of suspected ANFO samples.

CONCLUSIONS

ANFO is the most popular industrial explosive and is used as an oxidizing agent. It is also used as the main charge in improvised explosive devices by the group of terrorists due to its high stability and easy availability. In various states of India, like West Bengal and Madhya Pradesh, most of the blasting incidents has been reported due to this low-cost explosive. This work has aimed to develop a simple, accurate, and fast analytical method of detection based on ATR-FTIR spectroscopy to determine the ingredients of ANFO in crime exhibits with minimal use of chemical solvents for forensic science laboratories. The present study proved that ATR-FTIR analysis can be one of the best choices to identify ANFO-based explosives from different crime scenes and arrange intelligence information by collecting the data for forensic research and analysis. Since the application of the ATR-FTIR methodology is capable of cutting down costs and boosting considerably the classification frequency; FTIR analysis also gains attention due to minimal sample preparation, minimal use of solvents and chemical reagents without generating any harmful waste. For this study to be useful, each forensic science laboratory could use this method of analysis for ANFO/Explosive samples and develop the same method to establish selective identification in criminal cases.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Technical Note

Assessment of Banknotes as a Matrix for Detecting Post-Explosion Residues of Fuel-Oxidizer Explosive Mixtures Using Ion Chromatography

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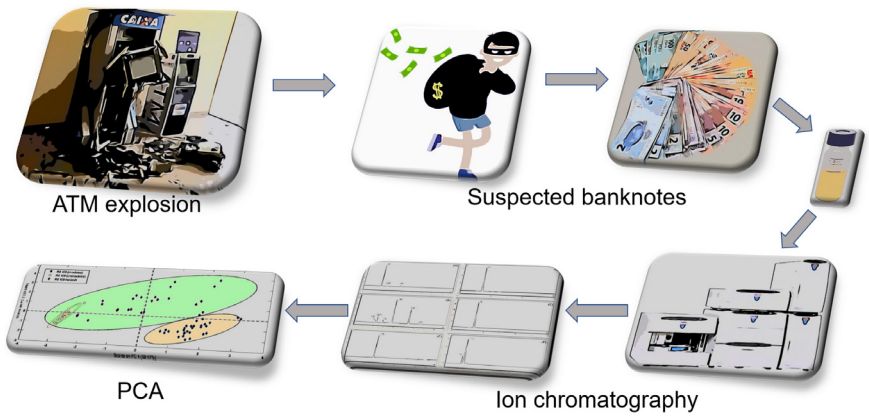

Banknotes are commonly subjected to chemical analysis in forensic laboratories in the search for post-explosion residues. This matrix presents unique challenges due to the potential presence of target analytes resulting from everyday use, as well as the lack of control samples for comparison. In addition to their relevance in attacks against Automated Teller Machines (ATMs), banknotes are of significant interest when confiscated from suspicious individuals, vehicles, and locations, as they can provide valuable evidence in establishing a connection to this type of crime scene. In such cases, the absence of bulk particles, alternative materials, and control samples is common. This study employed ion chromatography to analyze uncirculated, circulated, and seized banknotes, aiming to determine their ionic profiles. This investigation provides insights into the background levels of target ions in banknotes and aids in the analysis of post-explosion residues. A simple, fast, and precise extraction method was proposed, yielding RSD values below 10% for most analytes in uncirculated banknotes. The study revealed the presence of various ions of interest, some in significant concentrations, even in uncirculated banknotes. PCA analysis demonstrated a clear separation of uncirculated notes based on their banknote value. However, this clustering behavior was not observed in circulated banknotes due to natural variations in analyte concentrations. Interestingly, when uncirculated, circulated, and seized R$ 100 banknotes were analyzed together, the seized samples from an ATM robbery showed a distinct separation from the other groups, indicating the potential for developing classification models.

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INTRODUCTION

The analysis of explosives and post-explosion residues is a critically important area in forensic chemistry, providing crucial insights into various incidents involving the use of explosives. This type of analysis plays a key role in addressing fundamental questions related to the crime, including: i) Was there an explosion? ii) What was the cause of the explosion? iii) Who was responsible for the explosion? iv) Are there any indications of a clandestine explosives production facility? v) Can any patterns or trends be identified?[1,2]

According to a previous study conducted by our research group, in Brazil, most cases involving the use of explosives are targeted against Automated Teller Machines (ATMs). This issue is also prevalent in several other countries worldwide.[2] The study further revealed that these crimes predominantly involve the use of Improvised Explosive Devices (IEDs) based on black powder, mixtures containing chlorate and/or perchlorate salts, and explosive emulsions.[2]

Figure 1 depicts the unpredictable nature of scenarios resulting from this type of crime, which can vary from the failure of the explosive device with minimal damage to the complete destruction of bank branches, causing significant structural damage. This unpredictability arises from various factors, including the type, quality, and containment of the explosive used, the expertise of the perpetrator, and the structural characteristics of the ATM location.[3]

Figure 1. Unsuccessful ATM explosion attempt (left),[4] effective ATM explosion with relative level of destruction (center)[5] and ATM explosion with high level of destruction of a bank branch with damage including its building structure (right).[6]

All these diverse variables introduce varying levels of complexity in both the investigation of explosion scenes and the subsequent laboratory analysis of post-explosion residues. When bulk material is available, a wider range of analytical techniques can be employed, resulting in more robust findings and mitigating potential issues related to matrix interference. Examples of analytical techniques commonly used in these cases include Raman microscopy,[2,7-9] FTIR,[2,10,11] GC-MS,[2] and DART-MS.[12]

Nevertheless, in some cases of explosions, only invisible residues may be left at the crime scene, necessitating solvent extractions for analysis and using a more limited set of techniques. Among these techniques, ionic chromatography with conductivity detection (IC-CD) is the most employed worldwide for the identification of inorganic components present in explosives.[13-15]

In the analysis of post-explosion residues using IC-CD, the objective is to identify the ions present in the original composition of the explosive mixture, as well as ions of substances formed during the explosive reaction. The interpretation of the ionic profile obtained from the analysis of cations and anions is evaluated to reach a conclusion regarding the identification of the explosive. Table I provides information on the original components and the main analytes of common fuel-oxidizer explosive mixtures frequently employed in ATM robberies in Brazil, including black powder, $KClO_3$-based mixtures, and explosive emulsion.[1,14-16]

Table I. Common explosives mixtures used in Brazil and major target ions in post-explosion residues

Explosives	Composition	Major target ions in post-explosion residues
Black Powder	KNO_3, C, S	SO_4^{2-}, NO_2^-, NO_3^-, $S_2O_3^{2-}$, SCN^-, OCN^-, K^+
$KClO_3$/sulfur-mixture	$KClO_3$, sulfur	Cl^-, ClO_4^-, ClO_3^-, K^+, SO_4^{2-}
Explosive emulsion	NH_4NO_3, fuel oil	NH_4^+, Na^+, NO_3^-

As depicted in Table I, except for explosive emulsion, the presence of potassium cation (K^+) is detected, which is prevalent in the majority of our cases. Potassium salts are commonly used in pyrotechnic products due to their low hygroscopicity. However, salts of other cations, such as lithium (Li^+), sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}), can also be utilized for this purpose and should be considered as possible analytes of interest, although less frequently encountered.[16]

As indicated in Table I, most analytes are common ions that can originate from sources other than explosives, including their natural occurrence in the environment,[1,14-16] materials commonly used in evidence collection and laboratory sample processing,[17] and even the materials that support post-explosion residues themselves. For this reason, when analyzing post-explosion residues, especially those involving fuel-oxidizer explosive mixtures, it is crucial to have knowledge about the various materials involved, such as matrices, sampling and laboratory materials. This understanding facilitates the interpretation of the obtained results. While some studies have investigated background levels of these target analytes in various locations/matrices,[18-21] and sampling materials commonly used in routine laboratory practices,[17,22] no specific study has been conducted to determine the background levels of these analytes in a crucial matrix for this type of analysis: banknotes. This is particularly relevant in regions where ATM explosions are frequent.

An ATM explosion often leaves various types of trace evidence, allowing for the collection of multiple materials at the post-explosion scene. These materials may include fragments of plastic, metal, and paper from Improvised Explosive Devices (IEDs) or objects/structures affected by the explosion. Additionally, it is possible to collect soil, clothing, banknotes, swabs, and cotton balls or disks impregnated with residues. The collection of multiple types of materials generally increases the chances of obtaining results that enable a simpler and more conclusive interpretation, as it allows for the selection of more suitable materials that are less prone to interference issues.

Nevertheless, there are instances when the banknotes seized for forensic analysis do not come directly from the explosion scene but are obtained from suspicious individuals, vehicles, properties, or found abandoned on the street. These banknotes can be submitted to the forensic laboratory with the aim of establishing a connection to criminal activities involving ATM robberies using explosives. Unlike in post-explosion scenes, in these cases, there is often not more than one type of material, but rather only the banknotes. In these situations, the lack of control samples (blanks), coupled with the inherent presence of target analytes even in banknotes unrelated to ATM robberies, poses a significant challenge in interpreting the results of the analysis. Figure 2 illustrates some real cases involving the collection of banknotes for post-explosion residue analysis.

Figure 2. Examples of banknotes seized in different situations and sent to the laboratory for post-explosion residue analysis: ATM post-explosion scene (left),[23] suspect vehicle (center),[24] suspect banknotes found on the street (right).[25]

Some studies have focused on identifying explosives in banknotes through direct analysis of bulk particles.[7,8,26] However, the analysis becomes considerably more challenging when bulk particles are absent, requiring prior extractions. In such cases, the background level of target analytes present in the banknote matrix becomes highly relevant. Based on our experience, this is the most common scenario, particularly when the banknotes are not directly obtained from post-explosion locations but from suspicious individuals, vehicles, or locations. As previously mentioned, the limited presence of residues and the impracticality of collecting control samples greatly hinder the interpretation of results through direct observation of ion chromatograms.

Chemometric models can be useful for data analysis of complex matrices containing interferences or variations from different sources.[27] The application of these models is quite popular in spectroscopy and have been applied to help explosive analysis in near infrared,[28] laser-induced thermal emission[29] and laser-induced breakdown spectroscopy data.[30] However, the application of chemometrics for the analysis of ion IC-CD data is scarce, probably because the chromatograms usually present very good resolution and a lower number of peaks in comparison with other chromatographic techniques, which leads to univariate analysis. To the best of our knowledge, no study has been reported applying multivariate analysis to explore the IC-CD data of banknotes involved in post-explosion cases.

In this context, this paper presents an exploratory study that examines the viability of using IC-CD analysis to identify banknotes containing post-explosion residues. Furthermore, a simple, fast, and precise water extraction method was proposed to enable the analysis of the target analytes usually present in post-explosion cases. The study clarifies the background level of target ions that can be detected through water extraction in uncirculated, circulated, and seized banknotes containing post-explosion residues. The exploratory and pattern recognition analysis of the data was performed using principal component analysis (PCA).

MATERIALS AND METHODS

Reagents and materials

A total of 166 real banknotes were included in the analysis. This comprised ten uncirculated banknotes and fifteen circulated banknotes for each denomination, including R$ 2, R$ 5, R$ 10, R$ 20, R$ 50, and R$ 100. Additionally, sixteen seized banknotes of R$ 100 were included in the study. The uncirculated banknotes were obtained from the Banco do Brasil branch of the Central Bank of Brazil, while the circulated banknotes were collected randomly from various tolls and commercial establishments in Brazil. The suspected banknotes were obtained through a seizure conducted by the Federal Police.

Standard ion solutions were prepared following the procedures of the Forensic Chemistry Laboratory of the National Institute of Criminalistics of the Federal Police, which is certified according to ISO 17025, including the analysis of post-explosion residues within its scope. Anion standard solutions were prepared from a 500 mg L^{-1} stock solution prepared from sodium salts (chloride (Vetec Ltd.), chlorite, perchlorate, nitrite (Sigma-Aldrich Ltd.), sulfate (Cinetica Ltd.), thiosulfate (Carlo Erba Ltd.), nitrate (QEEL Ltd.) and

chlorate (Baker Ltd.)) and potassium salts (thiocyanate (Vetec), cyanate (Sigma-Aldrich Ltd.)). Cation standard solutions were prepared from a commercial stock solution (Dionex Six Cation-II Standard (lithium 50 mg L^{-1}, sodium 200 mg L^{-1}, ammonium 250 mg L^{-1}, potassium 500 mg L^{-1}, magnesium 250 mg L^{-1} and calcium 500 mg L^{-1})). All standards solutions and extracted samples were prepared using ultrapure water (18, 2 MΩ cm at 25 °C) obtained from a Millipore Direct-Q5 purification system.

Instrumentation

Ion chromatography was performed with a Thermo Scientific Dionex ICS-5000 Ion Chromatography System with self-regenerating electrolytic suppression and AS-AP Autosampler. The anion separations were performed in a Dionex IonPacTM AS19 column (2 x 250 mm) using a EGC eluent generator configurated with a potassium hydroxide (KOH) eluent cartridge, operating in multi-step gradient mode, starting from 10 mM (0 min) to 45 mM (40 min), whereas the suppressor current was set to 28 mA. For cation separations, a Dionex IonPacTM CS12A column (2 x 250 mm) was operated under an isocratic condition, 20 mM (30 min) methane sulfonic acid (MSA) generated in-situ from reagent water with EGC eluent generator using an MSA cartridge, and the suppressor current was set to 15 mA. The temperatures of the column and detector cell were held at 30 °C and 25 °C, respectively. A loop size of 10 mL and constant flow rate of 0.25 mL min^{-1} for both methods. After each run, approximately 2 mL of pure milli-Q water (18,2 MΩ cm at 25 °C) was pumped into the column to avoid cross contamination. Instrument control and data acquisition were performed using Chromeleon® software.

Two calibration curves were prepared for each analyte (except for sodium and cyanate), one for low concentrations and another for higher concentrations. Only linear determination coefficients (R^2) over 0.99 were used. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated through linear regression method, using the Equation 1:[31]

$$\text{LOD or LOQ} = \frac{\text{F x SD}}{\text{b}} \qquad \text{Equation 1}$$

Where F are the factors 3.3 or 10 for LOD or LOQ, respectively; SD is the residual standard deviation of the linear regression; and b is the slope of the regression line, both for low concentrations curves. The resolutions were calculated by the Chromeleon software, according with Equation 2:

$$\text{R} = 2 \text{ x } \frac{\text{t}_{\text{ref peak}} - \text{t}_{\text{r}}}{\text{BW}_{\text{ref peak}} + \text{BW}_{\text{r}}} \qquad \text{Equation 2}$$

Where $t_{ref\ peak}$ is the retention time of the reference peak for the resolution; t_r is the retention time of the current peak; $BW_{ref\ peak}$ and BW_r are the widths of the two peaks.

The target analytes of this study were: chloride (Cl^-), chlorite (ClO_2^-), chlorate (ClO_3^-), perchlorate (ClO_4^-), sulfate (SO_4^{2-}), nitrite (NO_2^-), nitrate (NO_3^-), thiosulfate ($S_2O_3^{2-}$), thiocyanate (SCN^-), cyanate (OCN^-), potassium (K^+), ammonium (NH_4^+), sodium (Na^+), lithium (Li^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}).

Sample preparation

Initially, a study was conducted to establish a sample preparation methodology that would enable the use of a small volume of water and good reproducible results. Uncirculated banknotes were employed to evaluate various factors, including the type of plastic tube, the way the samples were placed in these tubes (folded or rolled), the water volume, and the sonication time (5, 15, and 30 minutes). The findings indicated that the optimal extraction condition was obtained using 2.500 mL of water in a 4.30 mL plastic tube with a rolled banknote. Interestingly, all tested time periods yielded comparable results, suggesting that the extraction of target analytes is rapid. The methodology used in sample preparation is described in detail below and depicted in Figure 3.

Each banknote was carefully rolled up and inserted into a 4.30 mL plastic tube. Subsequently, 2.500 mL of ultrapure water was added to the tube, which was then sealed with its respective lid. The tube was subjected to sonication for five minutes, followed by vortexing for one minute, and subjected to centrifugation at 4000 rpm for five minutes. The resulting solution was filtered through a 0.45 µm filter directly into an IC-CD vial. Additionally, a dilution step was performed by transferring a portion of the solution into another IC-CD vial at a dilution ratio of 1:20. Both vials, containing the original and diluted solutions, underwent IC-CD analyses in duplicate.

