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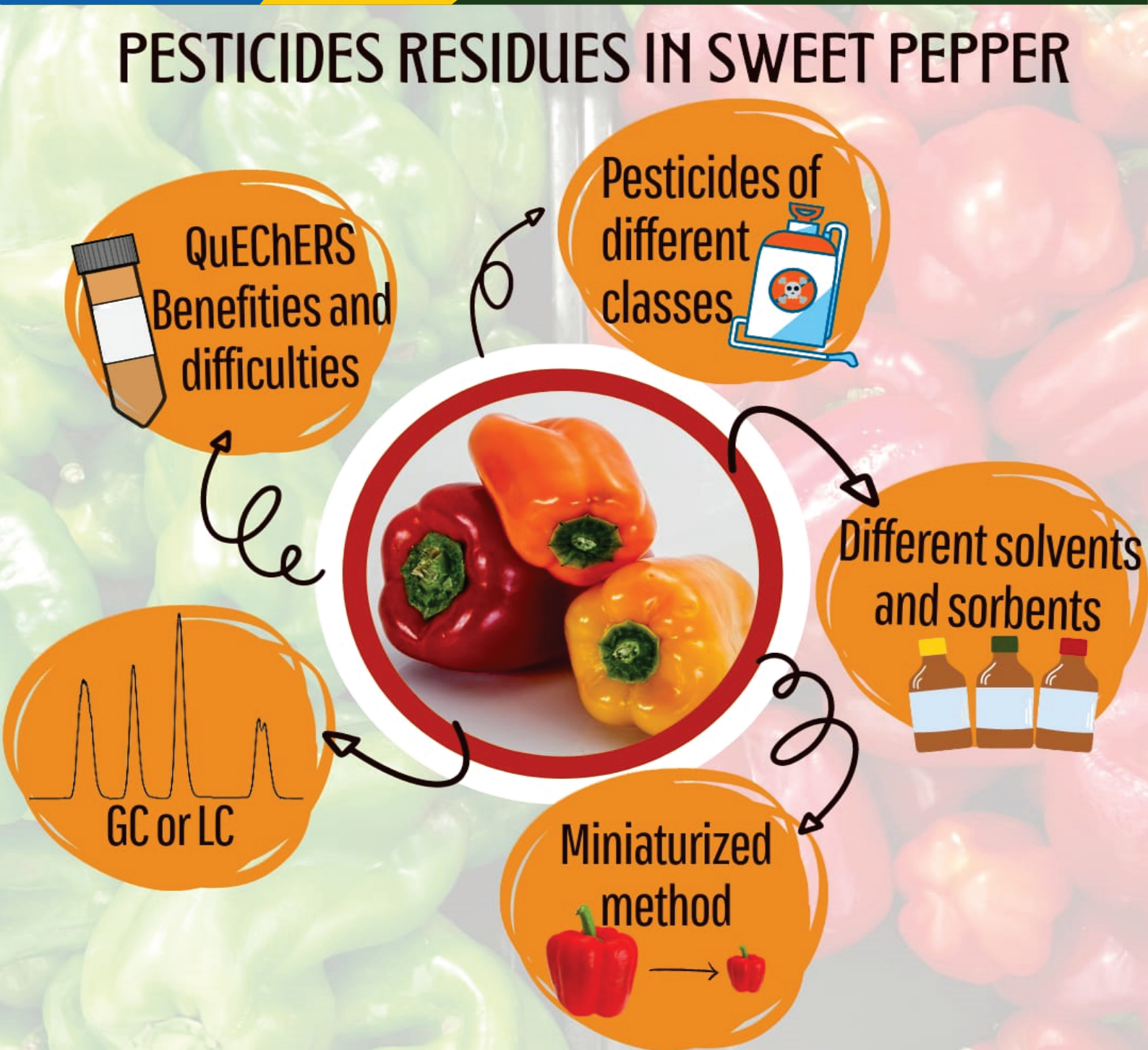

An Integrative Review on the Analysis of Pesticide Multiresidues in Sweet Pepper Samples using the QuEChERS Method and Chromatographic Techniques

Fabiane Ferraz Wisniewski, Elisandra Carolina Martins

October – December 2022 Volume 9 Number 37

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Scope

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

BrJAC is a quarterly journal that publishes original, unpublished scientific articles, reviews and technical notes that are peer reviewed in the double-blind way.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

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

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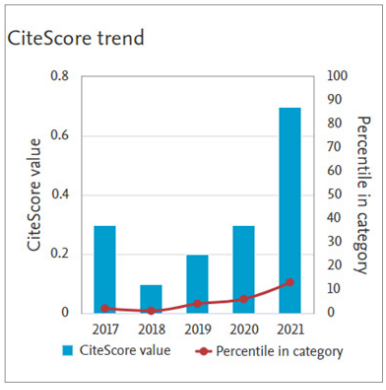

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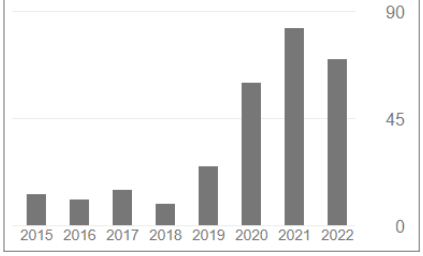

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CONTENTS

CONTENTS

CONTENTS

Brazilian Journal of Analytical Chemistry
2022, Volume 9, Issue 37, pp 1-2
doi: 10.30744/brjac.2179-3425.editorial.eagzagatto.N37

BrJAC

EDITORIAL

Go International

Elias Ayres Guidetti Zagatto
Full Professor at the Centre for Nuclear Energy in Agriculture, University of Sao Paulo, Piracicaba, SP, Brazil

The indissoluble teaching-research-extension triad is frequently mentioned in university and industrial environments, especially in relation to Analytical Chemistry (AC). This triad tends to be more expressive when it involves collaborations within different groups, and the presence of foreign scientists is welcome in the context. This is consistent with the expression “GO INTERNATIONAL”, endorsed by BrJAC.

With regard to teaching, the initial AC development in Brazil was strongly influenced by forthcoming foreign scientists, and IQ-USP, IQ-UNICAMP, and CENA-USP can be selected as examples. In BrJAC, expressions such as “… Ph.D. students affectionately called scientific children”[1] and “treat your students with special care”[2] have already been used. It is worth mentioning here the interview recently granted by Suzanna Rath to BrJAC[3]. She was born in Germany and came to Brazil at the age of seven, staying here until the end of her M.Sc. program. Thereafter, she received her Ph.D. in Germany and, after returning to Brazil, proved to be an excellent teacher and scientist. Asked for what she would like to be remembered, she replied: “for having had the privilege of sharing the little that I know”. This reflects the importance of GO INTERNATIONAL in her professional life. BrJAC teaching support through promotional materials, novel product presentations, articles, reviews, points of view, sponsor reports, scientific events, etc. should also be highlighted.

With regard to research, relevant scientific achievements and industrial production are currently noted, and the role of BrJAC is to disseminate them through published materials, organization of webinars, presentations in events, apparatus demonstrations, etc. A relevant aspect linked to GO INTERNATIONAL is the growing number of publications authored by researchers from other countries. This is evident in this BrJAC issue, which includes 01 review and 09 technical articles. Amongst them, 44% come from Morocco, India, or Uruguay, different countries with specific cultural lives and conditions. The review deals with chromatographic analysis of pesticides in sweet pepper, and the articles are focused on the following: extraction and evaluation of flamboyant (*Caesalpinia pulcherrima*) mirim gum as a viscosifying agent for enhanced oil recovery fluids; simultaneous obtaining and fractioning of essential oils by hydro-distillation; evaluation of a biodegradable system for Pb(II) and Hg(II) removal from waters; MS estimation of $\delta^{13}C$ and $\delta^{18}O$ in blueberries (*Vaccinium spp.*) for authenticity control and traceability purposes; multivariate analysis of mid-infrared spectrometric data for tracing the origin of Moroccan saffron; voltammetric behavior of 8-oxoguanine on a glassy carbon electrode modified with multi-walled carbon nanotubes/carbon black; ultrasound-assisted determination of copper in bovine and ovine livers, aiming at food surveillance and animal status monitoring; and an eco-friendly ultrasound-assisted procedure for simultaneous determinations of evogliptin and metformin hydrochloride in bulk and combined tablet dosage forms. Noteworthy aspects of the above-mentioned contributions are their excellent quality, originality, and relevance.

With regard to extension, it is worth noting that, with the continuous quality improvement of the journal, there is a better adherence of BrJAC with GO INTERNATIONAL, and this meets the objectives of this journal. The incentives for those engaged with AC have been increasing, and the development of novel products (e.g., instruments and reagents), training of staff, especially those associated with GAC (e.g., working in the analytical laboratories and/or developing new analytical methods/procedures), introducing

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and disseminating novel instruments, participation in scientific events, etc. have always be mentioned in the BrJAC. One can then infer that the indissoluble teaching-research-extension triad is a positive factor in the inception, development, and industrial production of modern AC instruments.

All above-mentioned aspects can be appreciated by reading this issue. So, enjoy reading.

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Elias Ayres Guidetti Zagatto has a degree in Agronomic Engineering from the University of São Paulo (1971), a master degree in Nuclear Energy in Agriculture from the University of São Paulo (1974) and a doctoral degree in Analytical Chemistry from the University of Campinas (1981). He is currently a Professor at the Center for Nuclear Energy in Agriculture, University of São Paulo, and a Member of the Brazilian Academy of Sciences. His research activities mainly include the design and development of flow analyzers, with applications on relevant samples in the agronomic, environmental, pharmaceutical and industrial areas.

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2022, Volume 9, Issue 37, pp 3–7
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INTERVIEW

Professor Susanne Rath, a researcher who has bravely faced challenges since childhood, kindly granted BrJAC an interview

Susanne Rath

Susanne Rath is an associate professor in the Institute of Chemistry at the University of Campinas (Unicamp), where she coordinates the "*Laboratório de Bioanalítica Paracelsus*". She graduated with a Bachelor's degree in Chemistry (1983) from the University of Brasília (UnB), a Master's degree in Chemistry (1986) from Unicamp, and a Ph.D in Pharmaceutical Chemistry (1990) from the Johann Wolfgang Goethe Universität Frankfurt am Main, Germany. So far, she has published 110 articles and seven book chapters, had four patents granted, and she has presented over 230 papers at scientific conferences. She supervised 17 master's students, 20 doctorate students and 10 post-docs. In addition, she coordinated 23 research projects supported by Brazilian funding agencies.

Prof. Dr. Rath's primary research is focused on toxic compounds in food, residue depletion studies of veterinary drugs in food-producing animals, development and validation of analytical methods, application of bidimensional chromatography and mass spectrometry, environmental impact assessment of veterinary drugs, antimicrobial resistance and N-nitrosamines in food, cosmetics and drugs.

Since 2007, Prof. Rath has been a member of the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). Since 2011, Prof. Rath has been a member of the Technical Group on Maximum Residue Limits for Veterinary Drugs in Food of the National Health Surveillance Agency (Anvisa) of the Ministry of Health of Brazil.

How was your childhood?

I was born in Munich, Germany, and my parents migrated to Brazil when I was only seven years old. My childhood was full of challenges and adventures. The first was to learn the Portuguese language and adapt to the new customs in the south of Brazil. Rio Grande do Sul was the place of my first Brazilian home but not the last. Many moves between different cities happened over the years, and, briefly, I can say that I had a nomadic childhood. Over the years, I fell in love with Brazilian culture and decided to build my life in this country.

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

I don't remember having in my live a specific event or person who guided or encouraged me to pursue a career in Chemistry. My scientific curiosity, my love for reading, and the pleasant feeling of learning new things always motivated me. Early on, I realized that my vocation was directed to science. Chemistry, Biochemistry, or Pharmacy would have been my primary choices, but it was Chemistry that I decided to study at the University of Brasilia.