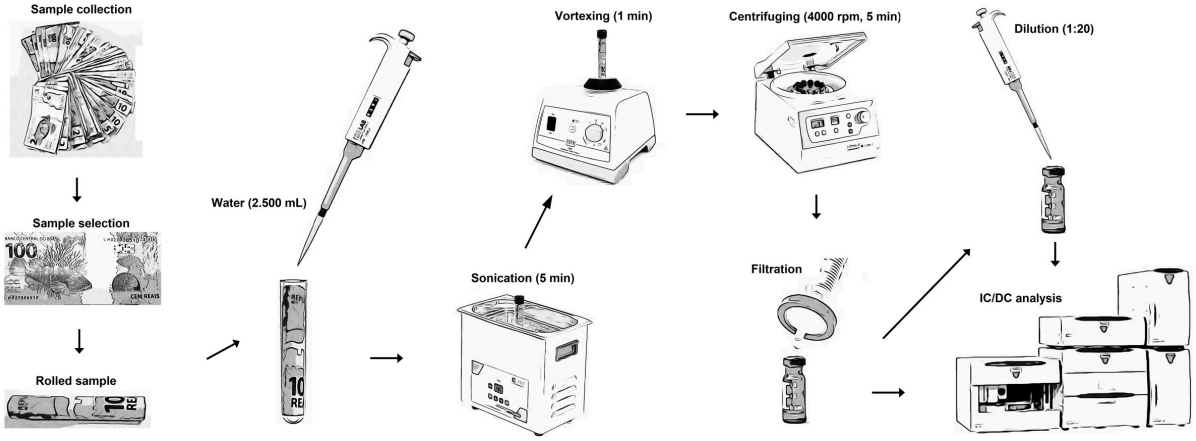

Figure 3. Schematic diagram of the sample preparation for the IC-CD analysis.

Statistical analysis

All chromatograms were processed in the Chromeleon software to obtain the peak areas for all analytes, data were autoscaled before analysis. Principal Component Analysis (PCA) were performed using PLS-Toolbox version 8.8.1 (2022) for MATLAB R2022a. The data matrix was composed by the peak areas of the sixteen target analytes previously described and the PCA was performed using Singular Value Decomposition (SVD) and 95% confidence level for Q residuals and Hotelling T^2 limits for outlier detection.

RESULTS AND DISCUSSION

Table II presents the figures of merit obtained for the method used to quantify the ten anions and six cations in the samples. All analytes demonstrated very good resolution, and the detection and quantification limits were deemed suitable for determining explosive residues in banknotes and other common forensic samples.

Table II. Figures of merit from anion and cation of interest standard samples

Analyte	t_R (min)	Range (mg L^{-1})		Resolution	LOD (mg L^{-1})	LOQ (mg L^{-1})
		Curve 1	Curve 2			
Cl^-	9.037	0.20-1.00	0.80-300.00	4.95	0.009	0.031
ClO_2^-	7.574	0.20-1.00	0.80-300.00	2.78	0.022	0.073
ClO_3^-	12.160	0.20-1.00	0.80-300.00	1.97	0.010	0.033
ClO_4^-	36.680	0.20-1.00	0.80-300.00	3.14	0.019	0.062
SO_4^{2-}	19.070	0.20-1.00	0.80-200.00	3.83	0.036	0.117
NO_2^-	10.847	0.20-1.00	0.80-150.00	1.34	0.009	0.031

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Table II. Figures of merit from anion and cation of interest standard samples (continuation)

Analyte	t_R (min)	Range (mg L^{-1})		Resolution	LOD (mg L^{-1})	LOQ (mg L^{-1})
		Curve 1	Curve 2			
NO_3^-	14.374	0.20-1.00	0.80-300.00	2.27	0.037	0.120
$S_2O_3^{2-}$	27.464	0.20-1.00	0.80-150.00	2.07	0.053	0.175
SCN^-	32.977	0.20-1.00	0.80-100.00	3.51	0.004	0.013
OCN^-	11.394	0.20-1.00	------	1.79	0.011	0.036
K^+	6.400	0.063-2.500	2.50-250.00	7.93	0.044	0.144
NH_4^+	5.193	0.031-1.250	1.25-125.00	4.37	0.015	0.050
Na^+	4.587	------	0.80-150.00	2.43	0.279	0.921
Ca^{2+}	12.970	0.063-2.500	2.50-250.00	3.13	0.023	0.076
Mg^{2+}	10.427	0.031-1.250	1.25-250.00	3.25	0.022	0.074
Li^+	3.900	0.006-0.250	0.25-25.00	3.09	0.005	0.015

The results in Table III show that the presence of several ions of interest was observed in all banknotes, including the uncirculated ones. These detected ions include chloride (Cl^-), sodium (Na^+), potassium (K^+), sulfate (SO_4^{2-}), calcium (Ca^{2+}), ammonium (NH_4^+), magnesium (Mg^{2+}), and nitrate (NO_3^-). However, most of the uncirculated banknotes presented considerably lower concentrations than the circulated banknotes and, as expected, the former showed a more homogeneous concentration (lower RSD) profile of the ions studied. This result highlights the relevance of knowing the ionic profile of materials commonly submitted to analysis, especially in the absence of a control/blank, in order to avoid false positive or inconclusive results. Furthermore, it emphasizes the importance of prioritizing the search for the minority ions, such as perchlorate (ClO_4^-), chlorate (ClO_3^-), chlorite (ClO_2^-), nitrite (NO_2^-), thiosulfate ($S_2O_3^{2-}$), thiocyanate (SCN^-) and cyanate (OCN^-). Depending on the composition of the explosive (Table I), these minority ions may represent valuable information to determine the presence of an explosive residue. However, in the samples used in this study, these minority target ions were not identified, further increasing the degree of difficulty in interpretation of the results.

Table III. Mean concentrations (mg L^{-1}) and RSD%, shown in parentheses, of identified target ions (mg L^{-1}) present in each set of uncirculated (u), circulated (c) and seized (s) banknotes extracts

Bank Notes/ Ions	Cl^-	SO_4^{2-}	Na^+	K^+	Mg^{2+}	Ca^{2+}	NH_4^+	NO_3^-
R$ 2 (u)	71.6 (3)	124.5 (3)	33.9 (5)	21.5 (3)	24.1 (4)	57.5 (6)	2.6 (8)	2.8 (34)
R$ 2 (c)	1631.7 (49)	205.2 (52)	1060.8 (51)	571.3 (55)	31.4 (42)	243.0 (110)	37.8 (73)	17.6 (56)
R$ 5 (u)	199.8 (4)	150.4 (5)	82.8 (4)	28.5 (4)	7.7 (4)	89.2 (4)	2.0 (166)	5.2 (178)
R$ 5 (c)	2083.1 (54)	234.6 (37)	1159.0 (50)	627.5 (51)	21.8 (45)	193.7 (66)	35.8 (86)	23.0 (55)
R$ 10 (u)	252.7 (4)	297.3 (5)	82.2 (5)	34.5 (3)	6.7 (4)	137.9 (4)	19.0 (148)	1.8 (96)

(continues on the next page)

Table III. Mean concentrations (mg L^{-1}) and RSD%, shown in parentheses, of identified target ions (mg L^{-1}) present in each set of uncirculated (u), circulated (c) and seized (s) banknotes extracts (continuation)

Bank Notes/ Ions	Cl^-	SO_4^{2-}	Na^+	K^+	Mg^{2+}	Ca^{2+}	NH_4^+	NO_3^-
R$ 10 (c)	2500.1 (39)	309.9 (47)	1438.4 (36)	794.8 (38)	30.9 (35)	249.6 (63)	48.7 (77)	26.5 (42)
R$ 20 (u)	198.3 (3)	171.5 (2)	85.9 (4)	40.3 (4)	7.7 (3)	134.0 (5)	1.1 (151)	2.9 (273)
R$ 20 (c)	2675.5 (24)	239.5 (40)	1559.8 (24)	863.4 (29)	32.6 (25)	274.9 (52)	70.6 (71)	28.6 (46)
R$ 50 (u)	224.5 (6)	168.1 (7)	74.3 (7)	39.7 (3)	8.1 (5)	115.9 (6)	9.4 (13)	3.2 (70)
R$ 50 (c)	2162.5 (38)	247.3 (28)	1395.8 (40)	779.1 (36)	31.6 (36)	414.5 (59)	59.6 (93)	16.1 (79)
R$ 100 (u)	293.8 (4)	303.9 (4)	81.1 (4)	50.1 (4)	10.3 (17)	159.9 (21)	37.2 (136)	1.2 (240)
R$ 100 (c)	1363.4 (59)	418.9 (28)	686.8 (62)	414.5 (65)	20.3 (46)	297.8 (79)	138.5 (51)	1.8 (192)
R$ 100 (s)	2269.1 (17)	238.4 (25)	1018.0 (16)	522.0 (17)	20.0 (17)	142.5 (28)	29.4 (110)	0.7 (397)

The results presented in Table III also draw special attention to the typical expected results for $KClO_3$/sulfur-based mixtures, as the most prevalent anions detected in all banknotes (Cl^- and SO_4^{2-}) align with the major anions found in the post-explosion residues of these mixtures. Consequently, they exhibit a closely matching anionic IC-CD profile. Additionally, in terms of cation analysis, the presence of potassium ions is also a complicating factor, as potassium is a very common counter-ion in explosive mixtures, as mentioned earlier.

It is important to clarify that, unlike the typical chromatogram example for a $KClO_3$-based mixture depicted in Figure 4, where the concentrations of the analytes enabled the identification of significant minor ions, such as ClO_3^-, there are instances where only the major ions are detected, further complicating the interpretation of results. This situation can arise due to various factors, including a more thorough explosive reaction, a minimal amount of residue, the utilization of a technique with a high detection limit, or an improper collection/sampling or extraction process.

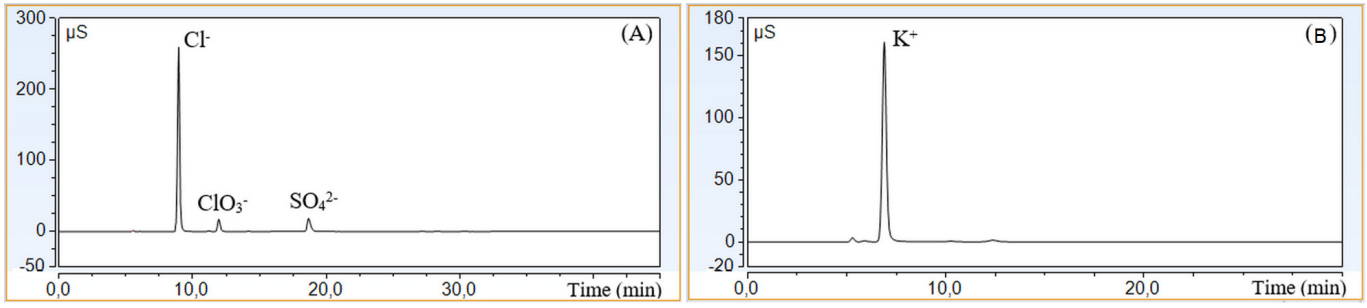

Figure 4. Typical anion (A) and cation (B) exchange ion chromatograms of post-explosion residues of an explosive mixture based on potassium chlorate and sulfur.

In order to illustrate the aforementioned challenge in result interpretation, Figure 5 demonstrates the striking similarity in IC-CD anionic profiles obtained from (1) a forensic case involving the analysis of post-explosion residue on a cotton ball collected at an ATM explosion scene (Figure 5A), (2) one of the circulated banknotes examined in this study (Figure 5B), and (3) residues from a controlled burning of a $KClO_3$/sulfur mixture, where the minor ions (such as chlorate and/or perchlorate) were not detectable due to their insufficient concentrations (Figure 5C). Regarding the results for cations, the analysis of the controlled burning residue reveals the presence of K^+ as the sole cation in the profile (Figure 5F), which generally facilitates the interpretation of the results. However, in real cases, the cationic profile is often not as clean as in controlled burning, with the detection of various other naturally occurring ions (Figure 5D). When this occurs, the resulting cationic profile for the banknotes (Figure 5E) and a real case can be quite similar as well.

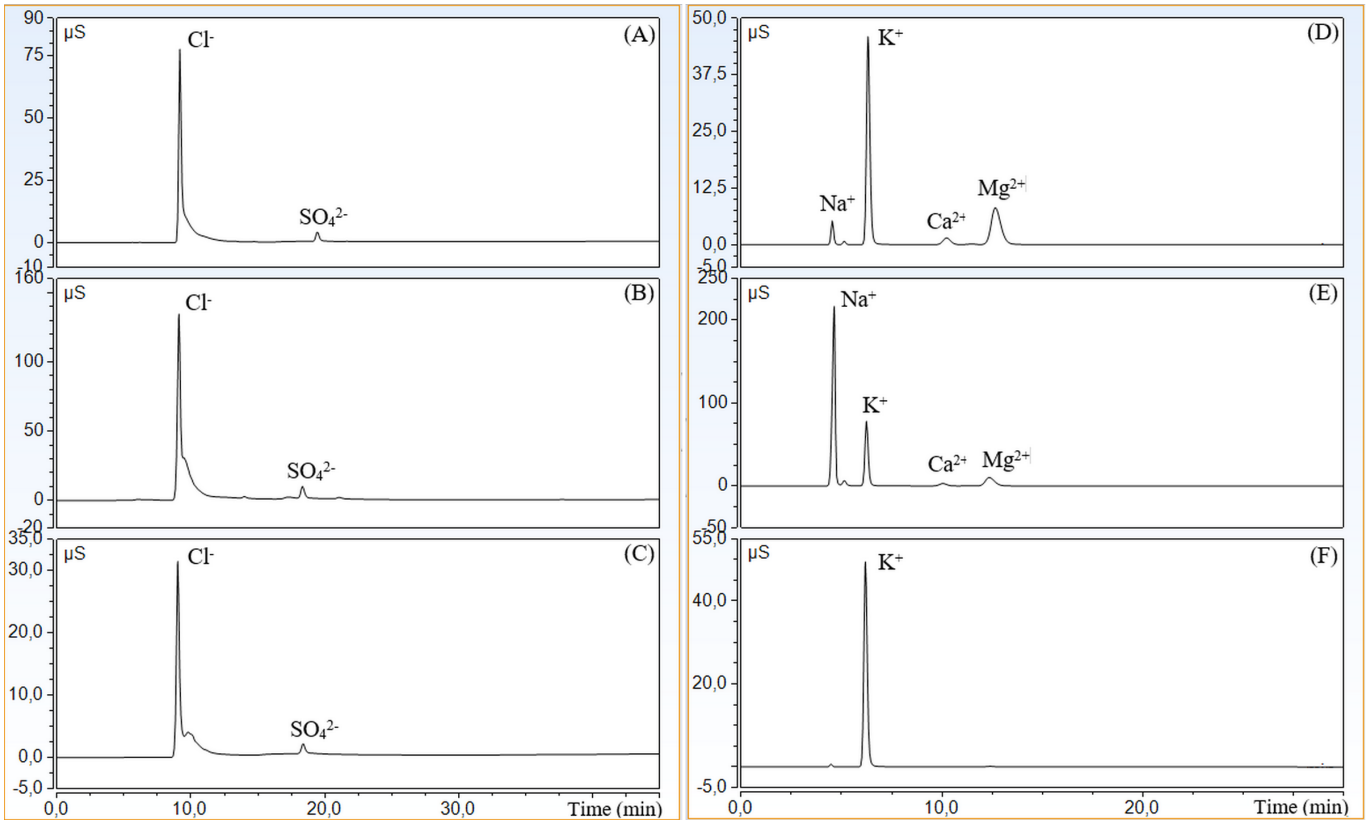

Figure 5. Anion (left) and cation (right) exchange ion chromatograms of post-explosion residue from a real post-explosion residue collected with a cotton ball (A, D), one of the circulated R$ 20 bank notes used in this study (B, E) and a controlled burning of a $KClO_3$/sulfur mixture (C, F).

Due to this difficulty of interpretation, that especially occurs in cases involving banknotes, unsupervised exploratory analyses based on PCA were carried out to verify the existence of patterns in this forensic problem. The results were obtained using the concentrations of six ions of interest (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-}) for three sets of data, as follows: i) uncirculated banknotes, ii) circulated banknotes and iii) uncirculated, circulated and seized R$ 100 banknotes. These analytes were selected because they were common ions observed in all three groups of data (uncirculated, circulated and seized banknotes).

The first two principal components explained 91% of the variance present in the ionic profile formed by the six ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-}) detected in the uncirculated banknotes. The score plot presented in Figure 6 show that the uncirculated banknotes form well defined clusters according to each of

the denominations studied (banknote value). This interesting pattern might be related to the ink and other materials used for the manufacture of each note value. Based on the loadings plot, it can be seen that: i) all ions are important for both principal components (PC), except for Cl^- in relation to PC2; ii) magnesium has the opposite sign to the other ions, appearing to explain a significant part of the different behavior of the R$ 2 notes; iii) K^+ and Ca^{2+} ions present a high correlation.

Although the presence of distinct clusters in non-circulating banknotes (Figure 6A) may not appear to be of forensic interest, it is still an interesting finding that also highlights the accuracy and precision of the proposed method. Additionally, it reveals that banknotes have two categories of interferents: intrinsic ones related to their manufacturing process and extrinsic ones related to their circulation. The latter can be better observed in the following results presented for circulating banknotes.

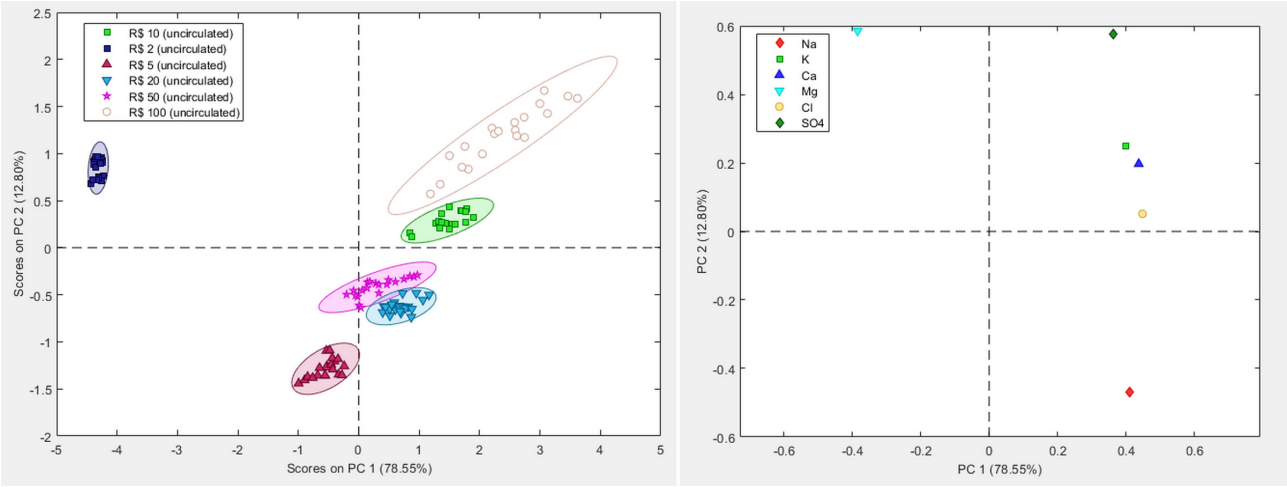

Figure 6. Scores plot (left) and loadings plot (right) for the first two principal components for uncirculated banknotes.

The two-dimensional PCA model for the circulating banknotes using the same ions explained only 78% of the variance, which may suggest the presence of a more complex variance structure in the data. As shown in Figure 7, the clear presence of clusters is no longer observed in the score plot. Furthermore, the loadings distribution is also significantly different from the one observed in the uncirculated banknotes. This result shows that, due to their circulation, the concentration of the analytes in each note value is changed and ionic profile becomes indistinguishable, obfuscating the intrinsic differences observed in uncirculated banknotes. Even with the use of the third and fourth PCs no clear pattern of class separation is observed.

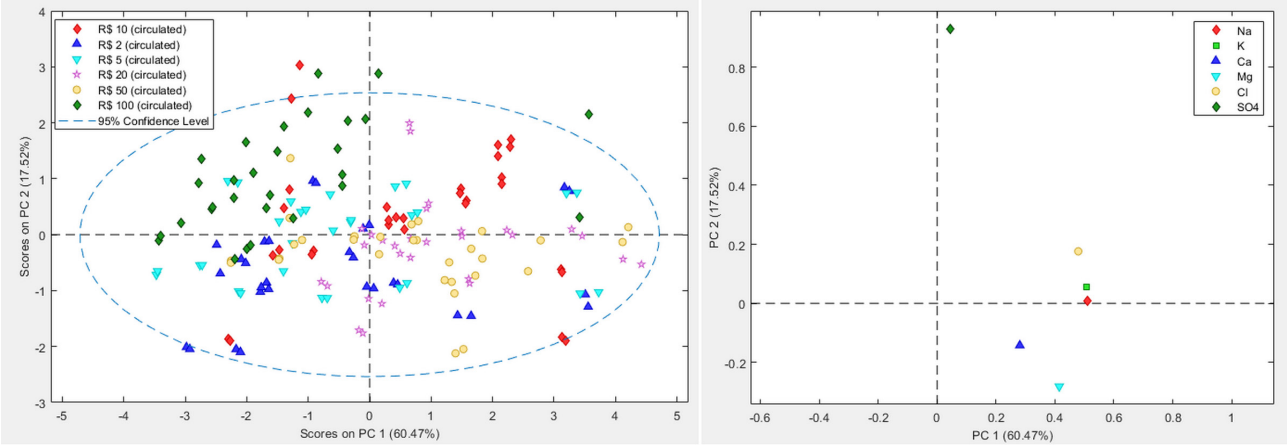

Figure 7. Scores plot (left) and loadings plot (right) for the first two principal components for circulated banknotes.

In the third dataset the first two PCs explained 90% of the variance and the score plot presented in Figure 8A shows that uncirculated/circulated and seized R$ 100 banknotes tend to form distinct groups, in which PC2 seems to be the most important component for the separation between the groups of seized and circulated banknotes. The uncirculated samples presented the most negative values in PC1, but were very close to the circulated samples. This proximity or overlap between uncirculated and circulated notes is, in a way, expected, since among the circulated banknotes there may be the presence of banknotes with just a little circulation, therefore, making them close to the uncirculated ones. Similarly, among the seized banknotes, there may be the occurrence of banknotes that suffered negligible contact with post-explosion residues, thus approaching to the non-suspicious circulated banknotes. After removing the 4 outliers (highlighted by red circles in Figure 8A), two referring to seized banknotes and two referring to circulated banknotes, the scores plot presented in Figure 8C is obtained, where a considerable separation between seized banknotes and the others two groups is observed, considering the 95% confidence ellipses.

Based on the loadings and scores plots (Figure 8B-C), apparently there is a correlation between the seized banknotes and higher concentrations of the ions Cl, K^+ and Na^+ that seems to be important for the distinction of the seized banknotes. Thus, considering that potassium chloride (KCl) is the main product in the explosive reaction of explosives based on chlorate and/or potassium perchlorate and that this type of explosive is widely used in criminal actions in Brazil,[2] this result indicates that the seized banknotes may have come into contact with residues of this type of material. Regarding the importance observed for the Na^+ ion, it may be related to the fact that these types of explosives are often not composed of pure potassium salts, but often with the presence of sodium salts.

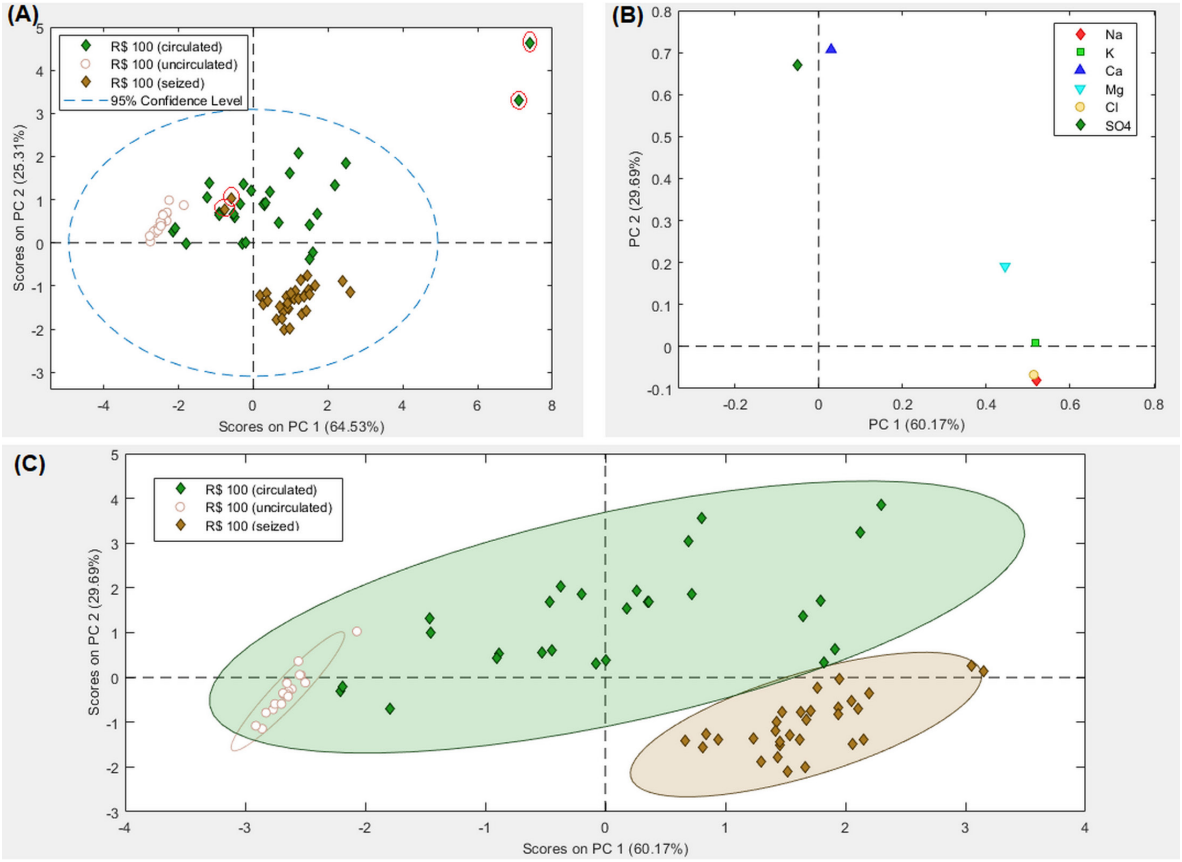

Figure 8. Score plot before removing the outliers (highlighted by red circles) (A), loadings and score plots (B and C, respectively) after removing the outliers for the first two principal components for uncirculated, circulated and seized R$ 100 banknotes. (ellipses) 95% confidence regions of each class.

CONCLUSIONS

The assessment of background levels for the main analytes usually detected in post-explosion residues was demonstrated, wherein their presence in both non-circulated and circulated banknotes was observed, with higher concentrations and greater variability in the latter. This finding highlights the importance of prioritizing alternative materials with lower susceptibility to interferences, whenever feasible, for such purposes.

On the other hand, the study also revealed the absence of highly relevant target ions, such as chlorite (ClO_2^-), chlorate (ClO_3^-), perchlorate (ClO_4^-), thiosulfate ($S_2O_3^{2-}$), thiocyanate (SCN^-), cyanate (OCN^-). Therefore, the absence of these ions in the analyzed banknotes is an important finding, as their potential presence in real cases within this context can be indicative of contact with post-explosion residues.

The chemometric analysis performed by PCA showed promising results for the identification of explosives on banknotes in the absence of bulk particles. In spite of the utilization of a limited dataset in this study, consistent results were attained in this case study, indicating that discrimination between unsuspected and suspected banknotes can be achieved through a straightforward water extraction process followed by IC-CD and PCA. Furthermore, a formal and robust classification model can be developed by investigating a larger sample of unsuspected circulating banknotes and validating this model with real-world cases and/or conducting controlled explosions using banknotes as matrices. This second study is currently underway in our laboratory and will be presented subsequently.

Although this study specifically focuses on Brazilian banknotes, it introduces, for the first time, the analysis of banknotes for the identification of post-explosion residue. This not only highlights the relevance within the context of Brazil but also draws attention to other countries facing similar challenges. It serves as an encouragement for them to conduct similar studies on their own banknotes.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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FEATURE

7th EspeQBrazil and 16th RSAS Discussed Advances in Chemical Speciation and Atomic Spectrometry

The 7th Brazilian Meeting on Chemical Speciation (7th EspeQBrasil) was held on November 26-27, 2023, and the 16th Rio Symposium on Atomic Spectrometry (16th RSAS) was held on November 28-30, 2023. Both meetings take place every two years and this time the 7th EspeQBrasil and the 16th RSAS were held together at the Dall'Onder Grande Hotel, in the city of Bento Gonçalves, Rio Grande do Sul (RS), Brazil. This joint meeting aimed to bring together experts from universities, research institutes, companies, laboratories, and industry for a worldwide discussion on the latest advances in various areas related to chemical speciation and atomic spectrometry.

All participants had the opportunity to explore emerging trends and their applications in various fields such as environmental, food, pharmaceutical, biological, geological, industrial materials, and other interesting areas.

Opening ceremony of 7th EspeQBrazil and 16th RSAS. Photo: Lilian Freitas

The events featured nearly 30 renowned speakers from more than 14 countries, including Germany, England, France, USA, Poland, Spain, Argentina, Uruguay, and Canada, among others, in the fields of chemical speciation and atomic spectrometry.

The two events were coordinated by researchers from the Chemistry and Chemical Engineering Postgraduate Programs of the Federal University of Santa Maria (UFSM, RS, Brazil).

Participants also had the opportunity to visit an exhibition of the companies sponsoring the events. Some of the companies present were Nova Analitica, Thermo Fisher, Analytik Jena, Agilent, SENS, and Vert.

Exhibition area of the event. Photo: Lilian Freitas

Source: 7th EspeQBrazil and 16th RSAS

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FEATURE

Pittcon 2024: 75 Years of Scientific Breakthroughs

The Pittcon Conference and Expo 2024, held February 24-28 in San Diego, California (USA), has once again provided a platform for companies to showcase their advances in laboratory technology. Pittcon is one of the largest annual meetings for analytical chemistry and scientific instrumentation.

Pittcon is a dynamic, international conference and exposition on laboratory science. Source: Pittcon

The 75th Pittcon received around 6,000 people and more than 1,000 participants gave technical talks. About 470 vendors filled the exhibit hall, joined by nonprofit organizations, government labs and media groups.

The Wallace H. Coulter keynote lecture was on “Sustainable nanomaterials for sensing human health and the environment” presented by Professor Omowunmi (Wunmi) Sadik (BioSMART Center and New Jersey Institute of Technology). The lecture discussed the use of sustainable nanomaterials for understanding reaction mechanisms, small-scale synthesis and biosensing. Sustainable nanotechnology is the research and development of nanomaterials with economic and social benefits and little or no negative environmental impact. In this presentation, a new frontier for safe nanomaterials with tunable surface and electrocatalytic properties, high sorption capacities and excellent reactivities was discussed. Sensing applications of these tunable materials for biomarkers of pain and persistent pollutants were presented.

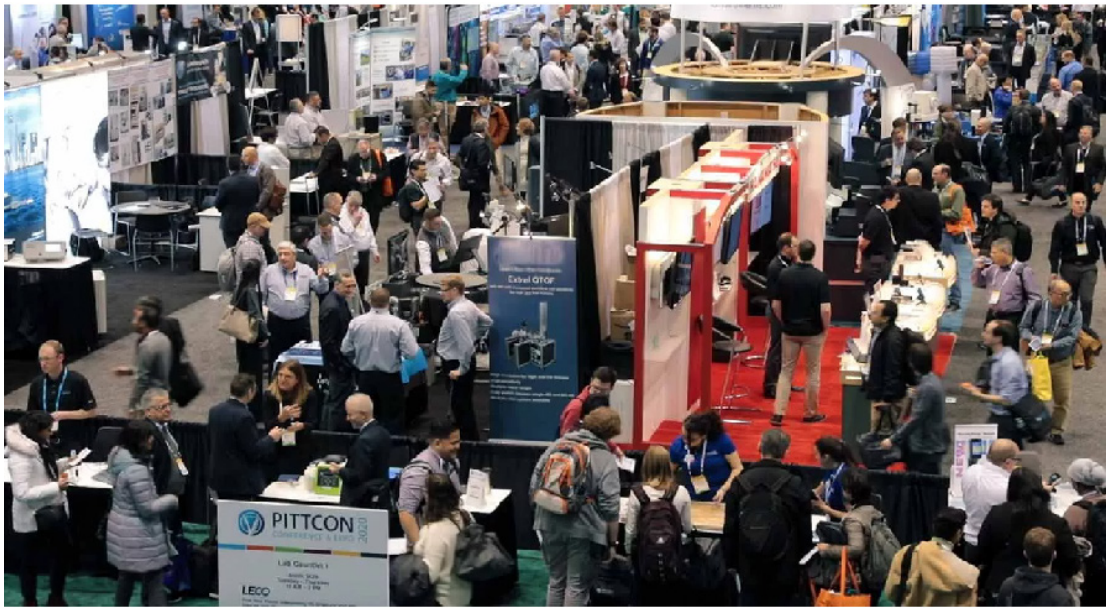
Pittcon’s technical program is comprised of more than 1,100 sessions and provides direct access to the latest research and developments from an international assembly of cutting-edge scientists and research pioneers. An event to showcase the latest advances in analytical research and scientific instrumentation and a platform for continuing education and opportunities to improve science.