When did you decide to go into the field of chemistry? What motivated you? How was the beginning of your career in chemistry?

In the first years of my undergraduate degree in Chemistry, I was sure I would follow an academic career. I was motivated by my passion for research and admiration for lecturing. After graduation, I decided to do a master's in Analytical Chemistry at the University of Campinas (Unicamp). I developed work in electrochemistry, which proved to be an exciting challenge. However, to reach my goal of becoming a professor, I required further specialization. I had the opportunity to do a Ph.D. in pharmacy, biochemistry, and food science at the Johann Wolfgang Universität in Frankfurt, Germany. The return to my home country reaffirmed my roots and allowed me to study pharmaceutical chemistry. I had great master's and many opportunities in Germany, but I was determined to return to Brazil.

My academic and teaching life began at the University of Brasília, first as a visiting professor and then as an associate professor. Driven by personal choices and searching for new challenges, I decided to leave Brasília and pursue a career in the Institute of Chemistry at Unicamp.

What has changed in the students' profile, ambitions, and performance since the time you started your career?

The student profile has changed over the years, and it has been a constant challenge to keep up with these transformations. In general, students have enormous ease in using technology for learning, but at the same time, they are more impatient, which seems to reflect in their scientific curiosity. They are questioning less, and they have more difficulty in defining the paths they would like to follow in their education and professional lives.

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

Analytical chemistry, as well as science in general, has evolved impressively with the advancement of technology and instrumentation. Today, the challenges are very different from those I faced at the beginning of my career. Having a research group at Unicamp is and has been a great privilege both because of the excellent infrastructure available and the ease of scientific cooperation with colleagues from the most diverse areas.

Indeed, my most significant contribution is not only focused on Analytical Chemistry but also in the areas of food toxicology and environmental chemistry. I can highlight my work in the area of food safety. It is more specifically focused on the residues of veterinary drugs in food. For over a decade, I have actively participated in the Joint FAO/WHO Expert Committee on Food Additives (JECFA) on risk assessments and, in particular, in the establishment of maximum residue limits for veterinary drugs in food, which were subsequently adopted by the Codex Alimentarius and by the governmental agencies of the member countries, which includes Brazil. This experience of interacting with our governmental agencies, such as the National Health Surveillance Agency (ANVISA) and the Ministry of Agriculture, Livestock, and Supply (MAPA), as well as other international agencies, and with the pharmaceutical industry has allowed me to share knowledge and experiences.

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

My research areas have followed my nomadic profile. I started in organic electrochemistry, and today I am developing research in food toxicology, pharmacy, and environmental chemistry. My research group has been working on developing analytical methods by using different separation techniques, emphasizing two-dimensional chromatography associated with mass spectrometry. This research aims to determine compounds with toxic potential in foods, drugs, and environmental matrices at ppb and ppt levels. In recent years, our group's main research has focused on veterinary drugs, including studies on the mobility of veterinary drugs in the environment and on the depletion of veterinary drugs in food-producing animals. Also, the emergence of bacterial resistance resulting from the use of antimicrobials in aquaculture has been addressed. Finally, we have focused on determining N-nitrosamines in personal care products, cosmetics, and medicines to understand where contamination by these compounds, considered a "group of concern", occurs in the process. With the knowledge acquired over the years, our laboratory has collaborated to find solutions to these problems.

Most of the results developed with the collaboration of students and researchers over the 25 years have been published or presented in scientific events. I had the great privilege of having students in our group who graduated in different areas of knowledge: it is always an enriching experience for everyone. Within the most recent work we developed, I would highlight one that came from a cooperative project with the research group of Professor Michael Schloter from the Helmholtz Zentrum in Munich. This work aimed to evaluate the emergence of resistant bacteria resulting from using antimicrobials in aquaculture. This cooperation extended into a new challenge: discussing the use of veterinary drugs from the "One Health" perspective. Now, this project involves researchers from five countries and received funding from the European Community Horizon 2020.

Good moments in the laboratory with students (Caio, Rafael, Alyne, Gabriela, Natalia and Andreza).

What is your opinion about the current progress of chemistry research in Brazil? What are the recent advances and challenges in scientific research in Brazil?

Due to the cuts in funds allocated to research, we are experiencing a delicate and challenging moment in Brazil. This scenario requires great effort from researchers to maintain their research activities.

Due to the cuts in funds allocated to research, we are experiencing a delicate and challenging moment in Brazil. This scenario requires great effort from researchers to maintain their research activities. Research in Brazil is closely associated with postgraduate education, so I understand that it is also crucial to discuss the situation of postgraduate scholarships. It is impossible to turn a blind eye to the financial difficulties that our postgraduate students are currently experiencing. Staying in large cities, such as Campinas-SP Brazil, to develop their thesis with exclusive dedication has been quite tricky. Training qualified human resources are one of our most important missions and our most significant contribution to society and should be prioritized by our public policies.

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?

The pandemic has imposed many changes in our day-to-day life. A situation that seemed to be temporary and controlled now extends over a long period of time. We have spent two years with many challenges that have required dedication in learning how to use technology to our favor, both in teaching classes and in discussing research projects, in an environment that was not always ideal. The pandemic years have been a period of understanding one another and ourselves.

I think that the greatest scientific advances that took place in this period were in the area of healthcare, the highest point being the surprisingly rapid development of vaccines. Scientists from different fields around the world have been researching with a common goal, which is the quintessential environment for any breakthrough research.

In Analytical Chemistry, I would point out to the ohmic sciences as a major milestone. Currently, the biggest challenge is not to develop an analytical method but to extract information from a large set of complex data that can be obtained with enormous speed, which can only be done by using cutting-edge technologies (i.e., big data).

What is the importance of awards for the development of science and new technologies?

There is a difference between receiving recognition and receiving awards. There seems to be a reversal of values in this regard. “Awards” ended up being part of one of the items in a researcher`s *curriculum*, and it lost its prominence, its real purpose. The development of science does not depend on awards. Scientists dedicate their lives to science because they breathe scientific curiosity, not because they expect to be recognized. As Albert Einstein once said – “the greater the knowledge, the smaller the ego”.

For you, what is the importance of the national funding agencies for the scientific development of Brazil?

Funding agencies are essential for the scientific development of any country. History clearly showed us this as a fact. Highly developed countries have invested and still invest in science. Brazilian funding agencies are the foundation for developing research and training qualified human resources in the country.

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?

The reduction of investments in research is a great setback for the development of Brazil. Unfortunately, our public policies have not had this perception, and the universities and research centers have suffered directly and continuously from funding cuts that impact scientific production.

To the young researchers, I would say not to give up and use their creativity to find alternatives for financing for their research. I have experienced, although modestly, the investment of some industries

in research projects. The interaction between the private sector and the academy benefits everyone and contributes to the country's development. We need to look for alternatives.

There are in Brazil, and around the world, several conferences on Chemistry. To you, how important are these meetings to the chemistry scientific community? How do you see the development of national chemistry meetings in Brazil?

Scientific meetings are particularly useful if there is an in-depth scientific discussion on relevant topics relevant to the development of science. I specifically remember attending an international event where representatives from academia, government, and industry were present. It was a small event but certainly, the most interesting one that I had the opportunity to attend. National meetings have greatly increased the number of participants, which, in my opinion, limits more in-depth discussions on specific topics. However, it is a valuable opportunity for students to present their scientific work, meet other research groups, and establish interactions for possible future collaborations.

Consultant's Meeting at the International Atomic Energy Agency in Vienna, 2019, discussing "Food safety and radiometric analysis".

What advice would you give to a young scientist who wants to pursue a career in chemistry?

Chemistry is simply fascinating! I would even say, immerse yourself in Chemistry, explore associated areas, expand your knowledge, and never get complacent. Run after your dreams, and when one challenge is overcome, look for new ones. Be creative and persistent and use all of your knowledge to ensure a sustainable and better world for the future generations.

For what would you like to be remembered?

For having had the privilege of sharing the little that I know.

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POINT OF VIEW

Paper-based microfluidics: What can we expect?

Wendell K. T. Coltro[1,2]

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In the last three decades, the scientific community has observed exponential growth in the development of microfluidic platforms and their use for applications in different fields. The noticeable advances are attributed to the advantages provided by miniaturization.[1] In summary, the downscaling of analytical devices has offered attractive features, including reduced consumption of samples and reagents, short analysis time, and minimal waste generation. In addition, the possibility to perform multiplexed assays in portable devices without bulky instrumentation is another attractive feature that boosted the investigation of miniaturized devices with the capability to be tested directly in the point-of-care (POC). Due to the sample volume required to proceed with a chemical analysis on a microscale (typically in the µL range), a complete understanding of the fluid control and handle on channels defined in micrometric dimensions was necessary, giving rise to the science known as microfluidics.[2] Many platforms including rigid and flexible materials can be explored for manufacturing microfluidic networks. Among all the substrates reported in the literature, the "paper" is by far the simplest and cheapest material currently employed for the development of microfluidic devices dedicated to analytical, bioanalytical, biomedical, environmental, food, and forensics applications.[3] For many readers, the first question is why paper is used instead of other materials such as glass. Well, glass is a rigid material, and microchannel engraving requires cleanroom facilities, photolithographic patterning, developing steps, and thermal sealing. This standard protocol makes use of sophisticated instrumentation, and it is not readily available to most researchers. In this way, paper emerges as a simple and alternative material to be used for microfluidics.

One of the major benefits of microfluidics refers to the sample-in-answer-out capability, which requires a fully automated fluid control to allow sample preparation, analytical separation, and detection stages. The fluid-controlled handling inside microchannels opens the possibility to integrate multiple analytical tasks in parallel into a high-throughput device. Considering these possibilities, it is worthwhile reflecting on how paper can be used to transport and handle a fluid.

Paper is currently one of the most widely used raw materials in research laboratories. Its use has been explored for over a century. In 1949, a paper containing barriers made of paraffin was exploited to successfully demonstrate the elution of pigments within a channel based on the sample diffusion process.[4] In 2007, paper was reinvented by the Whitesides group as a globally affordable substrate material for the development of miniaturized analytical platforms.[5] Since this period, paper has become an increasingly popular platform for multipurpose applications. Probably, its broad use is associated with advantages over other conventional substrates, as well as the fabrication technologies and the concept of "do-it-yourself microfluidics".[6] In comparison with other conventional materials, like glass and silicon, paper is relatively inexpensive, globally affordable, lightweight, bioactive, and easy to transport and store. Furthermore, paper-based products can be easily found as kitchen towels, coffee filters, blood separation paper, filter paper, office paper, and others.

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How does one create an analytical device on paper? This question is a common inquiry of undergraduate and graduate students when starting to study microfluidics. Initially, it is important to emphasize that paper substrates have a porous structure, which facilitates the spontaneous transport of fluid by capillarity. The wicking speed of liquid on a microchannel defined on paper depends on pore size and paper thickness. Microfluidic networks can be created on paper using hydrophobic barriers or defined by cutting approaches, which make it possible to obtain single paper strips or more complex designs containing interconnected microchannels for multiplexed assays.[3] In this regard, lithography-based fabrication methods were first employed to demonstrate the potential of paper substrates for developing microfluidic structures. However, due to the contradictory view in terms of cost, many other alternative approaches were developed to make affordable and popular the concept and potential of paper-based microfluidics. Thanks to the researchers´ creativity and paper versatility, the fabrication of microfluidic paper-based analytical devices is feasible through direct printing using wax, inkjet, or laser printing processes or even by manual protocols (freehand drawing or spraying) involving pens, pencils, stamps, scissors, scholar's glue, or lacquer resins.

Paper-based microfluidic devices, including examples of simple spot test arrays, chemosensors, biosensors, electrochemical sensors, wearable devices, and lateral flow assays, have been found in the main scientific Journals associated with analytical and bioanalytical chemistry.[7-10] In the academy, most of the advances seen in the recent literature have demonstrated improvements in terms of durability, shelf life, reproducibility, robustness, and analytical reliability, making paper-based microfluidic devices promising and emerging candidates to gain space in the market as alternatives to other materials. In this way, entrepreneurship and innovation deserve to be highlighted and emerge as the focus of many researchers interested in opening their businesses or company. The bridge between the academy and the productive sector depends on investment and engagement to overcome administrative and legal bureaucracies not only to open a company but also to maintain it in full operating mode.

The commercialization of microfluidic devices has been constantly growing. In the last three years, for example, many companies located in different countries have shipped over five hundred million units/year, clearly demonstrating the potential of microfluidic devices for different application areas including drug delivery, flow chemistry, analytical devices, pharmaceutical and life science, point-of-care diagnostics and clinical and veterinary settings.[11]

Considering the advantages of paper-based materials, what can we expect in the coming years? Commercially available products with sample-in-answer-out capabilities are highly desirable to be found more and more in the market. Due to the global affordability of paper as well as its attractive features to create microfluidic and sensor prototypes, it is possible to see a real niche full of possibilities for success. In this view, it is time to try our best and make commercially available paper-based products like wearable sensors or lateral flow devices to monitor clinically relevant compounds in different biological fluids like blood, urine, serum, sweat, saliva, and tears. This may be accelerated by spin-offs or startups independently or in partnership with well-established companies. In other words, it is time to innovate and transform an idea into a commercial product with a societal impact. The interface between rapid tests and immediate responses directly by the end user are highly desirable features in the market and risk analysis.

The SARS-CoV-2 worldwide outbreak is the most recent example that science can offer the possibility to obtain clinical diagnostics in a matter of minutes, allowing one to decide on the ideal treatment or, in this case, social isolation to prevent the virus transmission. Tens of self-diagnostics kits based on paper strips for SARS-CoV-2 are already commercially available for society in drug shops, hospitals, or healthcare clinics. Similar strategies may be seen shortly for Monkeypox or other global outbreaks.

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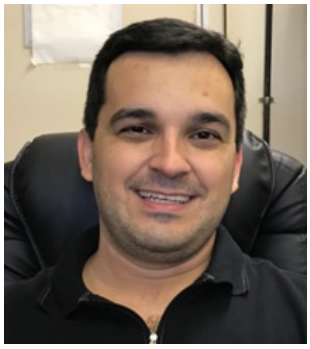

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LETTER

Chemometrics Reveals Not-so-Obvious Analytical Information

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The application of chemometric tools in analytical chemistry or other areas of chemistry has become essential. This is mainly due to the large amount and nature of the generated data[1,2] and the need to extract useful information from these and optimize steps throughout a process. It allows the quick decision-making visualization of interactions among variables, such as synergism or antagonism between parameters, during the development of a method,[3] as shown in Figure 1.

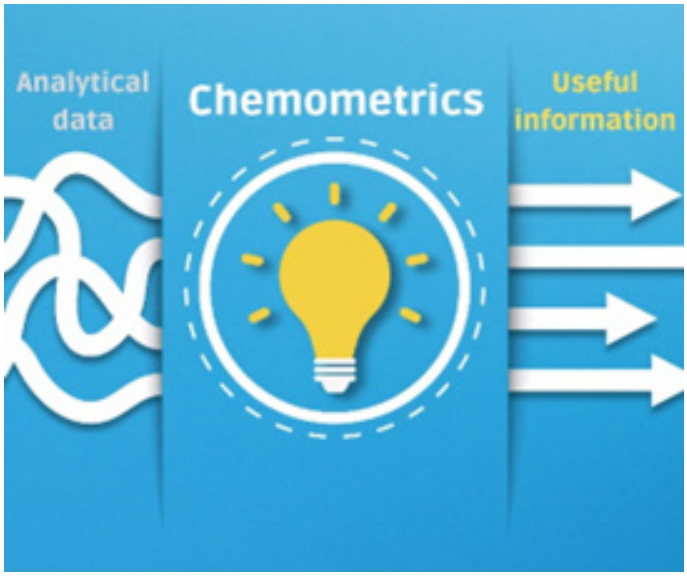

Figure 1. Pictorial of chemometrics directing analytical information.

Classical chemometric techniques have been disseminated and can be divided according to the study approach, among which exploratory data analysis stands out. Principal component analysis (PCA) is one of the most accessible and well-established ways to perform an initial exploration and extract relevant information from a given dataset and has been used quite successfully in various spectroscopic techniques.[4] Principal component analysis consists of projecting the data in a smaller dimension, enabling the detection of anomalous samples (outliers), the selection of essential variables in a given system, and unsupervised classification.[1,2,4]

Another branch of chemometrics involves the design of experiments (DoE). The primary purpose of the factorial design is to study the influence or effect of a given variable and its interactions in a specific system.[5-9]

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Multivariate calibration is another aspect of chemometrics, where several variables are used to calibrate one (or more) property or the concentration of a given chemical analyte.[10,11] Since the first publications of chemometric tools, numerous variations of these techniques, proposals for data fusion strategies, and applications using hyphenated instrumental techniques have been proposed.[12-14]

Industrial quality control and development (R&D) laboratories require an approach addressing adequate quality by design (QbD). The QbD strategies consider four steps that include an analytical target profile (ATP), a risk assessment, a design space (DS), and control strategy and validation based on figures of merit, for instance.[9]

Principal component analysis is the most widely multivariate technique used for data analysis. Jolliffe wrote a review reporting his wonderful experience with PCA in the last 50 years.[15] Indeed, PCA is an invaluable method for data, and I agree with it. PCA is the algorithm of choice for numerous chemometric techniques.[16]

Other computational languages, such as Python, are currently experiencing a rise in popularity in the field of chemistry. The R language has also become more popular than it was ten years ago. The scripts, functions, or codes are easily written with fewer lines and specific commands that minimize steps and help speed up calculations. The dissemination of free software has also become popular, and the sharing of codes through publications, social media, communities, or websites has become relatively easy.

From my point of view, chemometrics is no longer faced as a giant monster or a way to become scientific papers fancier without helpful content. Chemometrics extract information that is not easy to visualize at first through univariate evaluations or using simple plots. Nowadays, thousands of instrumental data can provide important chemical information, and we must use them for significant proposals. Indeed, QbD is proof of that, since Industry 4.0 is a reality.

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IN MEMORIAM

Carol Hollingworth Collins
1931/03/21 – 2022/09/18

BrJAC mourns the death of Prof. Dr. Carol Collins and recognizes her great contribution to the Analytical Chemistry in Brazil

Professor Carol Collins graduated in Chemistry from Bates College (1952) and obtained her PhD in Organic Physical Chemistry from Iowa State University of Science and Technology (1958), when she was introduced to the recently developed gas–liquid chromatography. She conducted postdoctoral research at the University of Wisconsin and later worked on radiochemistry and nuclear medicine at the Brookhaven National Laboratory and the Western New York Nuclear Research Center in Louvain (Belgium) and Southwest Asia.

Professor Collins came to the State University of Campinas (Unicamp) with her husband, Kenneth Collins, in July 1974, during the university's first decade, and played a leading role in consolidation of the Institute of Chemistry at Unicamp and in the growth of chemistry and analytical chemistry in Brazil. Her first line of research in Brazil was radioanalytical chemistry, later focusing on chromatographic techniques, initially applied to the products of radiochemical reactions and radiation chemistry. Subsequently, her attention was directed to the preparation of stationary phases for liquid chromatography. She gained remarkable achievement in the area of chromatography that allowed her to publish two books that are very popular in Brazil: "Introduction to Chromatographic Methods" (1987) and "Fundamentals of Chromatography" (2006).

Her scientific and technological contributions have been recognized through several awards, including the "Marie Curie Award" from the American Association of University Women and the "Simão Mathias Medal" from the Brazilian Chemical Society (SBQ). She also received honors in recognition of the contributions of Unicamp's 40th anniversary, SBQ's 30th anniversary, a tribute from the Journal of the Brazilian Chemical Society, the National Meeting of Analytical Chemistry, the School of Separations and the Brazilian Symposium on Chromatography and Related Techniques (SIMCRO) Medal.

For her outstanding performance and leadership in the creation and consolidation of the Analytical Chemistry Division of the Brazilian Chemical Society, her name was recognized in the Carol Collins Medal given to each National Meeting of Analytical Chemistry since the 2018 edition.

Professor Collins was also a full member of the Brazilian Academy of Sciences and the Academy of Sciences of São Paulo, and received the title of Professor Emerita of Unicamp on 14 May 2012, in addition to being Emeritus Researcher at the National Council for Scientific and Technological Development (CNPq).

Apart from her scientific competence, some characteristics shaped her personality and made her very popular among her colleagues and students: her vast gourmet knowledge, keen taste for caipirinha and coffee, infallible memory, great love for her work and for Brazil, dedication to science, analytical chemistry/chromatography, kindness as a person and her incessant search for justice. She was always receptive

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to clarifying the doubts of students, teachers and interested parties, which she did with great pleasure, characteristic of those who like to teach and transmit knowledge.

The contributions of Professor Collins to the training of human resources, the consolidation and development of the Institute of Chemistry at Unicamp and the analytical chemistry/chromatography sector in Brazil and abroad are immeasurable. Her brilliant trajectory will leave a huge legacy that is difficult to measure and she will remain forever in the memory of those who were fortunate enough to live with her.

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REVIEW

An Integrative Review on the Analysis of Pesticide Multiresidues in Sweet Pepper Samples using the QuEChERS Method and Chromatographic Techniques

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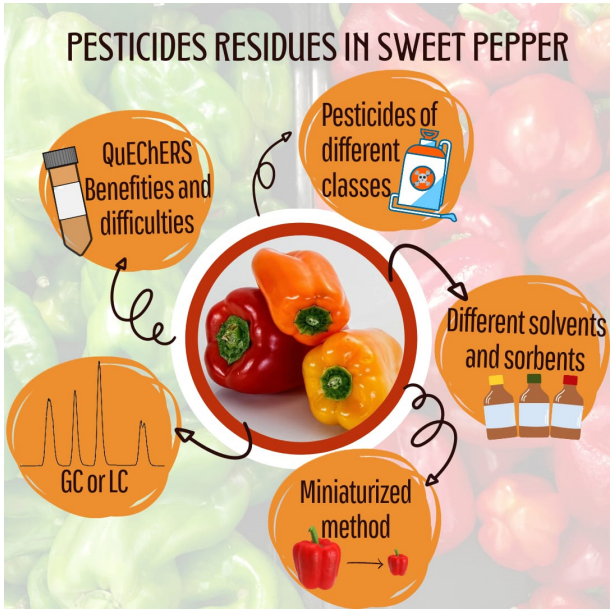

Since foods are complex matrices that contain pesticides of different classes, multiresidue sample preparation methods such as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) are used and modified to obtain more accurate and sensitive results. This work was developed through an integrative review in the journal databases Capes, Science Direct, Scielo, and Scientia Chromatographica, from 2011 to 2021, to answer the following question: "What is the most efficient and advantageous sample preparation method for the determination of multiresidue pesticides of interest in sweet pepper samples, when chromatographic techniques are used for detection?" The sweet pepper was chosen because the Pesticide Residue Analysis in Food Program (PARA) suggests that it is the sample with the highest percentage of irregularities related to active ingredients not allowed or above the Maximum Residue Limit (MRL). A total of 391 articles were found, 11 of which met the inclusion criteria established. Several analyses were studied. The organophosphates were the most studied class of pesticide, with seven articles. In addition, there was a predominance of nonpolar analytes (log K_{ow} > 1). The use of different extracting solvents, such as methanol, acetonitrile, ethyl acetate, and acetone, was observed, with acetonitrile presenting the best analytical parameters in most cases. The use of different sorbents such as secondary and primary secondary amine (PSA), ocatadecyllane (C18), graphite carbon (GCB), and carbon black was noted, as well. The authors highlight the difficulties in the analysis when the matrix effect is significant (except for fensulfothion, tensulfothion, flonicamid, and its metabolite TFNA-AM) and the degradation of analytes through the analysis process

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(flonicamid, captan, folpet, thiophanate methyl and benomyl). Finally, the statistical test Analysis of Variance (ANOVA) was used to identify if there was a significant difference between the different methods used for the same analysis or when the same method used for the different analytes.