Pittcon’s international exhibition offers the opportunity to see the latest laboratory instruments, participate in product demonstrations and seminars, speak directly with technical experts, and find solutions to your laboratory challenges.

During the event, participants had the opportunity to interact with partner suppliers. Their most recent innovations were presented and conversations with new ideas and solutions also took place.

Pittcon's short courses have brought meaningful topics in bioanalytics, cannabis, energy, environmental science, food science, forensic science, industry, biological sciences, nanotechnology, materials science, pharmaceuticals and more.

About Pittcon



The laboratory industry showcases cutting-edge innovations at Pittcon 2024. Source: Pittcon

Pittcon is not just a conference, it is a space where the boundaries between industries blur and the possibilities for connection are limitless. Pittcon is a community where analytical chemists from all walks of professional life converge.

Whether you're a seasoned professional looking for future employees, a newcomer looking for mentors to guide your journey, or someone in need of innovative solutions, Pittcon is where connections happen.

Pittcon was born in the city of Pittsburgh, which in the 1940s was the nucleus of a huge industrial center that pulsed with the flow of raw materials and manufactured products. Everyone needed testing and analysis laboratories to check their incoming and outgoing goods - and the scientists who ran these laboratories founded the Spectroscopy Society of Pittsburgh (SSP) and the Society of Analytical Chemists of Pittsburgh (SACP). The two societies merged their annual symposia in 1950, giving rise to the first Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. This first conference had 800 attendees and 14 exhibitors displaying the latest in laboratory equipment - Fisher Scientific Company (now part of Thermo Fisher Scientific, one of the largest exhibitors at the 2009 show) being one of them.

The conference first moved out of Pittsburgh in 1968 due to an employee strike at the planned venue, the Penn-Sheraton Hotel, but the move to the Cleveland (Ohio) Convention Center was so successful that the show remained there for next 11 years. When the conference moved to Atlantic City in 1980, it attracted nearly 16,000 attendees and 369 exhibiting companies, and the number of visitors surpassed the 30,000 mark in 1987. In 1988, the Pittcon 'road show' began, with the conference host changing city almost every year, taking place in Atlanta, New York, Chicago and New Orleans. The 1996 Chicago conference had the largest number of visitors ever - with 34,079 participants and 1,116 exhibitors on their way to the "windy city".

Next Pittcon

The Pittcon Conference and Expo 2025 will be held in the city of Boston, Massachusetts (USA), between March 1st and 5th, 2025.

The Boston Convention and Exposition Center (BCEC) is the most user-friendly and technologically advanced convention center in the world. It is located in the Seaport District, Boston's newest and fastest-growing neighborhood, quickly becoming its new core. It has hundreds of new restaurants, trendy hotels, state-of-the-art spaces for technology companies and biotechnology companies, and is highly walkable.

Source: Pittcon

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SPONSOR REPORT

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Quantitation of per- and polyfluoroalkyl substances (PFAS) in aqueous samples by LC-MS/MS following EPA Draft Method 1633

Kevin J. McHale
Thermo Fisher Scientific, Somerset, NJ

This report was extracted from the Thermo Scientific Application Note 002348

Keywords: EPA Method 1633, per- and polyfluoroalkyl substances (PFAS), environmental contaminants, solid phase extraction, Vanquish Flex Binary UHPLC, TSQ Quantis Plus triple quadrupole mass spectrometer

Goal: To demonstrate the measurement of 40 per- and polyfluoroalkyl substances (PFAS) in 500 mL water samples at or below the method detection limits (MDLs) reported in U.S. EPA Draft Method 1633 by LC-MS/MS on the Thermo Scientific™ TSQ Quantis™ Plus mass spectrometer.

INTRODUCTION

PFAS are per- and polyfluoroalkyl substances. They comprise a hydrophobic chain of C- F bonds and a hydrophilic end group. The chemical nature of the C-F bonds makes these compounds extremely stable. Hence, PFAS have been given the term “forever compounds”. They have been in use for decades in a wide variety of industrial uses and for many everyday consumer products. Because of their ubiquitous nature and chemical stability, PFAS have made their way into all aspects of the environment, including the water and soil and some even in the air. With contact with the environment, PFAS become integrated into plants, animals, and humans. Once in biological organisms, PFAS do not efficiently breakdown. This leads to bioaccumulation of PFAS, which has shown evidence of certain health effects in humans, including possible increased risk of cancer and infertility.[1]

The U.S. EPA has taken a more active approach to monitoring PFAS in the environment in recent years. In March 2023, the EPA proposed the National Primary Drinking Water Regulation (NPDWR) to establish legally enforceable levels of six PFAS in drinking water, including PFOA and PFOS at 4 ng/L.[2] Previously developed methods EPA 537.1 and EPA 533 were established to measure PFAS in drinking water, including the six PFAS designated under the NPDWR. More recently, EPA Method 1633 was developed, in conjunction with the Department of Defense, to measure PFAS in non-potable water, (bio)solids, and tissue samples for the intended use of regulating PFAS via the Clean Water Act (CWA). The third draft of EPA Method 1633 was released in December 2022 following a multi-laboratory validation study in spiked wastewaters.[3]

This application note will present data for measuring 40 PFAS in fortified water samples following the third draft of EPA Method 1633. An MDL study was conducted in reagent water to demonstrate that equivalent or better performance can be attained using the Thermo Scientific™ Vanquish™ Flex Binary UHPLC system and Thermo Scientific™ TSQ™ Quantis Plus mass spectrometer.

EXPERIMENTAL

Consumables

A list of materials used is included in Table A1 in the Appendix.

Sample preparation

High-density polyethylene (HDPE) bottles were thoroughly rinsed with Thermo Scientific™ UHPLC-MS grade methanol and air-dried prior to preparation of all water samples and sample processing solutions. Solid phase extraction (SPE) eluting solution was prepared on the day of sample extractions owing to the volatility of ammonium hydroxide.

PFAS standards were obtained from Wellington Laboratories (Guelph, ON), stored at 4 °C until needed, and used as received.

500 mL water samples (Optima™ LC-MS grade, Fisher Scientific™) were fortified with target PFAS analytes at concentrations consistent with a mid-level calibration point and at concentrations near the method's limit of quantitation for MDL determinations.

Shortly before adding water samples to the conditioned SPE cartridges, 25 µL extracted internal standards (EIS) solution was spiked into each water sample and mixed by inverting bottles numerous times for approximately 30 seconds.

Solid phase extraction (SPE) of water samples was accomplished according to the protocol detailed in Sections 11.2, 12.1, and 12.2 of EPA Draft Method 1633.

Calibration solutions were prepared according to Table 4 of EPA Draft Method 1633. Due to the sensitivity of the TSQ Quantis Plus mass spectrometer, two additional calibration solutions at concentrations equivalent to 25% and 50% of the lowest calibration solution (i.e., CS1) were also used for the LC-MS/MS calibration procedure. The Calibration Verification Standard (CV) used herein was the CS3 standard rather than the suggested CS4.

Liquid chromatography

To prevent interferences from PFAS attributable to the liquid chromatography (LC) system, the Vanquish Flex Binary UHPLC system was modified with the PFAS Upgrade Kit. This kit includes PEEK tubing and a PFAS delay column to shift any residual PFAS in the LC system away from the target PFAS compound injected onto the analytical column. Fresh mobile phase was prepared after every five days of use. The LC method details are shown in Table 1.

Table 1. LC method parameters

Parameter	Value	
Analytical column	Thermo Scientific™ Acclaim™ 120 C18, 2.1 × 50 mm, 2.2 um	
Delay column	Thermo Scientific™ Hypersil GOLD™, 3.0 × 50 mm, 1.9 µm	
Column temperature	40 °C	
Injection volume	5 µL	
Autosampler temperature	20 °C	
Mobile phase	(A) H_2O + 2% ACN + 2 mM ammonium acetate + 0.1% acetic acid (B) ACN + 2% H_2O + 2 mM ammonium acetate + 0.1% acetic acid	
Flow rate	0.4 mL/min	
Gradient	Time (min)	% B
	0.0	10
	1.0	30

Table 1. LC method parameters (continued)

Parameter	Value	
Gradient	Time (min)	% B
	5.0	46
	10.0	76
	10.5	86
	10.9	86
	11.0	10
	13.0	10

Mass spectrometry

All PFAS target analytes, extracted internal standards (EIS), and non-extracted internal standards (NIS) for EPA Method 1633 were detected using timed SRM (t-SRM) on the TSQ Quantis Plus mass spectrometer. Table 2 provides the ion source and TSQ Quantis Plus mass spectrometer detection settings used for data acquisition. The SRM transitions table of measured PFAS is included in Table A2 in the Appendix.

Table 2. TSQ Quantis Plus mass spectrometer parameters

Parameter	Value
Ion source	H-ESI
Polarity	Negative
Spray voltage	-1,000 V
Sheath gas	50 a.u.
Aux gas	12 a.u.
Sweep gas	0.5 a.u.
Ion	225 °C
Vaporizer temperature	300 °C
Q1, Q3 resolution	0.7 FWHM
CID gas	2.5 mTorr argon
SRM cycle time	0.4 s

Data analysis

All LC-MS/MS data were acquired and processed using the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.

RESULTS AND DISCUSSION

Separation of PFOS and bile acids

The third draft of EPA Method 1633 includes a requirement that certain bile acids, such as taurodeoxycholic

acid (TDCA), taurochenodeoxycholic acid (TCDCA), and tauroursodeoxycholic acid (TUDCA), must be analyzed to ensure that they do not elute within a 1-minute window of PFOS linear and branched isomers, even in aqueous samples. This is because PFOS and TDCA (and its isomers TCDCA and TUDCA) have precursor ions that differ by 0.64 u, which cannot be differentiated with a quadrupole mass filter at unit resolution, and the same product *m/z* 79.96. Hence, if these compounds are not sufficiently separated chromatographically, these bile acids would cause a positive bias in the measurement of PFOS.

The initial LC method employed for EPA Draft Method 1633 used methanol as the organic solvent in the mobile phases, as it is also used in EPA Methods 537.1 and 533. However, during the bile acid check experiments, it was observed that PFOS could not be sufficiently separated from TDCA, TCDCA, and TUDCA (data not shown). When methanol was changed to acetonitrile in the mobile phases, these bile acids shifted to much earlier retention times relative to PFOS. Figure 1 shows TDCA is separated from the branched isomers PFOS by more than 2 minutes using the LC method in Table 1. Furthermore, TCDCA and TUDCA have retention times of 3.2 and 4.1 minutes, respectively, using the same method (data not shown).

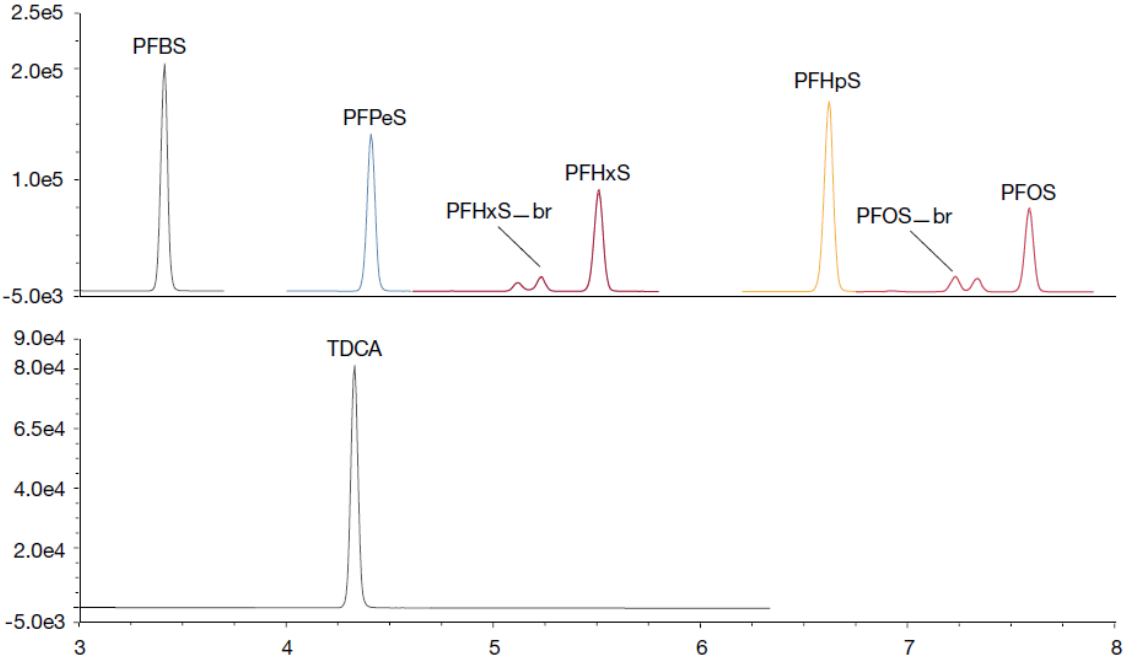

Figure 1. Chromatograms for PFAS separation, including PFOS shown in top chromatogram, compared to the analysis of bile acid TDCA in bottom chromatogram. The LC method uses acetonitrile as the organic mobile phase instead of methanol according to EPA Draft Method 1633 to ensure separation of PFOS and TDCA.

Calibration data

Following the procedure described in Section 10.3 of EPA Draft Method 1633, a total of nine calibration solutions were used for the purpose of LC-MS/MS system calibration on the TSQ Quantis Plus mass spectrometer. Calibration curves for all target PFAS were fit using 1/x (concentration) weighting and not forced through zero. Target PFAS had linear regression fits with the exceptions of 5:3FTCA, 7:3FTCA, and the three x:2FTS compounds, which used quadratic regression curves. $R^2 > 0.997$ was achieved for all compounds. Relative standard errors (RSE) were calculated for all method analytes, accounting for the calibration curve type in the calculations. The vast majority of RSE values were <10%, while six native PFAS compounds had RSEs between 10% and 16%.

Precision and recovery data

Reagent water samples were fortified with native PFAS at concentrations consistent with a mid-level ongoing precision and recovery (OPR) standard. Table A3 in the Appendix shows the native PFAS spiked concentrations, mean percent recovery, and precision results for N=5 fortified water samples. With the exception of 6:2FTS, very good precision and recovery data are obtained.

Extracted internal standards (EIS) had mean percent recoveries of 77–110% and RSDs of 2.3–11.9%, with median values of 106% and 4.2%, respectively. Not surprisingly, the lowest recovery and poorest precision came from the most hydrophobic compounds, D5-N-EtFOSA and D9-N-EtFOSE.

6:2FTS was observed in the extraction method blanks at varying amounts, leading to its biased high percent recovery and poor precision values. Because of these results, an investigation into the potential sources of the contamination was conducted. After a thorough examination of all reagents and materials used during the SPE process, it was discovered that 6:2FTS contamination was from the polypropylene stopcocks used to control the sample flow through the SPE cartridges.

Method detection limits data

To determine the overall quantitative performance, an MDL study was conducted. Table A4 in the Appendix presents MDL values for the native PFAS measured on the TSQ Quantis Plus mass spectrometer and results from EPA Draft Method 1633 in aqueous samples. MDLs on the TSQ Quantis Plus mass spectrometer are equivalent or better for all but two analytes – the aforementioned 6:2FTS and PFBA.

PFBA was fortified in water samples at 4 ng/L in this MDL study. However, PFBA was observed in the extracted method blanks between 0.9 and 1.8 ng/L. The relatively high concentration of PFBA in the method blanks contributed to the higher MDL concentration.

CONCLUSIONS

Following the protocols in 1633, the TSQ Quantis Plus mass spectrometer has demonstrated MDLs at, or in most cases, below those listed in EPA Draft Method 1633 for aqueous samples. For extractions of mid-level fortified samples, results well within the recovery range of 70–130% and RSDs <20% were obtained, with the exception of 6:2FTS.

PFBA, which had slightly higher MDL value than in EPA Draft Method 1633, is notoriously challenging to quantify at or below 1 ng/L owing to cross-contamination issues. While many sources of PFBA contamination have been identified, further investigations are needed.

The unsatisfactory results for sample extractions of 6:2FTS, which was later found to be caused by contamination of the SPE stopcocks, reinforces the need to evaluate all reagents and materials, as well as thoroughly clean all equipment touched by the samples, to achieve the validation criteria in EPA Draft Method 1633. A selection of suggested Thermo Scientific branded materials for use in EPA Method 1633 are listed in Table A1 of the Appendix.