Keywords: pesticides, multi-residue, sweet pepper, sample preparation, QuEChERS.

INTRODUCTION

Sweet pepper (*Capsicum annuum*) belongs to the *Solanaceae* family. It is rich in vitamin C when green and in vitamin A when ripe, while being a source of iron (Fe), potassium (K) and phosphorus (P).[1] According to the Brazilian Institute of Geography and Statistics (IBGE) it is estimated that, in 2017, Brazil produced 224,286 tons of sweet pepper, with São Paulo being the main producing state.[2]

The cultivation of sweet peppers has economic and social relevance, since a large part of the national production comes from family farming, as it allows quick economic return in addition to complementing the diet. However, the number of surveys and data on the crop are limited, making access to information about it difficult for those who produce or are interested in sweet pepper production. In addition, few pesticides are authorized for the crop,[3] which can make pest control difficult.

Pesticides are understood to be "products and agents of physical, chemical or biological processes, [...] whose purpose is to change the composition of flora or fauna, in order to preserve them from the harmful action of living beings considered harmful".[4] In the context of agricultural production, the use of pesticides from different agronomic classes (herbicides, fungicides, insecticides, etc.) and different chemical groups (organophosphates, triazines, pyrethroids, etc.) is commonplace, aiming to increase productivity by minimizing the occurrence of plant pests and diseases. However, the indiscriminate use of these substances can generate negative external effects, both for the environment, contaminating soil and water, and for human health, which can cause acute or chronic intoxication and bioaccumulation of some substances, due to the ingestion of waste that may be present in these foods. This is why Maximum Residue Limits (MRL) have been established, corresponding to the maximum amount of pesticide residues that can be found in food without being a concern to human health.[5]

According to the National Health Surveillance Agency (ANVISA), until the first quarter of 2021, 504 pesticides are authorized in Brazil for 154 crops. 57 of these pesticides are authorized for the sweet pepper crop[6] and are listed in Table I, together with their MRL, chemical group, and the agronomic class they belong to.

Table I. Pesticides allowed for the sweet pepper crop[6]

Authorized pesticide	MRL (mg kg^{-1})	Chemical group	Agronomic class
Abamectin	0.040	Avermectins	Acaricide, insecticide, nematicide
Acetamiprid	0.700	Neonicotinoid	Insecticide
Alpha-cypermethrin	0.020	Pyrethroid	Insecticide
Azoxystrobin	0.500	Strobilurin	Fungicide
Bifenthrin	0.300	Pyrethroid	Insecticide, formicide and acaricide
Boscalida	0.500	Anilide	Fungicide
Buprofezin	0.500	Thiadiazinone	Fungicide
Kasugamycin	0.030	Antibiotic	Fungicide and bactericide

(continues on the next page)

Table I. Pesticides allowed for the sweet pepper crop[6] (continuation)

Authorized pesticide	MRL (mg kg^{-1})	Chemical group	Agronomic class
Cyantraniliprole	0.150	Anthranilamide	Insecticide
Cymoxanil	0.100	Acetamide	Fungicide
Cletodim	0.500	Cyclohexanedione Oxime	Herbicide
Clomazone	0.050	Isoxazolidinone	Herbicide
Chlorantraniliprole	0.300	Anthranilamide	Insecticide
Chlorfenapyr	0.300	Pyrazole Analogue	Insecticide and acaricide
Chlorothalonil	5.000	Isophthalonitrin	Fungicide
Kresozym-Methyl	0.050	Strobilurin	Fungicide
Deltamethrin	0.060	Pyrethroid	Insecticide and formicide
Diafenthiuron	3.000	Phenylthiumea	Acaricide and insecticide
Difenoconazole	0.500	Triazole	Fungicide
Dimethomorph	0.200	Morpholine	Fungicide
Epinephrine	0.500	Spinosines	Insecticide
Spiromesifen	0.700	Ketoenol	Insecticide and acaricide
Ethofenproxy	0.700	Diphenyl Ether	Insecticide
Phenamidone	0.200	Imidazolinone	Fungicide
Fenpyroximate	0.100	Pyrazole	Acaricide
Phenpropatrine	0.200	Pyrethroid	Insecticide and acaricide
Fluazinam	0.070	Phenylpyridinylamine	Fungicide and acaricide
Fluensulfone	0.200	Heterocyclic Fluoroalkenyl Sulfone	Nematicide
Fluopicolide	0.200	Benzamide Pyridine	Fungicide
Flupiradifurone	0.600	Butenolide	Insecticide
Flutriafol	0.200	Triazole	Fungicide
Fluxapyroxad	0.100	Carboxamide	Fungicide
Formatanate	2.000	Phenyl Methylcarbamate	Insecticide and acaricide
Imidacloprid	0.500	Neonicotinoid	Insecticide
Indoxacarb	0.050	Oxadiazine	Insecticide, termite and formicide
Iprodione	4.000	Dicarboximide	Fungicide
Iprovalicarb	0.050	Carbamate	Fungicide
Lambda-Cyhalothrin	0.200	Pyrethroid	Insecticide

(continues on the next page)

Table I. Pesticides allowed for the sweet pepper crop[6] (continuation)

Authorized pesticide	MRL (mg kg^{-1})	Chemical group	Agronomic class
Mancozeb	3.000	Alkylenebis (Dithiocarbamate)	Fungicide and acaricide
Metconazole	0.100	Triazole	Fungicide
Methiocarb	0.050	Phenyl Methylcarbamate	Insecticide
Put In	3.000	Alkylenebis (Dithiocarbamate)	Fungicide
Pyraclostrobin	1.000	Strobilurin	Fungicide
Piridaben	0.500	Pyridazinone	Acaricide and insecticide
Pyrimethanil	1.000	Anilinopyrimidine	Fungicide
Pyriproxyfen	0.500	Pyridyloxyprophilic Ether	Insecticide
Propamocarb	2.000	Carbamate	Fungicide
Propineb	3.000	Alkylenebis (Dithiocarbamate)	Fungicide
Tebuconazole	0.200	Triazole	Fungicide
Teflubenzuron	0.150	Benzoylurea	Insecticide
Thiabendazole	2.000	Benzimidazole	Fungicide
Thiacloprid	0.200	Neonicotinoid	Insecticide
Thiamethoxam	0.200	Neonicotinoid	Insecticide
Thiophanate-Methyl	0.100	Benzimidazole (Precursor Of)	Fungicide
Trifloxystrobin	0.100	Strobilurin	Fungicide
Trifluralin	0.050	Dinitroaniline	Herbicide
Zoxamide	0.100	Benzamide	Fungicide

Source: ANVISA, 2021.[6]

In order to control and ensure safer food, regulatory bodies have carried out analyses to detect pesticides in food since 1960.[7] In Brazil, this monitoring has been carried out since 2001 by the Pesticide Residue Analysis in Food Program (PARA), coordinated by ANVISA. The program aims to identify whether the number of residues detected is in accordance with the MRL prescribed by legislation and whether they are authorized for cultivation.[8]

PARA prioritizes the most consumed foods according to the Family Budget Survey (POF), which is carried out by the IBGE (the Brazilian Institute of Geography and Statistics), as well as foods that are likely to present risk, according to previous reports of the program. Since PARAs was established, sweet pepper was the sample with the highest percentage of irregularities in the different sampling cycles regarding the presence of pesticide residues above the MRL established by the legislation and of pesticides not allowed for the crop.[8,9] The average percentage of irregularities during each sampling cycle is represented in Figure 1.

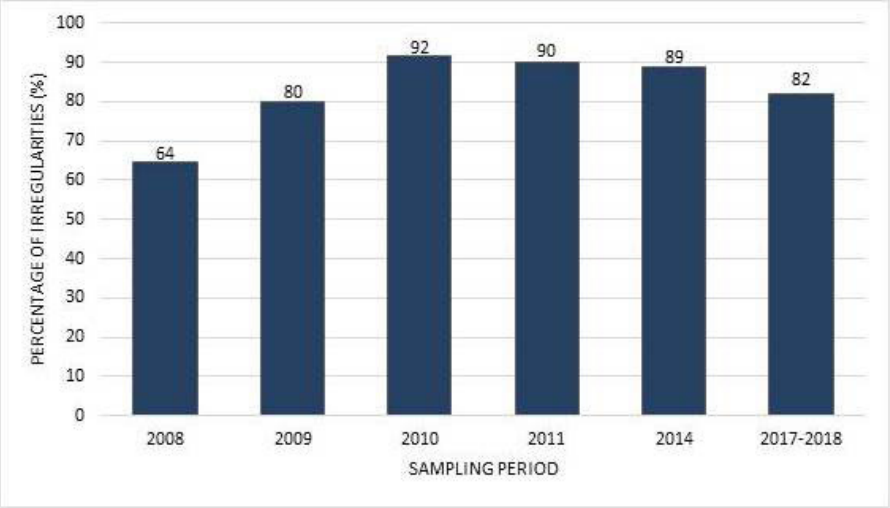

Figure 1. Mean percentage of irregularities in sweet pepper samples, according to PRAFP/ ANVISA. (Adapted from ANVISA 2009, 2010, 2011, 2013, 2016, 2019.[10-15])

Figure 1 shows that from 2008 to 2010 the number of irregularities has been growing, reaching 92% in 2010 (of a total of 146 analyzed samples). From then on, a decrease in irregularities has been observed, year after year. However, the percentage of irregularities in the last biannual sampling cycle (2017-2018) is still quite high, reaching 82% of a total of 326 analyzed samples.

In addition, in Paraná, through the SESA Resolution Nº. 217/2011, the State Pesticide Residue Analysis in Food Program PARA/PR was established, coordinated by the Division of Food Sanitary Surveillance of the State Center for Sanitary Surveillance and by the Central Public Health Laboratory of Paraná (Lacen/ PR).[16] In the state, samples are collected at Supply Centers (CEASA) units and schools in the state network. They are selected according to consumption data from the POF carried out by IBGE for the population of Paraná and the historical records of PARA residues maintained by ANVISA.[17]

In 2020, the first report from PARA/PR was released, in which twenty samples of sweet peppers collected at CEASA units were analyzed, with a total of 70% of unsatisfactory samples, including 17 pesticides not allowed for the crop and 6 others above the MRL. On the other hand, a sample collected from school meals showed no irregularities.[17]

The determination of pesticide residues in food is relevant in estimating human exposure to these compounds, due to the adverse effects that these substances can have. Since foods are complex matrices, samples must go through a previous preparation stage to extract and concentrate their analytes with subsequent sample clean-up. In this stage, it is common to use multiresidue methods, capable of simultaneously extracting large amounts of pesticides, since foods generally contain residues of different types of pesticides.[18]

Therefore, in order to comply with the strict MRLs prescribed by legislation and overcome the limitations of current methods, generating extracts that can be analyzed by Liquid Chromatography and Gas Chromatography-Tandem Mass Spectrometry (LC-MS/MS and GC-MS/MS), the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe), a multiresidue method was developed in 2003 by Anastassiades *et al.*[19] The main steps of the method are presented in Figure 2.

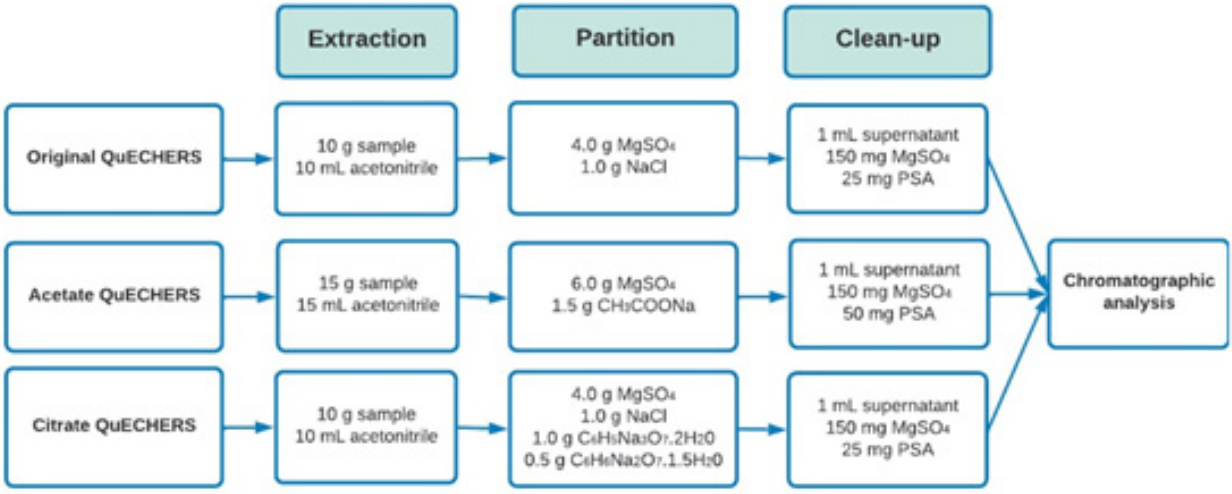

Figure 2. Original, Acetate and Citrate QuEChERS methods steps. [Adapted with permission from: (39) Zanella, R.; Prestes, O. D.; Adaime, M. B.; Martins, M. L. QuEChERS (Chapter 24). In: Borges, K. B.; Figueiredo, E. C.; Queiroz, M. E. C. *Preparo de amostras para análise de compostos orgânicos*. LTC, **2015**. License granted by LTC Publisher, GEN Group, on May 25, 2022.]

The original QuEChERS method consists of extracting the residue with acetonitrile, partitioning it through the addition of magnesium sulfate and sodium chloride, and clean-up using dispersive solid phase extraction (d-SPE).[19] Several modifications were studied in order to improve the analytical parameters, also increasing the percentage of recovery and the scope of analytes and samples. Two of these modifications stand out: acetate QuEChERS, where the medium is buffered at pH 4.8 after the socdium acetate is added, and citrate QuEChERS, which uses a mixture of sodium citrate dihydrate and hydrogen citrate sesquihydrate with a buffering effect at a pH from 5.0 to 5.5.[20]

In addition, different solvents can be used in the extraction step and different compounds can be used as sorbents in the clean-up step. The choice depends on the characteristics of the analyte and on the sample of interest. For example, methanol can be used as a solvent in the determination of polar analytes, while acetonitrile is used in the recovery of analytes with different polarities.[18] Regarding sorbents, the use of primary secondary amine (PSA) removes polar compounds, including pigments, sugars and fats, in addition to being indicated for the determination of nonpolar organophosphate pesticides.[21] In turn, graphite carbon black (GCB) removes pigments such as chlorophyll, while octadecyl (C18) removes nonpolar interference from the matrix and is indicated for a determination of polar analytes.[22]

Considering the above, correlating the characteristics of the analytes studied here with the analytical parameters, efficiency, and toxicity of the reagents used in the original QuEChERS method and its modifications would be extremely relevant for further research, as it would allow investigators to select the most appropriate methodology and/or experimental conditions for their studies, enabling them to identify which is the most efficient and/or advantageous sample preparation method to determine the pesticide multiresidues of interest in sweet pepper samples, using chromatographic techniques for detection.

METHODOLOGY

The integrative review is divided into six stages. The first step is the definition of the guiding question. Therefore, we determined that the question that should be answered was: “What is the most efficient and/or advantageous sample preparation method for the determination of multiresidue pesticides of interest in sweet pepper samples when chromatographic techniques are used for detection?”

In the second stage, the period from 2011 to 2021 was defined for the search of relevant data in the databases Scielo, Science Direct, Portal de Periódicos Capes, and Scientia Chromatographica, with

the following keywords: “QuEChERS and multiresidues” associated with “bell pepper, sweet pepper and *capsicum annuum,* and *pimentão*” (the latter being the Portuguese word for “sweet pepper”). At this stage, inclusion criteria were also defined:

i) Full text in Portuguese or English;
ii) Presence in the title or abstract of the terms “QuEChERS or multiresidues” associated with “bell pepper, sweet pepper, *pimentão*, or *capsicum annuum*”;
iii) Articles must have used chromatographic techniques for detection;
iv) Articles must have presented an analytical validation for the sweet pepper sample.

Articles that did not meet one or more inclusion criteria were excluded. Review articles and duplicates were also excluded.

In the third step, we verified whether the articles met inclusion criterion *i*. Then, after reading the title and abstract, we included articles that met inclusion criteria *ii* and *iii*. The articles included in this stage were listed in a data collection table, including their titles, authors, years of publication, objectives, samples, analytes, and sample preparations used.

In the fourth step, duplicates were removed and the articles left were read in their entirety, to verify whether they met all inclusion criteria, especially the analytical validation for the sweet pepper sample (criterion *iv*). Then, a critical analysis of the articles included was carried out, showing similarities between the documents, and listing, on a table, data from the analytical validation of the proposed methods, which were: limit of detection (LOD), limit of quantification (LOQ), percentage of recovery, standard deviation, linearity and whether the method had a matrix effect.

In the fifth stage, the discussion of the results was carried out, seeking to identify possible shortcomings in the methods as well as to identify the advantages and disadvantages of each proposed modification.

Finally, the sixth stage of the integrative review consisted of concluding the study, comparing the efficiency of each method, the analytical parameters, as well as other advantages and disadvantages of each method, considering the characteristics of their respective analytes. The statistical test used to verify if there was a significant difference between the percentages of recovery was the Analysis of Variance (ANOVA), with a confidence level of 95%. In this step, the recovery values obtained for the same analyte were compared, from different sample preparation methodologies (original QuEChERS and its modifications) and to verify whether the recovery values in a given method were significantly different in different analytes. In cases where a significant difference was observed in ANOVA, the test of least significant difference (LSD) was performed, with 95% confidence, according to the equation represented in Figure 3.[23]

$$LSD = t\sqrt{\frac{2\ X\ MSE}{N_g}}$$

Figure 3. Equation used to calculate LSD. (Source: Skoog, D. A.; Holler, F. H.; Crouch, S. R. *Fundamentos de química analítica*. Thompson, 2006.[23])

Where *MSE* corresponds to the mean squared error; *t* is the tabulated value with N-1 degrees of freedom and *Ng* is the number of replicas.[23]

Unfortunately, a statistical comparison for all analytes in the articles could not be carried out, and nor was it possible to compare all methods used for the same analyte, since the precision of the methods differs greatly depending on the analyte, and, consequently, the variance cannot be considered equal. Thus, only analytes that presented the smallest variances and could be considered statistically equal were compared.[23]

RESULTS AND DISCUSSION

Integrative reviews are the broadest form of review. It surveys current knowledge on a specific topic so the results of independent studies are identified, analyzed, and synthesized to contribute to the resolution of a specific question.[24] To start the integrative review protocol, the guiding question was determined, as mentioned in the previous section: "What is the most efficient and/or advantageous sample preparation method for the determination of multiresidue pesticides of interest in sweet pepper samples, when chromatographic techniques are used for detection?"

After the question was determined, the review protocol was developed in order to answer the guiding question. Sampling criteria must guarantee the representativeness of the sample as they are important indicators of the reliability of the results.[24] Figure 4 shows the number of articles selected and excluded in each stage.

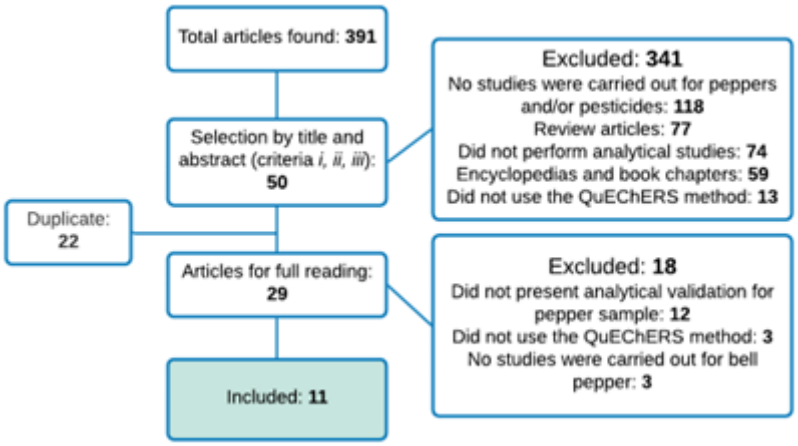

Figure 4. Diagram of identification and selection of integrative review articles. (Figure created by the authors.)

The selected articles were published in journals with an impact factor ranging from 0.68 to 7.51. Table II presents the QuEChERS method and the modifications used in the selected articles, the analysis technique, the samples, the number of analytes studied, the chemical group they belong to, and the impact factor of the journal in which they were published.

Table II. QuEChERS method and its modifications for the determination of pesticide multiresidues in sweet pepper samples

QuEChERS method	Analysis technique	Sample	Amount of Analytes	Chemical group	Impact factor
Original[25]*	GC-NPD	Sweet pepper	11	Organophosphate	0.68
Original without clean-up[22]*	UHPLC-Orbitrap-MS	Sweet pepper	5	Nicotinoid and organophosphate	3.06
Original without clean-up. Acetonitrile acidified with 1% formic acid. Optimization of grinding with addition of ascorbic acid.[26]*	SFC-MS/MS GC-MS/MS LC-MS/MS	Sweet pepper and tomato	2	Phthalimide and Dicarboximide	6.06

(continues on the next page)

Table II. QuEChERS method and its modifications for the determination of pesticide multiresidues in sweet pepper samples (continuation)