Despite the challenges presented from cross-contamination of PFBA and 6:2FTS, the combination of the Vanquish Flex UHPLC system and the TSQ Quantis Plus mass spectrometer is more than capable to fulfill the requirements of EPA Draft Method 1633 for aqueous samples delivering excellent value and productivity.

REFERENCES

1. Potential health effects of PFAS chemicals | ATSDR (cdc.gov)
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3. U.S. EPA 3rd Draft Method 1633, Analysis of Per- and Polyfluoroalkyl Substances (PFAS) in Aqueous, Solid, Biosolids, and Tissue Samples by LC-MS/MS, December 2022. https://www.epa.gov

APPENDIX

Table A1. Suggested materials for EPA Draft Method 1633. All products are from Thermo Fisher Scientific unless specifically noted.

Item	Product	Part number
PFAS delay column	Hypersil GOLD, 3.0 × 50 mm, 1.9 µm	25002-053030
Analytical column	Acclaim 120 C18, 2.1 × 50 mm, 2.2 µm	068981
Guard column	Acclaim 120 C18, 2.1 × 10 mm, 5 µm	069689
Guard column kit	Acclaim guard kit (holder and coupler) V-2	069707
Mobile phase chemicals	Water, UHPLC-MS grade, 1 L	W8-1
	Acetonitrile, UHPLC-MS grade, 1 L	A9561
	Ammonium acetate, LC-MS grade, 50 g	A114-50
	Acetic acid, LC-MS grade, 1 mL ampoules	A113-10X1AMP
Other reagents	Methanol, UHPLC-MS grade, 1 L	A458-1
	Ammonium hydroxide, ACS Plus grade, 500 mL, glass bottle	A669-500
	Formic acid, LC-MS grade, 1 mL ampoules	A117-10X1AMP
	Optima™ LC-MS grade water, 4 L, Fisher Chemical™	W64
Centrifuge tubes	15 mL conical polypropylene centrifuge tubes	05-539-12
Syringes	Luer-slip syringes, PE barrels, PP plungers, 5 mL	S7510-5
Filters	Disposable syringe filters, 25 mm, 0.2 µm, nylon membrane	CH4513-NN
SPE cartridges	Biotage™ EVOLUTE™ PFAS, WAX, 150 mg/6 mL, 30/pk	614-0015-CP
Autosampler vials	Polypropylene, 1.5 mL, screw-top, Level 1	6ESV9-1PP
Autosampler caps	Polypropylene caps, 9 mm, screw-thread	C5000-50

Table A2. Timed SRM on the TSQ Quantis Plus mass spectrometer

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
PFBA	1.1	2.3	213	169	9	72
M3PFBA	1.1	2.3	216	172	9	72
MPFBA	1.1	2.3	217	172	9	72
TDCA	1.1	8	498.29	80	67	250
TDCA	1.1	8	498.29	124	53	250
PFMPA	2	2.7	229	85	10.5	72
PFMPA	2	2.7	229	185	7	72
PFPeA	2.3	3	263	219	8.5	77
M5PFPeA	2.3	3	268	223	8.5	77

Table A2. Timed SRM on the TSQ Quantis Plus mass spectrometer (continued)

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
PFMBA	2.5	3.15	279	85	10.5	80
PFMBA	2.5	3.15	279	235	7.5	80
4:2FTS	2.7	3.35	327	81	28	160
4:2FTS	2.7	3.35	327	307	20	160
M2-4:2FTS	2.7	3.35	329	81	28	160
M2-4:2FTS	2.7	3.35	329	309	20	160
NFDHA	2.9	3.5	295	85	22	63
NFDHA	2.9	3.5	295	201	8	63
PFHxA	2.9	3.6	313	119	19	92
PFHxA	2.9	3.6	313	269	9	92
MPFHxA	2.9	3.6	315	119	19	92
MPFHxA	2.9	3.6	315	270	9	92
M5PFHxA	2.9	3.6	318	120	19	92
M5PFHxA	2.9	3.6	318	273	9	92
PFBS	3	3.7	298.94	80	32	190
PFBS	3	3.7	298.94	99	29	190
M3PFBS	3	3.7	302	80	32	190
M3PFBS	3	3.7	302	99	29	190
HFPO-DA	3.2	3.9	285	169	7	80
HFPO-DA	3.2	3.9	285	185	17	80
13C3-HFPO-DA	3.2	3.9	287	169	7	80
13C3-HFPO-DA	3.2	3.9	287	185	17	80
PFEESA	3.4	4.1	314.95	83	19	135
PFEESA	3.4	4.1	314.95	135	22	135
PFHpA	3.7	4.4	363	169	17	102
PFHpA	3.7	4.4	363	319	9.5	102
M4PFHpA	3.7	4.4	367	322	9.5	102
3:3FTCA	3.9	4.8	241	117	32	82
3:3FTCA	3.9	4.8	241	177	7	82
PFPeS	4	4.7	348.94	80	35	200

Table A2. Timed SRM on the TSQ Quantis Plus mass spectrometer (continued)

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
PFPeS	4	4.7	348.94	99	32	200
ADONA	4	4.8	377	85	22	94
ADONA	4	4.8	377	251	10	94
6:2FTS	4.2	5	427	81	30	195
6:2FTS	4.2	5	427	407	22.5	195
M2-6:2FTS	4.2	5	429	81	30	195
M2-6:2FTS	4.2	5	429	409	22.5	195
PFOA	4.5	5.4	413	169	17	114
PFOA	4.5	5.4	413	369	10	114
PFHxS	4.7	5.8	398.94	80	38	220
PFHxS	4.7	5.8	398.94	99	34	220
M4PFOA	4.7	5.4	417	172	17	114
M8PFOA	4.7	5.4	421	376	10	114
M3PFHxS	5.1	5.8	402	80	38	220
M3PFHxS	5.1	5.8	402	99	34	220
MPFHxS	5.1	5.8	403	84	38	220
PFNA	5.55	6.35	463	219	17	122
PFNA	5.55	6.35	463	419	10.5	122
MPFNA	5.55	6.35	468	423	10.5	122
M9PFNA	5.55	6.35	472	427	10.5	122
PFHpS	5.9	6.8	448.93	80	40	240
PFHpS	5.9	6.8	448.93	99	37	240
8:2FTS	6.1	6.9	527	81	33	280
8:2FTS	6.1	6.9	527	507	26	280
M2-8:2FTS	6.2	6.9	529	81	33	220
M2-8:2FTS	6.2	6.9	529	509	26	220
PFOS	6.3	7.8	498.93	80	46	270
PFOS	6.3	7.8	498.93	99	40	270
5:3FTCA	6.6	7.5	341	217	25	102
5:3FTCA	6.6	7.5	341	237	13	102

Table A2. Timed SRM on the TSQ Quantis Plus mass spectrometer (continued)

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
PFDA	6.6	7.4	512.96	269	17	138
PFDA	6.6	7.4	512.96	469	11	138
MPFDA	6.6	7.4	515	470	11	138
M6PFDA	6.6	7.4	519	474	11	138
MPFOS	7	7.8	503	80	46	270
MPFOS	7	7.8	503	99	40	270
M8PFOS	7	7.8	507	80	46	270
M8PFOS	7	7.8	507	99	40	270
PFUdA	7.4	8.2	562.96	269	18	151
PFUdA	7.4	8.2	562.96	518.97	11	151
M7PFUdA	7.4	8.2	570	525	11	151
9Cl-PF3ONS	7.6	8.5	530.9	350.95	25	175
9Cl-PF3ONS_37Cl	7.6	8.5	532.9	352.95	25	175
PFNS	7.7	8.7	548.93	80	49	275
PFNS	7.7	8.7	548.93	99	43	275
N-MeFOSAA	7.8	9.2	570	419	18	220
N-MeFOSAA	7.8	9.2	570	483	16	220
N-MeFOSAA	7.8	9.2	570	512	19	220
PFDoA	8.2	9	612.95	169	25	163
PFDoA	8.2	9	612.95	569	11.5	163
MPFDoA	8.2	9	615	570	10.5	163
d3-N-MeFOSAA	8.3	9.2	573	419	18	220
N-EtFOSAA	8.4	10.1	584	419	20	200
N-EtFOSAA	8.4	10.1	584	483	18	200
N-EtFOSAA	8.4	10.1	584	526	20	200
PFDS	8.5	9.4	598.92	80	50	280
PFDS	8.5	9.4	598.92	99	46	280
7:3FTCA	8.6	9.5	441	317	20	129
7:3FTCA	8.6	9.5	441	337	11	129
d5-N-EtFOSAA	8.9	10.1	589	419	20	235

Table A2. Timed SRM on the TSQ Quantis Plus mass spectrometer (continued)

Compound	Start time (min)	End time (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
PFTrDA	8.9	9.7	662.95	169	26	174
PFTrDA	8.9	9.7	662.95	618.96	12	174
FOSA	9.1	9.9	497.95	78	30	240
FOSA	9.1	9.9	497.95	169	27	240
FOSA	9.1	9.9	497.95	478	23	240
M8FOSA	9.2	9.9	506	78	30	240
11Cl-PF2OUdS	9.2	10	630.9	450.94	27	163
11Cl-PF2OUdS_37Cl	9.2	10	632.9	452.94	27	163
PFTeDA	9.6	10.5	712.95	169	28	188
PFTeDA	9.6	10.5	712.95	668.96	12.5	188
M2PFTeDA	9.6	10.5	715	670	12.5	188
PFDoS	9.8	10.8	698.9	80	53	280
PFDoS	9.8	10.8	698.9	99	48	280
NMeFOSE	9.9	10.9	616	59	16	133
D7-NMeFOSE	9.9	10.9	623	59	16	133
NMeFOSA	10.2	11.1	512	169	26	222
NMeFOSA	10.2	11.1	512	219	24	222
D3-NMeFOSA	10.3	11.1	515	219	24	222
NEtFOSE	10.5	11.4	630	59	16	137
D9-NEtFOSE	10.5	11.4	639	59	16	137
NEtFOSA	10.8	11.8	526	169	26	227
NEtFOSA	10.8	11.8	526	219	23	227
D5-NEtFOSA	10.8	11.8	531	219	23	227

Table A3. Precision and recovery of native PFAS from fortified water samples

Analyte	Spiked conc. (ng/L)	Mean %Recovery (N=5)	%RSD (N=5)	Analyte	Spiked conc. (ng/L)	Mean %Recovery (N=5)	%RSD (N=5)
PFBA	50.0	91.2%	3.4	6:2 FTS	50.0	232.9%*	52.4
PFPeA	25.0	92.4%	2.8	8:2 FTS	50.0	89.5%	1.4
PFHxA	12.5	91.3%	3.7	PFOSA	12.5	85.9%	3.9
PFHpA	12.5	88.5%	3.1	N-MeFOSA	12.5	85.6%	4.2
PFOA	12.5	89.8%	3.3	N-EtFOSA	12.5	83.2%	3.8

Table A3. Precision and recovery of native PFAS from fortified water samples (continued)

Analyte	Spiked conc. (ng/L)	Mean %Recovery (N=5)	%RSD (N=5)	Analyte	Spiked conc. (ng/L)	Mean %Recovery (N=5)	%RSD (N=5)
PFNA	12.5	87.8%	4.7	N-MeFOSAA_branched	3.0	94.4%	10.5
PFDA	12.5	89.0%	1.9	N-MeFOSAA	9.5	90.5%	3.2
PFUdA	12.5	87.0%	3.5	N-EtFOSAA_branched	2.8	87.7%	5.2
PFDoA	12.5	93.3%	3.3	N-EtFOSAA	9.7	87.0%	2.4
PFTrDA	12.5	88.0%	3.2	N-MeFOSE	125.0	90.5%	3.7
PFTeDA	12.5	93.6%	2.5	N-EtFOSE	125.0	92.6%	3.1
PFBS	12.5	86.8%	5.5	HFPO-DA	50.0	94.1%	2.0
PFPeS	12.5	92.7%	3.4	ADONA	50.0	102.5%	4.5
PFHxS_branched	2.4	86.6%	5.4	PFEESA	25.0	93.4%	3.3
PFHxS	10.1	86.9%	5.7	PFMPA	25.0	84.3%	3.4
PFHpS	12.5	82.7%	1.6	PFMBA	25.0	89.9%	3.1
PFOS_branched	2.6	85.8%	4.6	NFDHA	25.0	97.3%	1.7
PFOS	9.9	87.8%	2.3	9Cl-PF3ONS	50.0	97.1%	1.5
PFNS	12.5	90.0%	5.8	11Cl-PF3OUdS	50.0	110.5%	6.1
PFDS	12.5	92.4%	1.9	3:3FTCA	62.5	86.2%	5.3
PFDoS	12.5	116.8%	6.6	5:3FTCA	312.5	71.8%	2.8
4:2 FTS	50.0	97.6%	4.1	7:3FTCA	312.5	101.7%	2.4

*Biased high recovery from cross-contamination. See text for details.

Table A4. MDLs of native PFAS in fortified water samples

Analyte	TSQ Quantis Plus mass spectrometer MDL (ng/L, N=7)	EPA 1633 Draft 3 aqueous MDL (ng/L, pooled)	Analyte	TSQ Quantis Plus mass spectrometer MDL (ng/L, N=7)	EPA 1633 Draft 3 aqueous MDL (ng/L, pooled)
PFBA	1.92	0.80	6:2 FTS	135.26**	2.52
PFPeA	0.20	0.53	8:2 FTS	2.27	2.58
PFHxA	0.21	0.48	PFOSA	0.11	0.32
PFHpA	0.05	0.39	N-MeFOSA	0.36	0.41
PFOA	0.15	0.55	N-EtFOSA	0.36	0.43
PFNA	0.12	0.46	N-MeFOSAA	0.27	1.04
PFDA	0.15	0.53	N-EtFOSAA	0.23	0.80
PFUdA	0.15	0.44	N-MeFOSE	1.66	3.93
PFDoA	0.16	0.37	N-EtFOSE	1.53	5.13
PFTrDA	0.08	0.46	HFPO-DA	0.28	1.54
PFTeDA	0.14	0.51	ADONA	0.14	1.47
PFBS	0.13	0.37	PFEESA	0.21	0.79
PFPeS	0.07	0.53	PFMPA	0.23	0.54
PFHxS	0.13	0.56	PFMBA	0.19	0.53
PFHpS	0.21	0.87	NFDHA	0.21	1.92

Table A4. MDLs of native PFAS in fortified water samples (continued)

Analyte	TSQ Quantis Plus mass spectrometer MDL (ng/L, N=7)	EPA 1633 Draft 3 aqueous MDL (ng/L, pooled)	Analyte	TSQ Quantis Plus mass spectrometer MDL (ng/L, N=7)	EPA 1633 Draft 3 aqueous MDL (ng/L, pooled)
PFOS	0.19	0.64	9Cl-PF3ONS	0.17	1.42
PFNS	0.37	0.49	11Cl-PF3OUdS	0.43	1.78
PFDS	0.36	0.90	3:3FTCA	1.30	2.54
PFDoS	0.55	0.64	5:3FTCA	3.07	9.92
4:2 FTS	0.45	1.74	7:3FTCA	3.83	9.14

**Biased high MDL from cross-contamination. See text for details.

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Determination of Chromium Species using Ion Chromatography coupled to Inductively Coupled Plasma Mass Spectrometry

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The goal of this report is to highlight a simple method to separate and quantify both major Cr species, Cr (III) and Cr (VI) in drinking water.

Keywords: Chromium speciation, Drinking water, Hexavalent Cr, IC-ICP-MS, IonPac AG7 column

INTRODUCTION

Chromium is found naturally in rocks, soil, plants and animals, but can also be introduced into the environment as a result of human activity. Like many elements, chromium is found in multiple oxidation states, which can vary significantly in their toxicity, nutritional value, bioactivity, and environmental mobility. In trace amounts, trivalent chromium (Cr (III)) is considered an essential nutrient that promotes insulin, sugar, and lipid metabolism. In contrast, hexavalent chromium (Cr (VI)) is toxic and can lead to respiratory tract, stomach, and intestinal irritation, anemia, and is known to be a human carcinogen [1]. Cr (VI) can leach into drinking water sources naturally, but drinking water can also be contaminated by industrial processes such as wood treatment with copper dichromate, leather tanning with chromic sulfate, and stainless steel cookware. Because of the varying toxicity attributable to the different oxidation states of chromium, simply knowing the total chromium concentration in a solution is not sufficient to determine its true toxicity following exposure, and therefore speciation analysis is required. While inductively coupled plasma mass spectrometry (ICP-MS) can readily determine the total amount of an element present, chromatographic separation prior to the ICP-MS system is required to separate the different elemental species. Because Cr (III) and Cr (VI) have different charges, ion chromatography (IC) using anion exchange is the ideal separation method for analysis of these species.