QuEChERS method	Analysis technique	Sample	Amount of Analytes	Chemical group	Impact factor
Original with different sorbents (PSA, PSA + C18 and GCB)[27]*	UHPLC-MS/MS	Sweet pepper	81	Aryloxyphenoxypropionic acid, anilide, anilinopyrimidine, anthranilamide, benzimidazole, benzofuranyl, carbamate, carboxamide, chloroacetamide, strobilurin, phenylamide, phenylurea, phosphorothiolate, imidazole, isoxazolidinone, methylcarbamate, morphine, neonicotinoid, organophosphate, pyrazole, pyrethroid, pyridine, thiadiazinone, thiocarbamate, triazole, triazolopyrimidamine, urea	3.37
Citrate with solvent variation (acetonitrile, acetone, ethyl acetate and methanol), sorbent (use and non-use of PSA graphited carbon) and use of dry ice in the partition step[18]*	LC-MS/MS	Sweet pepper	21	Benzimidazole, carbamate, strobilurin, isoxazolidinone, neonicotinoid, organophosphate	7.51
Miniaturized citrate 3 g sample + 1.3 g $MgSO_4$ + 0.33 g NaCl + 0.16 sodium citrate sesquihydrate + 0.33 sodium citrate → 1 min shake → 5 min centrifugation. 1.5 mL supernatant + 50 mg PSA + 15 mg ENVI-Carb + 300 mg $MgSO_4$ → 30 s stirring → 5 min centrifugation[28]*	GC-QqQMS	Sweet pepper, tomato, cucumber and lettuce	88	Substituted benzene, cyclodiene, bridged diphenyl, phosphorothiolate, chlorinated hydrocarbon, organochlorine, organophosphate	4.76
Citrate and acetate with varying proportion of sorbents ($MgSO_4$, PSA, C18 and GCB)[29]*	LC-MS/MS	Sweet pepper, rice, soy, apple, tangerine and cabbage	3	Diamide	n. i.
Acetate with d-SPE and DLLME[30]*	LC-MS/MS	Sweet pepper, lettuce, garlic and ginger	8	Carbamate, phenylamide, neonicotinoid, organophosphate, thiocarbamate	0.83
Acetate[31]*	LC-MS/MS	Sweet pepper and tomato	3	Benzamide and Triazole	4.22
Acetate[21]*	HPLC-MS/MS	Sweet pepper, banana and papaya	11	organophosphates	0.96

(continues on the next page)

Table II. QuEChERS method and its modifications for the determination of pesticide multiresidues in sweet pepper samples (continuation)

QuEChERS method	Analysis technique	Sample	Amount of Analytes	Chemical group	Impact factor
Acetate[32]*	LC-MS/MS HPLC-PDA	Sweet pepper	2	Diamide	1.91

*Superscript numbers refer to the article reference. n. i. = not informed; GC-NPD: Gas Chromatography with Nitrogen-phosphorus Detector; UHPLC-Orbitrap-MS: Ultra High Performance Liquid Chromatographyhigh coupled to Orbitrap Mass Spectrometry; SFC-MS/MS: Supercritical Fluid Chromatography–Tandem Mass Spectrometry, Gas Chromatography–Tandem Mass Spectrometry; GC-MS/MS: Gas Chromatography–Tandem Mass Spectrometry; LC-MS/MS: Liquid Chromatography–Tandem Mass Spectrometry; UHPLC-MS/MS: Ultra High Performance Liquid Chromatography–; GC-QqQMS: Comprehensive two-dimensional Gas Chromatography with flow Modulation–Triple quadrupole Mass Spectrometry; HPLC-MS/MS: High-Performance Liquid Chromatography–Tandem Mass Spectrometry; HPLC-PDA: High-Performance Liquid Chromatography–Photo Diode Array.

The presence of analytes of different classes was noted in the articles, with organophosphates being the most observed, present in seven articles.[18,21,22,25,27,28,30] There was a predominance of nonpolar analytes (154 analytes with log k_{ow} > 1), when compared to polar analytes (22 analytes with log k_{ow} < 1), in addition to 51 analytes whose polarity was not specified.

Table II shows the predominance of acetate QuEChERS over citrate and original QuEChERS, as well as the predominance of original QuEChERS over citrate QuEChERS. In the 11 selected articles there have been different modifications aimed at minimizing the degradation of compounds,[26] minimizing the matrix effect and increasing extraction efficiency by combining different solvents and salts in different proportions,[29] as well the use of reduced samples, solvents and sorbents, in order to propose methods that are in accordance with green chemistry.[28]

The complete list of analytes studied in the selected articles (Table II), the class they belong to, their polarity, and recovery at the lowest concentration studied by the authors can be found in the supplementary material.

Original QuEChERS method

Four of the eleven articles selected used the original QuEChERS method,[22,25-27] to a total of ninety-nine analytes of different classes with different polarities. The ANOVA statistical treatment could only be performed for the articles of López-Ruiz *et al.* and Kemmerich *et al.*;[22, 27] the others did not present the exact standard deviation values. Table III shows parameters such as the percentage of recovery and log K_{ow} values, which can be related to polarity, and the higher the log K_{ow} value, the more hydrophobic the compound. The same parameters for all analytes studied by the authors selected in this work are found in the supplementary materials.

Table III. Analytical parameters for the analytes studied using the acetate QuEChERS method, except those whose variances were not small enough to be considered equal

QuEChERS	Analysis technique	Concentration µg kg^{-1}	Analytes	log k_{ow}[33]	Recovery (%)
Original without clean-up[22]	UHPLC-Orbitrap-MS	10	Flonicamid[a]	-0.24	88 ± 11
			TFNG[a]		89 ± 10
			TFNA[a]		NR
			TFNA-AM[a]		NR
		100	Flonicamid[a]	-0.24	91 ± 7
			TFNG[a]		84 ± 9
			TFNA[a]		88 ± 6
			TFNA-AM[a]		87 ± 9

(continues on the next page)

Table III. Analytical parameters for the analytes studied using the acetate QuEChERS method, except those whose variances were not small enough to be considered equal (continuation)

QuEChERS	Analysis technique	Concentration µg kg^{-1}	Analytes	log k_{ow}[33]	Recovery (%)
Original with variation of sorbents[27]	UHPLC-MS/MS	10	Thiacloprid[b]	1.26	85 ± 4
			Imazalil[a]	2.56	74 ± 4
			Pyrimethanil[c]	2.84	88 ± 4
			Methomyl[b]	0.09	82 ± 4
			Pyrifenoxa[a]	3.40	75 ± 5
			Thiabendazole[c]	2.39	90 ± 5
		50	Atrazine[a]	2.70	84 ± 3
			Pyrimethanil[a]	2.84	84 ± 3
			Pencicuron[b]	4.68	102 ± 3
		100	Thiabendazole[b]	2.39	78 ± 3
			Mephospholane[e]	1.04	90 ± 3
			Metobromuron[d]	2.48	85 ± 3
			Metalaxyl[c]	1.71	83 ± 3
			Ametrine[d]	2.36	85 ± 3
			Azaconazole[f]	2.36	99 ± 3
			Phenpropimorph[c]	4.50	81 ± 3
			Pyridafenthion[a]	3.20	73 ± 3
			Profenophos[d]	1.70	86 ± 3
			Atrazine[c]	2.70	80 ± 4
			Azoxystrobin[c]	2.50	82 ± 4
			Mevinphos[f]	0.12	96 ± 4
			Simazine[f]	2.10	96 ± 4

NR = not recovered. Superscript letters were used to indicate a significant difference.

As observed in Table III, from the original QuEChERS without clean-up, proposed by López-Ruiz *et al.*,[22] flonicamid and its metabolites showed recovery percentages between 87 and 91%, with no significant difference at 95% confidence. The method optimized by the authors is based on acidified QuEChERS, using acetonitrile containing 1% formic acid and a mixture of salts (magnesium sulfate and sodium chloride). Such results obtained by the authors, presented in Table III, refer to the optimized method, which added to the process a stirring with a PT2100 *polytron* (Kinematica AG, Littan/Luzern, Switzerland) and excluded the clean-up step with sorbents. The clean-up stage, using PSA and a mixture of PSA and graphited carbon, resulted in a decrease in the recovery percentage, with the use of 50 mg of PSA, the recoveries ranged from 65% for TFNG to 85% for flonicamid (polar), while the mixture of 50 mg of GCB with 50 mg of PSA provided recoveries of 60% for TFNA-AM and 80% for the flonicamid. The authors justified this by the fact that the analytes are retained in the binding sites of the sorbents, being an unnecessary step in matrices with high water content. The method studied was shown to have good sensitivity, with LOQ values lower than the MRL, for sweet pepper samples.[22] The use of sorbents (PSA, C18 and GCB) was also associated with a decrease in sorption percentage for two broflanilide metabolites, S (PFOH)-8007 (using PSA, C18 and GCB as sorbent) and DM-8007 (using GCB as sorbent).[29] However, the authors obtained excellent extraction of pigments from the samples, using a mixture of sorbents ($MgSO_4$, PSA and GCB).

Examples of other works that used PSA and/or GCB as sorbents are the studies of Lemos *et al.*[21] and Figueiredo *et al*.[25] for nonpolar organophosphates and mevinphos and methamidophos (polar organophosphates), Morais, Collins and Jardim for analytes with different polarities,[18] and Figueiredo *et al*. for nonpolar analytes and for methamidophos.[25] Matadha *et al.* used a mixture of PSA and C18 was as sorbent in studies with apolar analytes. However, the authors did not perform comparative studies without the use of sorbents. And so, it is not possible to relate the results obtained with the use of sorbents.[31]

On the other hand, the use of original QuEChERS with varying sorbents, using UHPLC-MS/MS,[27] in studies with several classes of pesticides and original QuEChERS, using GC-NPD in studies with organophosphates,[25] obtained satisfactory results using the clean-up step with PSA. And of eleven analytes studied by Figueiredo *et al*.,[25] the use of sorbent was not efficient only for pirimiphos, obtaining a recovery of only 60%.

The use of original QuEChERS with varying sorbents by Kemmerich *et al.*[27] was validated for seventy-nine of the eighty-one analytes studied by the authors. The method could not be validated for two analytes of the benzimidazole class, benomyl (log K_{ow} = 1.4) and thiophanate methyl (log K_{ow} = 1.4), because they showed low percentages of recovery and determination coefficient lower than 0.99. The low percentages of recovery were associed whith degrading the analytes in carbendazin. Thus, benomyl and thiphanate methyl are calculated as carbendazim.

Among the analytes presented in Table III, for the initial concentration of 10 µg kg^{-1}, the method QuEChERS original with variation of sorbents presented by Kemmerich *et al*.[27] showed lower recoveries for imazalil (log k_{ow} = 2.56) and pyrifenox (log k_{ow} = 3.40), and greater recovery for thiabendazole (log k_{ow} = 2.39), in relation to the other analytes. For the concentration of 50 µg kg^{-1}, pencycuron (log k_{ow} = 4.68), a very nonpolar phenylurea, showed a higher percentage of recovery (102%), and for the initial concentration of 100 µg kg^{-1}, the lowest recovery was observed for pyridafenthione (log k_{ow} = 3.20), and the highest for azaconazole (log k_{ow} = 2.36), a nonpolar triazole.

In general, there was a significant difference between most analytes in study, but unfortunately it was not possible to establish a relationship between the recovery percentages and the log K_{ow}. The method showed higher LOD and LOQ for analytes with log K_{ow} greater than 2.9.

Furthermore, in the four articles that used original QuEChERS with and without modifications, a pronounced matrix effect was observed for the analytes, with the exception of captan, folpet,[26] and flonicamid and its metabolite TFNA-AM.[22] Another problem reported was the degradation of analytes such as captan (log K_{ow} = 2.5) and folpet (log K_{ow} = 3.02), which can be minimized using techniques and proper conditions. Supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS) is a good alternative for the determination of folpet and captan, which tend to degrade during injection by GC-MS/MS and LC-MS/MS.[26] The addition of ascorbic acid (3%) and dry ice in the milling step, allowed recoveries between 94 and 99%, respectively. Without the use of dry ice, captan and folpet could not be detected even at the highest concentrations. The use of ascorbic acid and dry ice inhibits hydrolysis and oxidation, common degradation processes during milling. These processes may occur due to the presence of pectinase enzymes, which cause the hydrolysis of pectin, present in peppers, being a matrix-dependent process.[26]

It was also observed that the use of sodium chloride (NaCl), in the partition step, had a positive influence on the recovery of polar compounds, such as acetamiprid (log K_{ow} = 0.8) and chlorotianidin (log K_{ow} = 0.905), since the NaCl added to the system makes the phase separation more complete. By varying the amount of NaCl added to the extract, the polarity range (selectivity) and the degree of cleanliness in the partition stage can be controlled.[19] In addition, the use of acetonitrile proved to be efficient for providing the recovery of analytes with different polarities, in addition to reducing the number of lipophilic co-extractives and matrix pigments.[27]

Citrate QuEChERS method

The use of QuEChERS citrate was observed in articles by Morais, Collins and Jardim,[18] Ferracane *et al*.[28] and Noh *et al*.,[29] which together totaled one hundred and four analytes of different classes and polarities. Table IV presents log K_{ow} and recovery percentages for some analytes studied by the authors

— the complete list can be found in the supplementary materials. For the articles by Morais, Collins and Jardim[18] and Noh *et al*.[29] it was not possible to perform statistical treatments because they did not present numerical standard deviation values, only estimates, based on graphs.