One of the challenges with chromium speciation is that Cr (VI) can be degraded to Cr (III) and Cr (III) can be converted to a precipitate ($Cr(OH)_3$), depending on the solution pH [2]. An additional difficulty in the accurate speciation analysis of Cr by ICP-MS are the numerous spectral interferences (e.g. $^{35}Cl^{16}O^{1}H^{+}$ or $^{40}Ar^{12}C^{+}$) on the most abundant chromium isotope, ^{52}Cr [3].

In this application note, the Thermo Scientific™ Dionex™ Aquion™ Ion Chromatography system was coupled with the Thermo Scientific™ iCAP™ RQ ICP-MS to determine the concentration of Cr (III) and Cr (VI) in drinking water.

MATERIALS AND METHODS

Sample preparation

The tap water was acidified with 10 µL of concentrated nitric acid per 10 mL aliquot to yield a pH of around 4. A fortified sample was spiked with 0.1 mL of a standard solution containing 10 µg L^{-1} of both Cr species to 10 mL of the sample to give a final concentration of 0.1 µg L^{-1}.

Instrument configuration

The ion chromatography system used for this work consisted of a Dionex Aquion IC system and a Thermo Scientific™ Dionex™ AS-AP autosampler. All components of the IC system were controlled using the ChromControl plug-in for Thermo Scientific™ Qtegra™ Intelligent Scientific Data Solution™ Software. The system was purged and equilibrated prior to the start of sample analysis on each day. Data evaluation was accomplished using the tQuant virtual evaluation module of Qtegra Software. The chromatographic method was developed and published elsewhere [4], in brief, an isocratic separation using 0.3 mol L^{-1} nitric acid was used to separate both Cr species using a Thermo Scientific™ Dionex™ IonPac™ AG7 anion exchange guard column. Using a guard column alone (length of only 5 cm) effectively reduces the analysis time per sample and therefore increases sample throughput. At the same time, the chromatographic resolution and sample capacity are sufficient for the analysis.

The iCAP RQ ICP-MS was operated using the conditions summarized in Table 1. After optimization of the instrument using the autotune routines delivered with the Qtegra ISDS Software, the outlet of the column was directly connected to the PFA-LC nebulizer using a zero dead volume connector. The instrument was operated using kinetic energy discrimination (KED) with He as a collision gas to effectively eliminate all potential polyatomic interferences on Cr.

Table 1. Instrument configuration

Ion Chromatography	
Column	Dionex IonPac AG7, 2 x 50 mm
Flow rate	0.4 mL min^{-1}
Eluent	0.3 mol L^{-1} Nitric Acid
Injection volume	25 µL
ICP-MS	
Spray chamber	Quartz cyclonic, chilled at 2.7 ºC
Nebulizer	PFA-LC
Injector	2.5 mm I.D., quartz
Interface	Nickel sampler and skimmer cone High matrix skimmer cone insert
Forward power	1550 w
Nebulizer gas	1.12 L min^{-1}
Collision cell gas	He at 4.5 mL min^{-1}
KED voltage	3 V
Dwell times	0.1 s
Total acquisition time	3 min 20 sec

RESULTS AND DISCUSSION

The second chromatogram (from top to bottom) in Figure 1 shows the separation of a solution containing Cr (III) and (VI) at a concentration of 0.1 µg L^{-1}. Both species are completely separated and complete elution is achieved within 120 s. To assure complete elution if a much higher concentration of Cr (III) is present in a sample, the total runtime of the method was extended to 200 s. For calibration of the system,

a three-point calibration curve was generated using standard solutions containing both Cr species at concentrations between 0.1 and 10 µg L^{-1}. The analytical figures of merit obtained are shown in Table 2. The stability of retention times was verified using 10 injections of tap water spiked with 0.1 µg L^{-1} of both species. The attainable detection limits were calculated based on the standard deviation of the peak area observed in the peak area of repeated injections (N = 15) of unspiked tap water. This allows for a rather conservative and realistic assessment of this parameter.

Table 2. Analytical figures of merit

	Cr (VI)	Cr (III)
Retention time (s)	36 ± 0.2	101 ± 1.2
Sensitivity (kcps/µg L^{-1})$^{-1}$	114	123
Detection Limit (ng L^{-1})	4.0	9.0

Next, the performance of the guard column was evaluated with a locally sourced drinking water. Drinking water typically contains a high amount of both different cationic (for example, alkaline and alkaline earth elements) as well as anionic species (for example carbonate, sulfate and chloride), leading to an increased column load and potentially compromising the separation efficiency for the species under investigation. As can be seen from the chromatograms in Figure 1, no difference in the elution profile is observed between the injection of a standard solution (in ultrapure water) or a spiked drinking water sample.

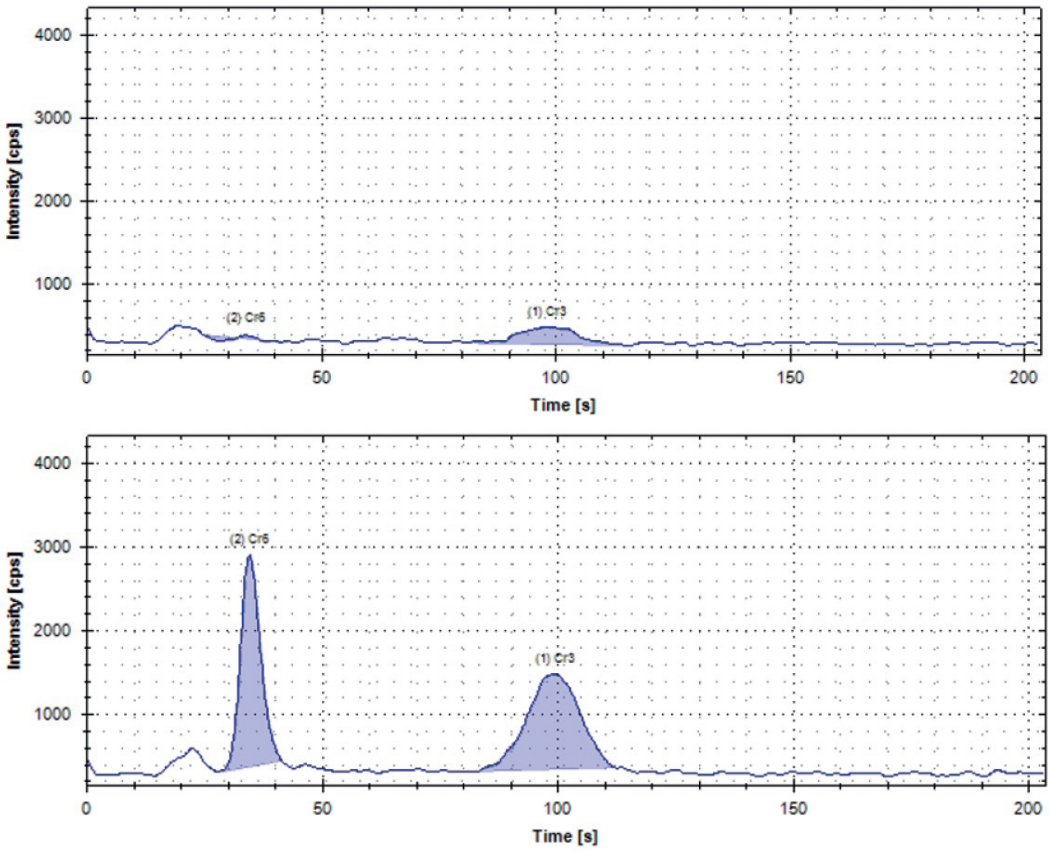

Figure 1. Chromatograms showing the injection of (from top to bottom) a blank, a standard solution containing 0.1 µg L^{-1} of both species (top), tap water, and spiked tap water (0.1 µg L^{-1}). For better comparability, all are scaled identically.

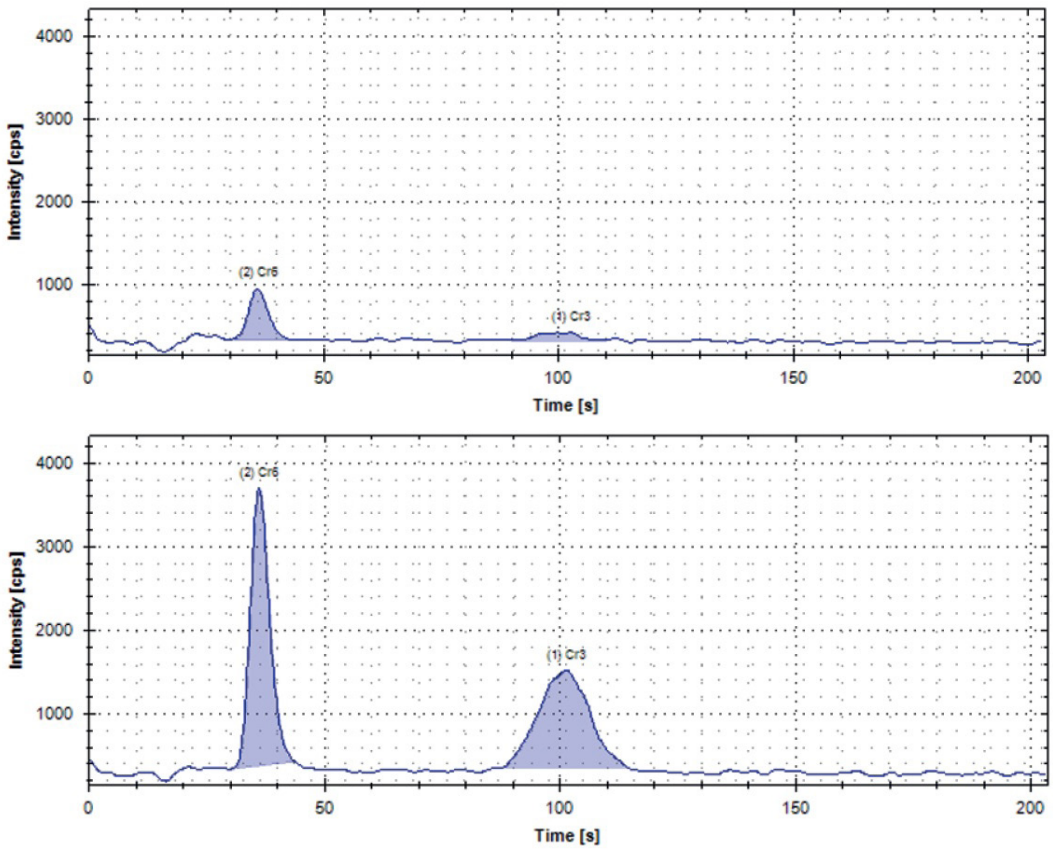

Figure 1. Chromatograms showing the injection of (from top to bottom) a blank, a standard solution containing 0.1 µg L^{-1} of both species (top), tap water, and spiked tap water (0.1 µg L^{-1}). For better comparability, all are scaled identically. (Continuation)

As can be seen, the blank did contain very low amounts of Cr (III) (approximately 20 ng L^{-1}) and the tap water sample contained a very low amount of Cr (VI), which was quantified to be approximately 25 ± 1 ng L^{-1}. To address the accuracy of the method, a sample was spiked with both Cr species at a concentration of 0.1 µg L^{-1} and the spike recovery was determined. In all cases, the spiked amount was recovered accurately with a recovery of 93 ± 1% for Cr (III) and 113 ± 5% for Cr (VI). The lower deviation for Cr (III) can be explained by the absence of naturally occurring Cr (III) in the samples, so that the recovery is based on the spiked amount only and excludes any variation from the actual content of water sourced from different taps.

CONCLUSION

In this report, a method was outlined that coupled the Dionex Aquion IC system with the iCAP RQ ICP-MS system, which demonstrated linear calibrations over three orders of magnitude, good stability (based on multiple injections), and suitable accuracy and Limits of Detection (LOD). The use of a guard column alone is sufficient for this application and allows reduction of runtimes to around 3 min and therefore improves sample throughput. With an eluent ideally suited to ICP-MS, superior column chemistry specifically designed to provide both anion and cation exchange sites, and dedicated hardware for ion chromatography, that completely eliminates trace metal contamination, IC-ICP-MS is the optimal combination for chromium speciation.

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2. Séby, F.; Charles, S.; Gagean, M.; Garraud, H.; Donard, O. F. X. *J. Anal. At. Spectrom.*, **2003**, *18*, pp 1386-1390.
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Brazilian Journal of Analytical Chemistry
2024, Volume 11, Issue 43, 137-143

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New Performance for High Volume Agriculture Laboratories

Enabling high throughput in elemental analysis of several agriculture matrices using Milestone's ETHOS UP with MAXI 24 HP

This report was extracted from the Milestone Industry Report Ethos UP – Maxi-24 HP | Agriculture

INTRODUCTION

Fertilizer is a fundamental component for the growing of plants. However, too much of the wrong nutrient can have adverse effects such as burning the roots. Characterizing the fertilizer content will indicate the formula's macronutrient content, as well as other nutrients such as calcium, magnesium, potassium, etc. Analysis on a fertilizer product gives the ratio of each macronutrient, which must be correct to ensure optimal efficacy. ICP analysis can provide a lot of information on fertilizer composition but the choice of the correct sample preparation technique is fundamental. Traditional sample preparation techniques include hot block digestion, closed vessel microwave digestion and ashing; each of them posing different challenges. Hot block digestions suffer from long run times, airborne contamination, poor digestion quality, and poor recovery of volatile compounds. Closed vessel microwave digestion has proven to be an effective technique with fast, complete digestions, a clean environment, and full recovery of volatile compounds. Milestone's ETHOS UP equipped with the MAXI 24 High Performance (HP) rotor incorporates all of the benefits of closed vessel microwave digestion while making sample preparation fast, easy, effective, and of the highest quality. This innovative solution perfectly integrates with the powerful ETHOS UP, matching both performance and throughput requirements of agricultural elemental analysis.

EXPERIMENTAL

In this industry report, a recovery study on certified reference materials has been performed to prove the efficacy of the ETHOS UP in sample preparation for metal analysis.

Instrument

The ETHOS UP is the most advanced microwave sample preparation equipment. It meets the requirements of modern analytical labs.

Figure 1. Milestone's ETHOS UP

Figure 2. MAXI-24 HP Rotor

The ETHOS UP used in this study was equipped with MAXI-24 HP rotor controlled via Milestone's easyTEMP contactless temperature. The superior temperature measurement of easyTEMP allows the processing of different samples of similar reactivities, thus reducing labor time and increasing the overall throughput.

MAXI-24 HP Rotor

The latest Milestone's development is the MAXI-24 HP, which combines performance and throughput within a single rotor-based platform. It completely innovates the rotor-base solutions providing high throughput without sacrificing the performance. Thanks to its 24 positions, it is the first high pressure and throughput rotor available in the market. The completely new design of its vessels allows to achieve conditions never seen for high throughput rotors. Thicker high purity PTFE-TFM vessels and caps, along with rugged PEEK shields are key ingredients to handle the conditions required to completely digest these samples.

Procedure

Table 1 reports the conditions used to prepare the sample.

Table 1. Sample amount and acid mixture used for the microwave digestion run

SAMPLE	SAMPLE AMOUNT	ACID MIXTURE
Multi-nutrient	0.2 g	8 mL of HNO_3 (65%), 0.5 mL of H_2SO_4 (96%), 2 mL of HF (48%)
Marine sediment (IAEA-457)*	0.5 g	9 mL of HNO_3 (65%), 3 mL of HCl (37%)
San Joaquin soil (NIST 2709a)*	0.5 g	9 mL of HNO_3 (65%), 3 mL of HCl (37%)
Tomato leaves (NIST 1573a)	0.5 g	5 mL of HNO_3 (65%) + 1 mL of H_2O_2 (30%)
Cabbage (IAEA-359)	0.5 g	5 mL of HNO_3 (65%) + 1 mL of H_2O_2 (30%)

* EPA 3051A was applied

All samples were weighted into the MAXI–24 HP vessel, approximately 0.5 g (as reported in Table 1). The acid mixture (trace metal grade) was added according to the data reported in Table 1 and the proper microwave method has been used as reported in Table 2.

Table 2. Microwave program

STEP	TIME	T2	POWER
1	00:10:00	160 °C	1500 W
2	00:15:00	210 °C	1800 W
3	00:10:00	210 °C	1800 W

After microwave digestion, the samples were diluted to 50 mL with deionized water and analyzed by ICP-OES.

Quantification

ICP-OES Instrumental Parameters: RF power (W): 1300; Plasma flow (L/min): 15.0; Auxiliary Flow (L/min): 1.5; Nebulizer Flow (L/min): 0.75; Replicate read time (s): 10; Instrument stabilization delay (s): 15; Sample Uptake Delay (s): 30; Pump Rate (rpm): 15; Rinse Time (s): 10; Replicates: 3.

RESULTS AND DISCUSSION

The performance of the Milestone ETHOS UP equipped with MAXI-24 HP rotor and easyTEMP technology was evaluated through a recovery study on multi-nutrient fertilizer (NIST SRM695), marine sediment (IAEA 457), San Joaquin soil (NIST 2709a), tomato leaves (NIST 1573a) and cabbage (IAEA 359) samples. The samples were digested with Milestone's ETHOS UP and subsequently analyzed via ICP-OES.

Table 3. Data of the recovery study on multi-nutrient fertilizer (NIST SRM695) sample

	Certified value	Recovery % (n=3)	RSD (%)
Al	0.61 ± 0.03%	89.3	2.3
As	200 ± 5 mg/Kg	96.7	2.6
Ca	2.26 ± 0.04%	103.5	2.8
Cd	16.9 ± 0.2 mg/Kg	93.5	1.7
Co	65.3 ± 2.4 mg/Kg	94.1	2.3
Cr	244 ± 6 mg/Kg	88.9	2.6
Cu	1225 ± 9 mg/Kg	91.7	1.7
Fe	3.99 ± 0.08%	89.7	2.3
Hg	1955 ± 0.036 mg/Kg	95.6	2.6
K	11.65 ± 0.13%	92.6	1.9
Mg	1.79 ± 0.05%	105.9	2.7
Mn	0.305 ± 0.005%	101.5	2.4
Mo	20.0 ± 0.3 mg/Kg	93.3	1.4
Na	0.405 ± 0.007%	95.6	2.2
Ni	135 ± 2 mg/Kg	90.0	2.3
Pb	273 ± 17 mg/Kg	102.3	2.6
V	122 ± 3 mg/Kg	99.6	1.0
Zn	0.325 ± 0.005 mg/Kg	101.3	2.1

Table 4. Data of the recovery study on marine sediment (IAEA-457) sample

	Certified value	Recovery % (n=3)	RSD (%)
Ag	1.93 ± 0.38 mg/Kg	94.1	1.4
Al	82660 ± 3430 mg/Kg	96.3	1.3
As	10.2 ± 1.0 mg/Kg	109.4	2.5
Cd	1.09 ± 0.08 mg/Kg	102.9	1.9
Co	14.7 ± 1.0 mg/Kg	90.0	2.1

Table 4. Data of the recovery study on marine sediment (IAEA-457) sample (Continuation)

	Certified value	Recovery % (n=3)	RSD (%)
Cr	144 ± 8 mg/Kg	89.9	2.0
Cu	365 ± 19 mg/Kg	91.3	1.4
Fe	41450 ± 2240 mg/Kg	91.6	0.9
Hg	0.143 ± 0.012 mg/Kg	93.2	1.3
Li	64.2 ± 5.5 mg/Kg	94.7	1.8
Mn	427 ± 30 mg/Kg	93.1	2.8
Ni	53.1 ± 2.7 mg/Kg	93.0	1.4
Pb	105 ± 7 mg/kg	91.4	1.2
Sn	27.40 ± 0.75 mg/Kg	93.7	2.1
Sr	137 ± 10 mg/Kg	94.1	1.1
V	87.4 ± 8.1 mg/Kg	101.3	2.0
Zn	425 ± 25.8 mg/Kg	96.8	2.5

Table 5. Data of the recovery study on San Joaquin soil (NIST 2709a) sample

	Certified value	Recovery % (n=3)	RSD (%)
Al	7.37 ± 0.16%	93.4	2.3
Ba	979 ± 28 mg/Kg	91.6	2.6
Ca	1.91 ± 0.09%	89.9	2.8
Cd	0.371 ± 0.002 mg/Kg	<LOQ	—
Co	12.8 ± 0.2 mg/Kg	94.2	2.7
Cr	130 ± 9 mg/Kg	90.7	2.4
Fe	3.36 ± 0.07%	83.3	1.4
K	2.11 ± 0.06%	92.8	2.2
Mg	1.46 ± 0.02%	102.8	2.3
Mn	529 ± 18 mg/Kg	98.1	2.6
Na	1.22 ± 0.03 %	93.6	2.8
P	0.0688 ± 0.0013%	113.1	1.7
Pb	17.3 ± 0.1 mg/kg	92.4	1.4
Sb	1.55 ± 0.06 mg/Kg	<LOQ	1.9
Si	30.3 ± 0.4%	96.3	1.2
Sr	239 ± 6 mg/Kg	95.7	2.3

Table 5. Data of the recovery study on San Joaquin soil (NIST 2709a) sample (Continuation)

	Certified value	Recovery % (n=3)	RSD (%)
Ti	0.336 ± 0.007%	91.3	2.6
V	110 ± 11 mg/Kg	102.0	2.8
Zr	195 ± 46 mg/Kg	94.4	1.7

Table 6. Data of the recovery study on tomato leaves (NIST 1573A) sample

	Certified value	Recovery % (n=3)	RSD (%)
Al	598.4 ± 7.1 mg/Kg	94.1	2.3
As	0.1126 ± 0.0032 mg/Kg	<LOQ	—
Ca	50450 ± 550 mg/Kg	96.7	1.0
Cd	1.517 ± 0.027 mg/Kg	92.1	2.1
Co	0.5773 ± 0.0071 mg/Kg	<LOQ	—
Cr	1.988 ± 0.034 mg/Kg	90.9	2.6
Cu	4.70 ± 0.14 mg/Kg	96.0	2.8
Fe	367.5 ± 4.3 mg/Kg	96.8	1.7
Hg	0.0341 ± 0.0015 mg/Kg	92.3	2.3
K	26760 ± 480 mg/kg	99.7	2.6
Mn	246.3 ± 7.1 mg/Kg	101.0	1.7
Na	136.1 ± 3.7 mg/Kg	97.1	2.3
Ni	1.582 ± 0.041 mg/Kg	93.5	2.6
P	2161 ± 28 mg/Kg	90.6	2.2
Rb	14.83 ± 0.31 mg/K.g	90.2	1.4
Sb	0.0619 ± 0.0032 mg/kg	<LOQ	—
Se	0.0543 ± 0.0020 mg/Kg	<LOQ	—
V	0.835 ± 0.034 mg/Kg	<LOQ	—
Zn	30.94 ± 0.55 mg/Kg	94.5	2.4

Table 7. Data of the recovery study on cabbage (IAEA-359) sample

	Certified value	Recovery % (n=3)	RSD (%)
As	0.10 ± 0.004 mg/Kg	<LOQ	—
Ba	11.0 ± 0.5 mg/Kg	91.6	1.2
Ca	18500 ± 510 mg/Kg	92.5	2.6

Table 7. Data of the recovery study on cabbage (IAEA-359) sample (Continuation)

	Certified value	Recovery % (n=3)	RSD (%)
Cd	0.12 ± 0.005 mg/Kg	<LOQ	—
Cr	1.30 ± 0.06 mg/Kg	90.3	2.0
Cu	5.67 ± 0.18 mg/Kg	94.8	2.1
Fe	148 ± 3.9 mg/Kg	95.2	1.6
Hg	0.013 ± 0.002 mg/Kg	94.1	0.7
K	32500 ± 690 mg/Kg	96.3	1.6
Mg	2160 ± 50 mg/Kg	91.4	1.7
Mn	31.9 ± 0.6 mg/Kg	98.9	2.9
Na	580 ± 21 mg/Kg	103.4	1.1
Ni	1.05 ± 0.05 mg/kg	95.5	1.4
Se	0.12 ± 0.011 mg/Kg	<LOQ	—
Sr	49.2 ± 1.4 mg/Kg	93.1	1.9
Zn	38.6 ± 0.7 mg/Kg	91.9	2.1

The analytical results were shown in Tables 3-7 with good recoveries of all elements and RSDs below 3%. This demonstrates the robustness and reproducibility of the digestion process with the ETHOS UP – MAXI-24 HP.

Figure 3 shows the temperature profile of the digestion as well as the multiple temperature visualization and recording for all the samples digest in the run.

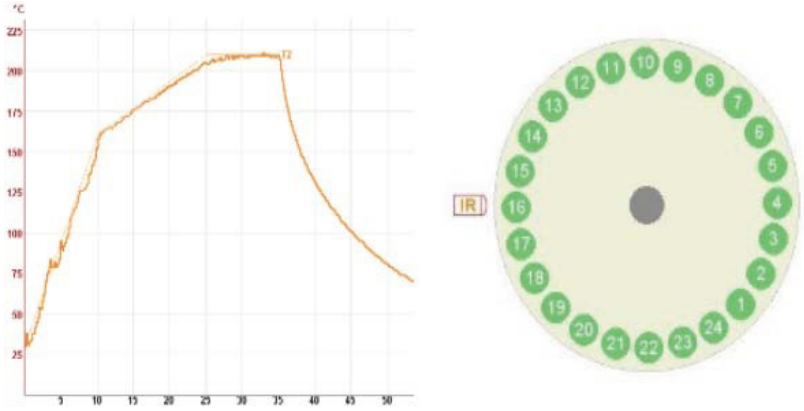

Figure 3. MAXI-24 HP Microwave Run Report and Multiple temperature traceability.

CONCLUSION

The data shown in this industry report demonstrates full recovery of the elements reported in the certificates of the reference material. Highly reactive samples such as fertilizer has been completely digested even in large sample amounts. The digestion process has been accurately controlled by the

easyTEMP sensor, ensuring same digestion quality and reliable results. In addition, microwave digestion using the Milestone ETHOS UP with easyTEMP control, provides the highest level of reproducibility and great ease of use, ensuring high quality digestion run after run.

Further reading here

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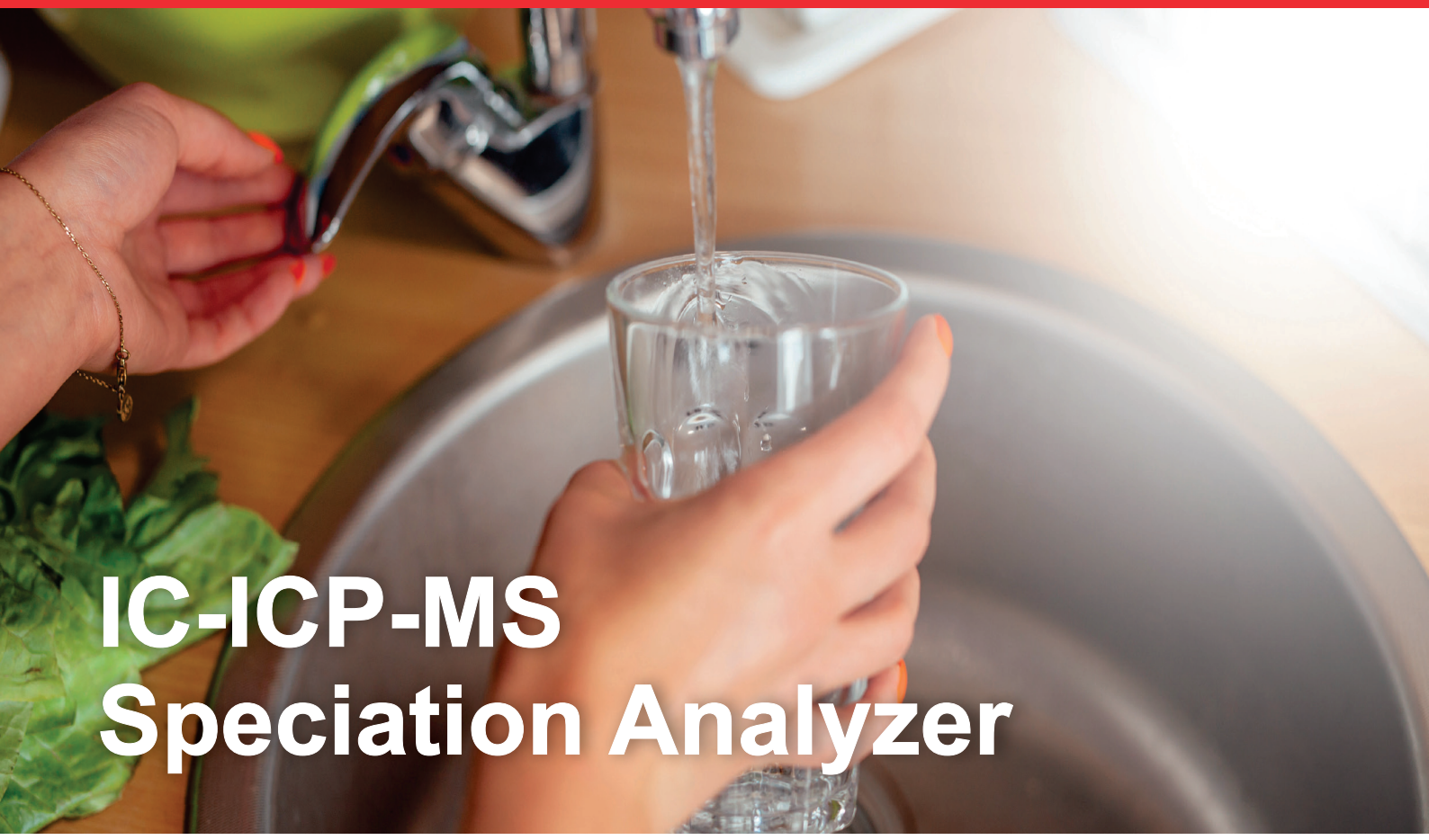

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- Metal-free IC separates the individual species without contributing trace metal contamination.
- IC-ICP-MS acts as a highly sensitive and element-specifc detection system.

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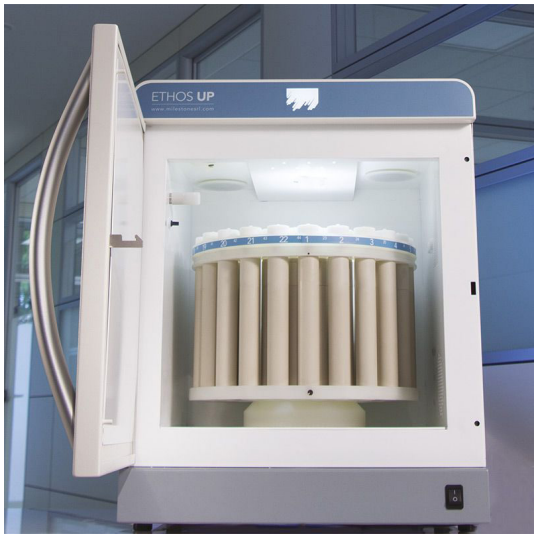

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The MAXI-24 High Performance (HP) and high throughput rotor was designed with double pressure limits and greater capacity than other high throughput rotors. It processes increased volumes of sample and a wide variety of matrices within a single rotor-based platform. Quick-assembly vessels are closed with a simple hand-tightening, improving your daily productivity.

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Flexibility

Rotors and pressure vessels are crucial components to ensure high digestion quality and safety. Milestone's rotors are build with high-purity PTFE-TFM, PEEK safety shields and a unique safety release mechanism.

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The ETHOS UP comes with a dedicated touch screen terminal with easyCONTROL software which incorporates our expertise and knowledge in microwave sample preparation. The ETHOS UP user-interface provides full control of all digestion parameters, complete documentation and improves the overall procedure.