Table IV. Analytical parameters for the analytes studied using the acetate QuEChERS method, except those whose variances were not small enough to be considered equal

QuEChERS	Analysis technique	Concentration (μg kg^{-1})	Analytes	Log K_{ow} [33]	Recovery (%)
Miniaturized citrate[28]	GC-QqQMS	10	4.4-DDD[a]		63 ± 2
			Chlorfenson[a]	4.21	66 ± 2
			Fention[b]	4.84	70 ± 2
			Methyl tolclofos[b]		71 ± 3
			Pentachlorothianisol[b]		78 ± 2
			Sulfotep[c]	3.99	108 ± 2
			4,4-Dichlorobenzophenone[d]		109 ± 2
			Terbufos[e]	4.51	117 ± 2
		50	Trans-chlordane[a]	2.78	88 ± 2
			Ethion[b]	5.07	93 ± 2
			Chlorbenside[b]	5.59	95 ± 2
			Methoxychlorine[b]		95 ± 4
			2,4-DDE [b]		96 ± 2
			Pyrimiphos-methyl[c]	4.20	99 ± 2
			4,4-DDD[c]		99 ± 2
			Tolclofos-methyl[c]	3.80	101 ± 2
			Endrin[c]	3.20	101 ± 2
			Bromophos-ethyl[d]	6.15	106 ± 2
Miniaturized citrate[28]	GC-QqQMS	100	Piperonyl[a]	4.51	95 ± 2
			4,4 methoxychloro[a] olefina[a]		96 ± 2
			Terbufos[a]	4.51	96 ± 2
			Dieldrin[a]	3.70	96 ± 2
			Hexachlorobenzene[a,b]	3.93	97 ± 2
			Sulfotep[a,b]	3.99	98 ± 2
			Chlorbenside[b]	5.59	100 ± 2
			Pentachlorobenzene[b,c]		101 ± 2
			Pirimiphos-methyl[b,c]	4.20	102 ± 2
			Chlorpyrifos[c,d]	4.70	104 ± 2
			Paration[d]		105 ± 2
			Quinalphos[d]	4.44	107 ± 2

Superscript letters were used to indicate a significant difference.

As seen in Table IV, there is a significant difference between most analytes studied, demonstrating that despite being a multi-residue method, its efficiency varies according with the analyte. At the initial concentration of 10 μg kg^{-1} the recoveries obtained for the 4,4-DDD (63 ± 2% and log K_{ow} = 6.02) and chlorfenson (66 ± 2% and log K_{ow} = 4.21) are significantly lower than those obtained for the other analytes, while terbufos (log K_{ow} = 4.61) recovery was the highest (117 ± 2%), even though both are quite nonpolar analytes.

For the concentration of 50 μg kg^{-1}, among the ten analytes compared, a significantly lower recovery was observed for trans-chlorinated (88 ± 2%), which is a nonpolar organochlorine (log K_{ow} = 2.78), and the highest recovery was obtained for bromophosethyl (106 ± 2%), which is a very nonpolar organophosphate (log K_{ow} = 6.15).

Among the twelve analytes presented in Table IV, for the concentration of 100 μg kg^{-1}, the percentage of recovery for piperonyl (95 ± 2), 4,4'-methoxychlor-olefin (96 ± 2), terbufos (96 ± 2 and log K_{ow} = 4.51), dieldrin (96 ± 2 and log K_{ow} = 3.70), and hexachlorobenzene (97 ± 2 and log K_{ow} = 3.93) are statistically equivalent.

Observing the recoveries obtained for the eighty-eight analytes investigated from the use of miniaturized QuEChERS citrate, it can be noted that the method was efficient in most cases, with recoveries greater than 70%. Furthermore, it was observed that the smallest variances and, consequently, the greatest precision, were obtained for the most nonpolar analytes.

The matrix effect was pronounced for all analytes investigated in the articles by Morais, Collins and Jardim, Ferracane *et al.* and Noh *et al*.,[18,28,29] being more pronounced for the more polar analytes (log K_{ow} < 1), such as methamidophos, acephate, thiamethoxam, methomyl, and imidacloprid.[18] Alternatively, the matrix effect can be minimized by preparing the analytical curve in the sample matrix,[21] by dilution and purification, which are basic methods for the removal of impurities and, consequently, tend to minimize the matrix effect.[34] However, Noh *et al.* it was observed that when the matrix effect is not high, there is no significant improvement when diluting or purifying the sample.[29] In a comparative study between citrate QuEChERS and acetate QuEChERS, it was observed that the lowest matrix effect was found performing the extraction by the citrate buffer method, followed by purification with d-SPE, using 25 mg of PSA.[29]

Furthermore, different sorbents were evaluated in citrate QuEChERS, such as the use and non-use of GCB, which did not present a significant difference in the chromatographic responses for most of the analytes investigated. Positively, the use of graphite carbon helped to clean the samples, reducing the matrix effect. It was also observed that the use of methanol solubilizes the clean-up salts and results in extracts with a cloudy appearance. In addition, ethyl acetate, as it is less polar than acetonitrile and acetone, tends to cause a significant decrease (<70%) in the recovery of polar compounds, such as acephate (log K_{ow} = -0.85) and methamidophos (log K_{ow} = -0.79). On the other hand, acetone caused increased recovery of methamidophos and acephate, but a decrease in recoveries of other analytes. Acetone also resulted in greener extracts, due to the extraction of a greater number of pigments, which causes a greater matrix effect, compared to acetonitrile or methanol.[18]

Finally, it can be said that the miniaturized citrate QuEChERS method is the most eco-friendly and cheapest method proposed. It enabled the validation of sixty-eight analytes out of the eighty-eight studied, with recoveries ranging between 70 and 120%. However, it did not allow the detection of thirteen analytes, namely: endrin aldehyde, azinphos-methyl, bromfenvinphos, bromfenvinphos-methyl, edifenphos, ethylene, endrin ketone, phosalone, fenamiphos, leptophos, pyraclophos, prothiophos, profenophos. The lack of detection for these compounds may be associated with the use of GCB, which causes elution problems for aromatic pesticides, such as leptophos (log K_{ow} = 6.31), which bind strongly during the clean-up step. However, this can be overcome by adding a larger amount of solvents, such as toluene or acetonitrile. In addition, the method achieved low recoveries for: triazophos (60 ± 4% and log K_{ow} = 3.55), 4.4-DDD (63 ± 2%), chlorfenson (66 ± 2% and log K_{ow} = 4.21) and chloroneb (56 ± 5% and log K_{ow} = 3.58).[28]

Acetate QuEChERS Method

The use of acetate QuEChERS was observed in five articles,[21,29,30-32] totaling twenty analytes of different classes. The use of acetate QuEChERS without modifications was observed for the articles by Lemos *et al.*, Matadha *et al.* and Buddidathi *et al.*[21,31,32] Noh *et al.* included the use of different sorbents (PSA, C18, GCB),[29] and Lawal and Low the use of additional steps in d-SPE and dispersive liquid-liquid microextraction (DLLME).[30]

Table V presents analytical parameters such as log K_{ow} and percentages of recovery for the analytes that could be compared using ANOVA. For the article of Noh *et al*.[29] it was not possible to perform the statistical treatment because it did not present standard deviation values.

Table V. Analytical parameters for the analytes studied using the acetate QuEChERS method, except those whose variances were not small enough to be considered equal

QuEChERS	Analysis technique	Concentration	Analytes	Log K_{ow}[33]	Recovery (%)
Acetate with *d-SPE* and DLLME[30]	LC-MS/MS	5 μg kg^{-1}	Thiobencarb[a]	4.23	91 ± 3
			Baycard[b]		102 ± 2
		100 μg kg^{-1}	Diazinon[a]	3.69	99 ± 3
			Thiamethoxam[a]	-0.13	99 ± 3
			Baycard[a]		100 ± 4
			Thiobencarb[a]	4.23	101 ± 3
		500 μg kg^{-1}	Thiobencarb[a]	4.23	98 ± 3
			Diazinon[a]	3.69	99 ± 4
			Propamocarb[a]	0.84	100 ± 3
			Baycard[a]		100 ± 4
Acetate[31]	LC-MS/MS	0.0005 mg kg^{-1}	Tebuconazole[a]	3.70	78 ± 6
			Fluopyram[a]	3.30	80 ± 7
			Fluopyram[a] benzamide[a]		84 ± 8
		0.01 mg kg^{-1}	Tebuconazole[a]	3.70	80 ± 6
			Fluopyram[a]	3.30	83 ± 7
			benzamed[a] Fluopyram[a]		85 ± 6
		0.025 mg kg^{-1}	Tebuconazole[a]	3.70	81 ± 5
			Fluopyram[a]	3.30	84 ± 5
			benzamine[a] Fluopyram[a]		88 ± 5
		0.05 mg kg^{-1}	Tebuconazole[a]	3.70	86 ± 4
			Fluopyram[a]	3.30	88 ± 4
			Fluopyram[a] benzamide[a]		91 ± 5
		0.1 mg kg^{-1}	Fluopyram[a] benzamide[a]		92 ± 4
			Fluopyram[a]	3.30	92 ± 3
Acetate[21]	HPLC-MS/MS	0.00625 mg kg^{-1}	Fensulfotion[a]	2.23	94 ± 7
			Mevinphos[a]	0.127	98 ± 7
			Diazinon[a,b]	3.69	102 ± 8
			Coumaphos[b]		106 ± 9

(continues on the next page)

Table V. Analytical parameters for the analytes studied using the acetate QuEChERS method, except those whose variances were not small enough to be considered equal (continuation)

QuEChERS	Analysis technique	Concentration	Analytes	Log K_{ow} [33]	Recovery (%)
Acetate[21]	HPLC-MS/MS	0.125 mg kg^{-1}	Fenthion[a]	4.84	84 ± 2
			Diazinon[b]	3.69	94 ± 3
		0.1 mg kg^{-1}	Azinphos-methyl[a]	2.96	99 ± 2
			Dichlorvos[a]	1.90	100 ± 2
			Fenthion[b]	4.84	103 ± 2
Acetate[32]	LC-MS/MS HPLC-PDA	0.05 mg kg^{-1}	Flubendiamide[a]	4.14	96 ± 8
			de-iodine Flubendiamide[a]		97 ± 6
		0.1 mg kg^{-1}	Flubendiamide[a]	4.14	98 ± 6
			de-iodine Flubendiamide[a]		100 ± 6
		1.00 mg kg^{-1}	Flubendiamide[a]	4.14	100 ± 4
			de-iodine Flubendiamide[a]		104 ± 5

Superscript letters were used to indicate a significant difference.

The use of acetate QuEChERS with d-SPE and DLLME by Lawal and Low[30] showed a significant difference between thiobencarb (log K_{ow} = 4.23) and baycarb at the lowest concentration studied, 5 µg kg^{-1}. Furthermore, the quantification of polar and nonpolar analytes did not show differences in the percentage of recovery.