Read more

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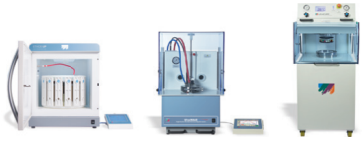

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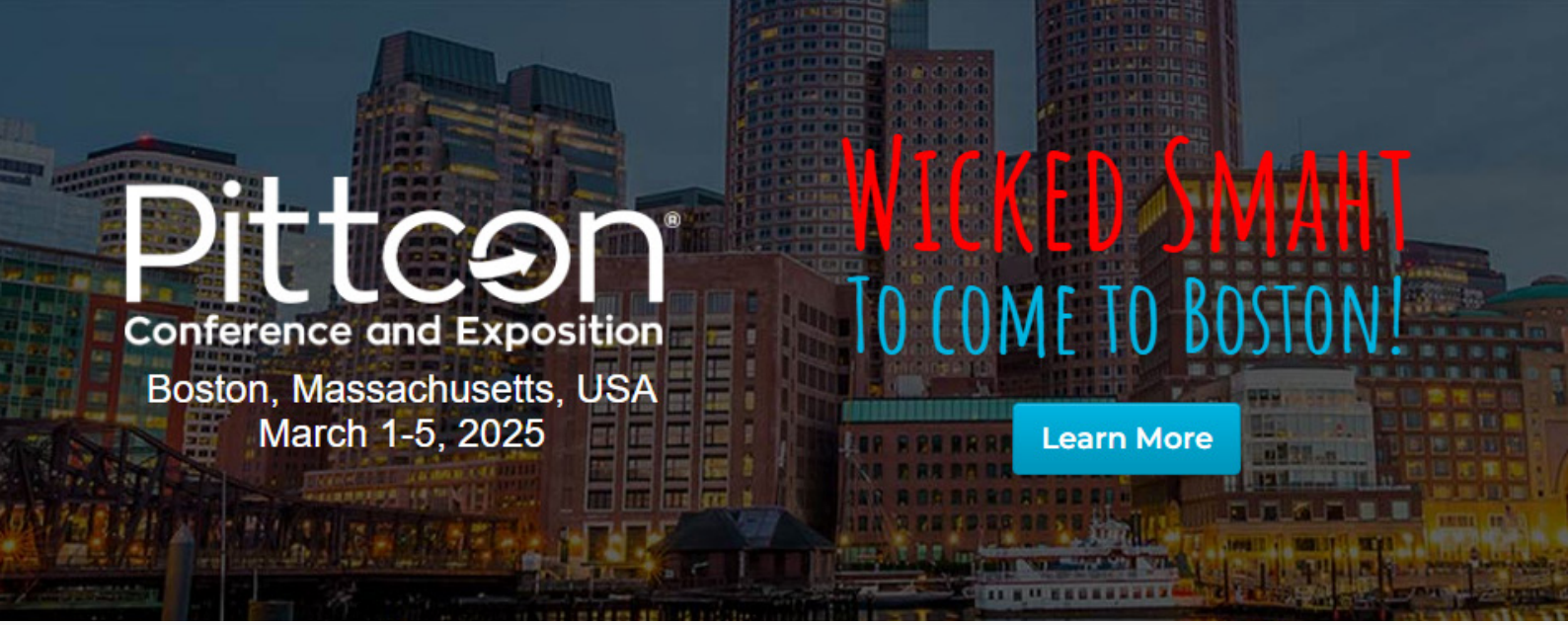

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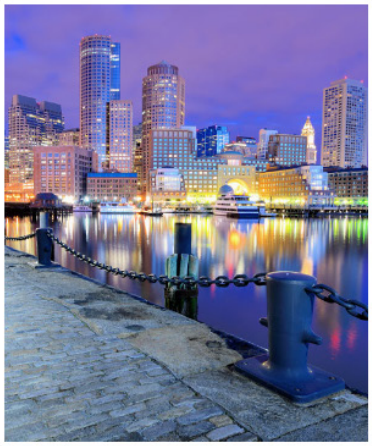

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Editorial Articles

Highly consolidated QC materials support EFLM Green Lab Initiative

A recent study from Ramón y Cajal University Hospital in Madrid, Spain, has shown that using a highly consolidated QC material can have a significant positive impact on lab efficiency and sustainability – without compromising analytical performance. Access **here.**

How to navigate the lab digitalization journey

Gain insights from industry experts on how to approach lab digitalization and ease the transition from paper to pixels. The message from laboratory professionals and digitalization experts who met to discuss the challenges and benefits of lab digitalization is clear: embracing digital technologies in laboratories is not just a trend, but a strategic imperative. Digital technologies will ensure operational excellence, help meet regulatory standards, and propel the industry forward. Access **here.**

Sustainable nanomaterials address PFAS and biosensing challenges

Sustainable nanomaterials are tackling PFAS contamination and pain biosensing whilst preventing harmful knock-on effects on the environment. From pain biosensors to innovations in environmental remediation, the unique properties of nanomaterials are helping to solve some of the most pressing environmental and human health-related challenges of today.

Driven by a passion for fabricating nanomaterials with minimal or zero carbon footprint, Dr. Omowunmi 'Wunmi' Sadik, a Distinguished Professor of Chemistry and Environmental Science at the New Jersey Institute of Technology (NJIT), focuses not just on nanomaterial fabrication but on their intended impact. Dr. Sadik's work involves the research and development of nanomaterials with economic and societal benefits with little or no negative environmental impact. Her team's focus extends beyond material fabrication to applications that address pressing environmental challenges and advance human healthcare. Access **here.**

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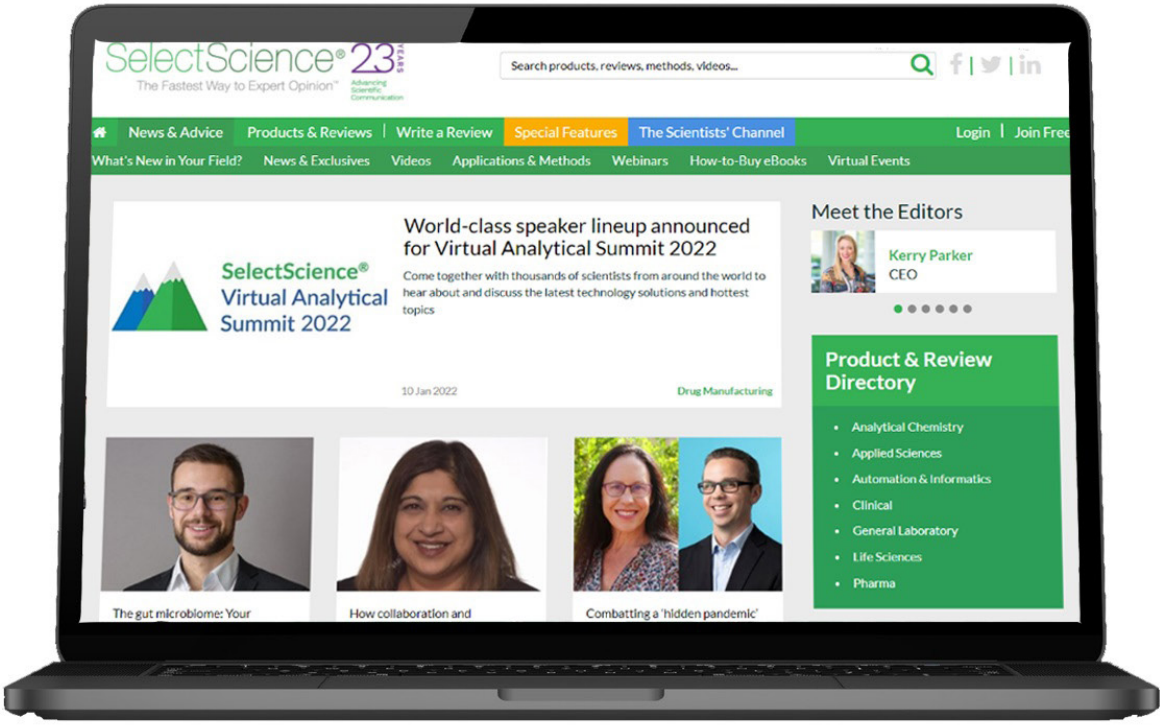

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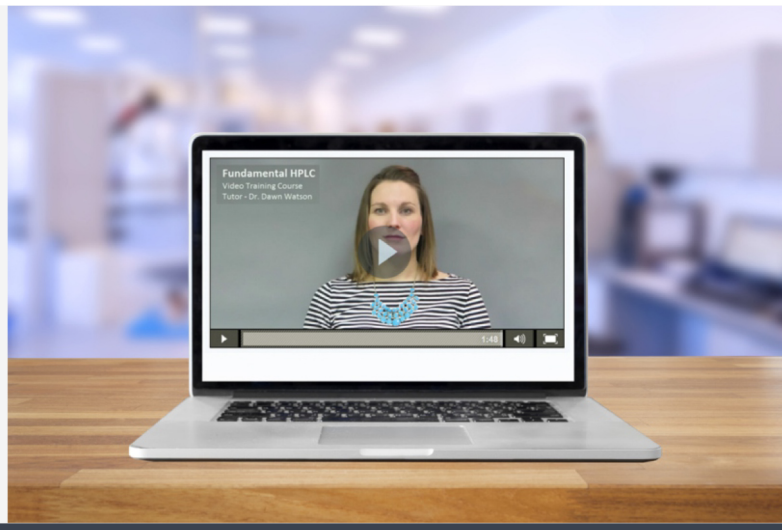

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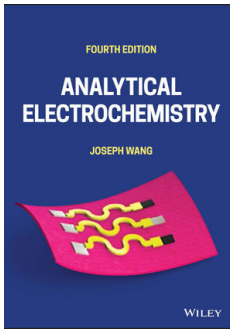
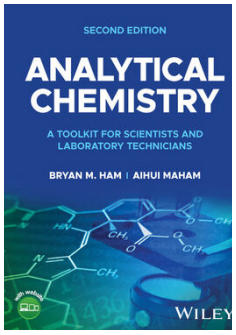


Analytical Electrochemistry, 4th Edition

Joseph Wang, Author

February 2023, Wiley

An accessible and robust text with comprehensive coverage of modern electroanalytical techniques and devices. With a strong focus on electroanalysis (as opposed to physical electrochemistry), the book offers readers a thorough grounding in the fundamentals of electrode reactions and the principles of electrochemical methods. It also demonstrates the solving of real-life analytical problems using the techniques discussed within. **Read more.**

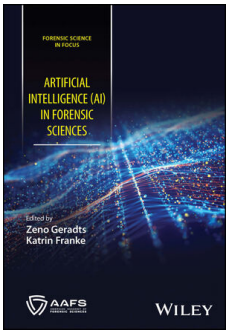


Analytical Chemistry: A Toolkit for Scientists and Laboratory Technicians, 2nd Edition

Bryan M. Ham, Aihui MaHam, Authors

February 2024, Wiley

This book begins with an introduction to the laboratory environment, including safety, glassware, common apparatuses, and lab basics, and continues on to guide readers through the fundamentals of analytical techniques, such as spectroscopy and chromatography, and introduce examples of laboratory programs, such as Laboratory Information Management Systems (LIMS). **Read more.**



Artificial Intelligence (AI) in Forensic Sciences

Zeno Geradts, Katrin Franke, Editors

August 2023, Wiley

This book covers issues of validation and emerging crimes that use AI; issues of triage, preselection, identification, reasoning, and explanation; demonstrates uses of AI in forensic science; and provides discussions of bias in the use of AI. It builds on key developing areas of focus in academic and government research, providing an authoritative and well-researched perspective. **Read more.**

Point-of-Care Biosensors for Infectious Diseases

Sushma Dave, Jayashankar Das, Editors

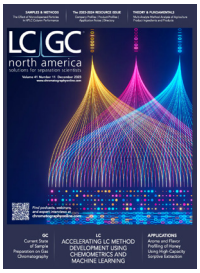
June 2023, Wiley

In this book, expert authors review current challenges in pathogen detection and the selection of suitable biomarkers, detail currently available biosensor platforms including electrochemical, piezoelectric, magnetic, and optical sensors, and cover technology development for point-of-care biosensors for viral, bacterial, and parasitic infections. **Read more.**

PERIODICALS & WEBSITES

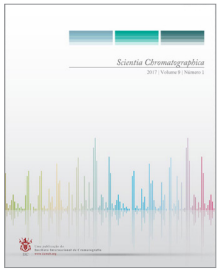
American Laboratory

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. **Featured Video:** ***Prof. Jemma Wadham Discusses using Ion Chromatography for Glaciology Studies***. **Watch the video.**



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. ***Multi-Active Method for the Analysis of Agriculture Product Technical Ingredients and Formulated Products.*** By Garvey J. et al. A collaborative multi-analyte method developed utilizes HPLC and UHPLC to analyse more than 70 active ingredients.



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.**

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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.

Feature article: ***What’s New in ICP-MS for Environmental Analysis?*** In this review article, the editors of *Spectroscopy* break down the most recent research and trends using inductively coupled plasma mass spectrometry (ICP-MS). **Read more.**

EVENTS in 2024

May 15 to 18
XXIII Brazilian Congress of Toxicology (CBTOX)
Convention Center Windsor Expo Center
Praia da Barra da Tijuca, Rio de Janeiro, RJ, Brazil
https://www.cbtox.com.br/

May 18 to 21
53rd Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq)
Convention Center of the Majestic Hotel
Águas de Lindóia, SP, Brazil
https://www2.sbbq.org.br/reuniao/2024/

May 22 to 25
47th Annual Meeting of the Brazilian Chemical Society (RASBQ)
Águas de Lindóia, SP, Brazil
https://www.sbq.org.br/reunioes-anuais

June 4 to 6
28th FCE Pharma
São Paulo Expo, São Paulo, SP, Brazil
https://www.fcepharma.com.br/

June 25 to 26
13th World Congress on Chromatography
Toronto, Canada
https://chromatographytechniques.conferenceseries.com

July 24 to 26
12th Countercurrent Chromatography Conference (CCC2024)
Jardim Botânico, Rio de Janeiro, RJ, Brazil
CCC2024-website

August 28 to 30
I Latin American Microfluidics Conference (LAMic 2024)
University of São Paulo, São Carlos, SP, Brazil
https://www.latammicro.org/

September 15 to 18
21st National Meeting on Analytical Chemistry (ENQA) & 9th Ibero-American Congress on Analytical Chemistry (CIAQA)
Hangar Centro de Convenções & Feiras da Amazônia, Belém, PA, Brasil
https://www.enqa2024.com.br//

October 6 to 10
34th International Symposium on Chromatography – ISC 2024
Liverpool, UK
https://isc2024.org/

EVENTS in 2024

October 20 to 25
SciX Conference 2024
Raleigh Convention Center, Raleigh, NC, United States
https://www.scixconference.org/scix-future-conferences

October 24 to 25
IV Reunião Bienal da Sociedade Brasileira de Eletroquímica e Eletroanalítica
Center for Agricultural Sciences (CCA), Federal University of São Carlos (UFSCar)
Araras, SP, Brazil
https://www.lsnano.ufscar.br/iv-sbee

November 9 to 12
29th Latin-American Capillary Electrophoresis, Microfabrication and Related Techniques Symposium and Brazilian Symposium on Metabolomics (LACE 2024 / BrMet)
Casa Grande Hotel Guarujá, Guarujá, SP, Brazil
https://www.lace2024.iqm.unicamp.br/

November 10 to 13
9th National Meeting of Forensic Chemistry (ENQFor) and 6th Meeting of the Brazilian Society of Forensic Sciences (SBCF)
Events Center of the Hotel Royal Tulip JP, Ribeirão Preto, SP, Brazil
https://www.enqfor.org.br/

AUTHOR GUIDELINES

Aims & Scope

Brazilian Journal of Analytical Chemistry is a double-blind peer-reviewed research journal dedicated to the diffusion of significant and original knowledge in all branches of Analytical and Bioanalytical Chemistry. It is addressed to professionals involved in science, technology, and innovation projects at universities, research centers and in industry. **BrJAC welcomes** the submission of research papers reporting studies devoted to new and significant analytical methodologies, putting in evidence the scientific novelty, the impact of the research and demonstrating the analytical or bioanalytical applicability. BrJAC **strongly discourages** those simple applications of routine analytical methodologies, or the extension of these methods to new sample matrices, unless the proposal contains substantial novelty and unpublished data, clearly demonstrating advantages over existing ones.

Additionally, there are other submission categories to BrJAC such as:

Reviews: They should be sufficiently broad in scope, but specific enough to permit an appropriate depth discussion, 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 and are handled by the Editor of Reviews.

Technical Notes: Concise descriptions of developments 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, and suggestions on issues related to Analytical Chemistry or Bioanalytical Chemistry. Letters are welcome and will be published at the discretion of the BrJAC Editor-in-Chief.

Point of View: This category is exclusively invited by the Editor-in-Chief.

See the next items for more information on the journal, the documents preparation, manuscript types, and how to prepare the submission.

Professional Ethics

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Manuscript submission

The BrJAC does not charge authors an article processing fee.

Manuscripts must be prepared according to the BrJAC manuscript template. Manuscripts in disagreement with the BrJAC guidelines are not accepted for revision.

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After the submitting author logs in to the system and enters his/her personal and affiliation details, the submission can be started.

All co-authors must be added to the Authors section.

Four documents are mandatorily uploaded by the submitting author: Cover letter, Title Page, Novelty Statement and the Manuscript. Templates for these documents are available for download **here**.

The four documents mentioned above must be uploaded 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.

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.

Cover Letter
The cover letter template should be downloaded and filled out carefully.
Any financial conflict of interest or lack thereof and agreement with BrJAC's copyright policy must be declared.
It is the duty of the submitting author to inform his/her collaborators about the submission of the manuscript and its eventual publication.
The Cover Letter must be signed by the corresponding author.

Title Page
The Title Page must contain information for each author: full name, affiliation and full international postal address, 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 (all submission categories)

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Review process

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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 BrJAC production 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.

Final Considerations

Whatever the nature of the submitted manuscript, it must be original in terms of methodology, information, interpretation or criticism.

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