On the other hand, the use of QuEChERS acetate by Matadha *et al*.[31] for the determination of tebuconazole (log K_{ow} = 3.70), fluopyram (log K_{ow} = 3.30) and fluopyram benzamide (both nonpolar analytes)[31] showed no significant difference, with 95% confidence, in any of the concentrations studied by the authors.

Furthermore, in acetate QuEChERS proposed for Lemos *et al*.[21] it was observed that the method presented recoveries in the range from 79 ± 4% (mevinphos at 0.1 mg kg^{-1}) to 112 ± 12% (azinphos-methyl at 0.00625 mg kg^{-1}), data available in the supplementary material. Therefore, it was noted that the only polar analyte analyzed by the author showed the lowest recovery; however, this fact was not observed for the other concentrations, and it cannot be said that the method is less efficient for polar analytes.

In the next article by Buddidathi *et al.*[32] which also used QuEChERS acetate, the determination of flubendiamide and its de-iodine metabolite flubendiamide was studied. Flubendiamide is a nonpolar diamide (log K_{ow} = 4.14) that tends to degrade in the field, forming de-iodine flubendiamide, with a half-life of 4.3 to 4.6 days in sweet pepper fruits produced in the open field, and from 5.7 to 6.6 days in sweet peppers produced in greenhouses. Thus, the detection of flubendiamide takes into account the presence of its metabolite to express the actual concentration of the substance. Both analytes studied by the authors showed no significant difference in their recovery percentages.

Furthermore, the matrix effect was pronounced for most analytes studied by the authors, with the exception of fensulfothion,[21] which showed neither suppression nor decrease in the chromatographic signal.

Comparison between the different methods used for the same analytes

The comparison of analyses of the same analyte using different QuEChERS sample preparation methods and the same range of enrichment was possible only for 2.65% of the analytes studied by the different authors, which corresponds six of 227, distributed in eleven articles. Table VI presents the concentration and analytical parameters for the analytes that could be compared.

Table VI. Analytical parameters for the analytes studied by different authors whose analysis could be compared, according with statistical treatment

QuEChERS	Analysis technique	Analyte	log k_{ow} [33]	Polarity	Concentration µg kg^{-1}	Recovery (%)	Significant difference
Miniaturized citrate[28]	GC-QqQMS	Diazinon	3.69	Nonpolar	100	96 ± 4	No
Acetate with d-SPE and DLLME[30]	LC-MS/MS					99 ± 3	
Miniaturized citrate[28]	GC-QqQMS	Mevinphos	0.127	Polar	10	110 ± 9	Yes
Original with different sorbents[27]	UHPLC-MS/MS					76 ± 7	
Miniaturized citrate[28]	GC-QqQMS	Pyridafenthion	3.20	Nonpolar	10	110 ± 10	Yes
Original with diferente sorbents[27]	UHPLC-MS/MS					84 ± 3	
Miniaturized citrate[28]	GC-QqQMS	Sulprophos	5.48	Nonpolar	100	97 ± 4	Yes
Acetate[21]	HPLC-MS/MS					81 ± 4	
Miniaturized citrate[28]	GC-QqQMS	Terbufos	4.51	Nonpolar	10	117 ± 2	No
Original with diferente sorbents[27]	UHPLC-MS/MS					117 ± 2	
Miniaturized citrate[28]	GC-QqQMS	Triazophos	3.55	Nonpolar	50	121 ± 7	Yes
Original with diferente sorbents[27]	UHPLC-MS/MS					86 ± 9	

From Table VI, it is possible to observe that diazinon was the only one to show no significant difference (comparison between the miniaturized citrate QuEChERS method and acetate QuEChERS with d-SPE and DLLME) and terbufos (comparison between the miniaturized QuEChERS citrate method and original QuEChERS with varying sorbents). For the determination of mevinphos, miniaturized citrate QuEChERS by Ferracane *et al.*[28] showed better recovery and lower accuracy, i.e., the standard deviation was higher compared to original QuEChERS with varying sorbents proposed by Kemmerich *et al.*[27] For the determination of pyridafenthione, miniaturized citrate QuEChERS of Ferracane *et al.*[28] showed high recovery with a higher standard deviation than the results found using the original QuEChERS with varying sorbents, which obtained lower recovery, but good accuracy, as indicated by a low standard deviation.[27] For the determination of sulprophos, miniaturized citrate QuEChERS[28] was shown to be more efficient when compared to acetate QuEChERS proposed by Lemos *et al.*[21] Finally, for the determination of triazophos, miniaturized citrate QuEChERS[28] showed recovery above the acceptable level of 70-120%, while the use of original QuEChERS with varying sorbents[27] showed recovery within the established range.

Methods currently used for sample preparation to determine pesticide multiresidues in food

Determining the amount of pesticide multiresidues in complex matrices, such as food, remains a challenge for the scientific community, due to the different properties of the analytes, as well as the interferences in the matrix. Therefore, the scientific community continually develops and improves sample preparation methods for the extraction of different pesticides from different matrices.

In addition to QuEChERS, other methods can be used for the extraction of pesticides from food matrices, such as gas-liquid microextraction (GLME) integrated with d-SPE, validated to determine residues of forty-seven pesticides, of different classes, in samples of apples, oranges, honey, and leek. The author reported that the method presented an equivalent or smaller matrix effect, compared to the QuEChERS method, which is commonly used.[35] Another method used as an alternative to QuEChERS is binary solvent liquid-phase microextraction (BS-LPME) for the determination of seventeen pesticides of different classes in red and rosé wines, which are a challenge for chemical analysis.[36]

Even with the use of different sample preparation methods for the determination of pesticide multiresidues in food, QuEChERS is still among the most used. According to the *Portal de Periódicos Capes*, until January 2022, QuEChERS was used as an extraction method in 6549 articles and in the Science Direct database, the method is found in 4194 articles, and the number of articles using it has been growing year after year.

There was a report on the use of the original QuEChERS method with an extra step of d-SPE for a better clean-up of extracts and reduction of the matrix effect for the extraction of triazole compounds from orange, grape and strawberry samples;[37] acetate QuEChERS was used for the determination of pesticides in samples of onion, watermelon, tomato, sweet pepper, cabbage, carrot, amaranth, cabbage, eggplant, beans, and okra.[38] Also, original and modified QuEChERS were used to determine different analytes in sweet pepper samples.[26,28,30]

CONCLUSIONS

The use of the QuEChERS method, with or without modifications proposed by the literature, showed good analytical parameters for most analytes investigated. However, even being a multiresidue method, its efficiency varies according to the analyte. In addition, miniaturized citrate QuEChERS by Ferracane *et al.*[28] stands out as the most eco-friendly method as it uses less reagents and, consequently, has a lower cost than methods for determining pesticide multiresidues in sweet pepper samples.

Due to the flexibility of the QuEChERS method, which is apt to be modified, different solvents and sorbents could be investigated by different authors. Acetonitrile proved to be the solvent with the best analytical parameters for analytes with different polarities,[18,27] while methanol is better for polar analytes.[18] On the other hand, among the sorbent extractors used, the use of PSA tends to remove polar compounds from the matrix, including pigments, sugars and fats, and is indicated for the determination of nonpolar pesticides.[21] In turn, C18 removes nonpolar compounds from the matrix and is indicated for the determination of polar analytes.[29] In addition, for flonicamide and its metabolites, the clean-up step (d-SPE), using PSA and GCB as sorbents, lowers the percentage of recovery, possibly because, in aqueous matrices (such as peppers), flonicamide and its metabolites are retained in PSA and GBC sites.[22] Therefore, the choice of solvent and/or sorbent for the optimization of the method depends on the characteristics of the analyte and the sample of interest.

Furthermore, other modifications can be used to improve efficiency or decrease matrix effects. Examples include the modified QuEChERS-dSPE Ionic Liquid-based DLLME method by Lawal and Low[30] and the optimization of the sample milling step through the addition of ascorbic acid and/or dry ice, allowing better recoveries for captan and folpet, which prevents hydrolysis and oxidation of analytes during milling.[26] Furthermore, the determination of captan and folpet by GC-MS/MS is problematic, due to the tendency of these analytes to degrade during the injection. Thus, other techniques should be investigated to this end, such as SFC-MS/MS.[26]

Although the instrumentation used for the detection of analytes is costly (GC-MS/MS, LC-MS/MS, UHPLC-MS/MS, among other chromatographic techniques), the ability to detect numerous analytes

simultaneously makes the investment in chromatographic systems worthwhile. We expect this study encourages future investigations regarding the determination of pesticide multiresidues in sweet peppers to provide cheaper, eco-friendly methods with good analytical parameters, in compliance with the strict international legislation.

Conflicts of interest

The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Table 1. Analytes studied using quEChERS, by different authors

Ref.	Analytes	Class[33]	Polarity	log K_{ow}[33]	Recovery (%)	Standard deviation
(25)	Chlorpyrifos	organophosphate	nonpolar	4.7	74.2	It did not present standard deviation values.
	ethion	organophosphate	nonpolar	5.1	70.0	
	phentoate	organophosphate	-	-	93.0	
	phorate	organophosphate	-	-	101.1	
	Melation	organophosphate	nonpolar	2.8	76.6	
	methamidophos	organophosphate	Polar	-0.8	86.6	
	paration	organophosphate	-	-	77.7	
	Pyrazophos	phosphorothiolate	nonpolar	3.8	77.4	
	Pyrimifos	organophosphate	-	-	60.0	
	terbufos	organophosphate	nonpolar	4.5	87.9	
	Triazophos	organophosphate	nonpolar	3.6	74.6	
(22)	Flonicamid	nicotinoid	Polar	-0.2	88.0	11.0
	TFNA	organophosphate	-	-	NR	NR
	TFNA-AM	organophosphorus	-	-	NR	NR
	TFNG	organophosphorus	-	-	89.0	10.0
(26)	capture	Phthalimide	nonpolar	2.5	96.0	It did not present standard deviation values.
	folpet	Dicarboximide	nonpolar	3.0	89.0	
(27)	3-hydroxy carbofuran	carbamate	-	-	88.0	14.0
	acetamiprid	neonicotinoid	Polar	0.8	98.0	9.0
	ametrine	triazine	nonpolar	2.6	90.0	6.0
	atrazine	triazine	nonpolar	2.7	101.0	12.0
	azaconazole	triazole	nonpolar	2.4	91.0	7.0
	azoxystrobin	strobilurin	nonpolar	2.5	83.0	12.0
	benomyl	benzimidazole	nonpolar	1.4	NR	NR
	Boscalida	carboxamide	nonpolar	3.0	82.0	13.0
	bromuconazole	triazole	nonpolar	3.2	91.0	16.0
	buprofezin	Thiadiazinone	nonpolar	4.9	88.0	7.0
	carbaryl	carbamate	nonpolar	2.4	79.0	11.0

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Table 1. Analytes studied using quEChERS, by different authors (continuation)

Ref.	Analytes	Class[33]	Polarity	log K_{ow}[33]	Recovery (%)	Standard deviation
(27)	Carbendazine	benzimidazole	nonpolar	1.5	105.0	12.0
	carbofuran	benzofuranil	nonpolar	1.8	110.0	8.0
	cyanazine	triazine	nonpolar	2.1	85.0	12.0
	cyproconazole	triazole	nonpolar	3.1	72.0	11.0
	Clomazone	isoxazolidinone	nonpolar	2.6	96.0	15.0
	Chloranthraniliprole	anthranilamide	nonpolar	2.9	85.0	12.0
	chlorbromuron	Urea	nonpolar	3.1	80.0	12.0
	Chlorpyrifos	organophosphate	nonpolar	4.7	75.0	11.0
	Clothianidin	neonicotinoid	Polar	0.9	106.0	18.0
	diazinon	organophosphate	nonpolar	3.7	92.0	14.0
	difenoconazole	triazole	nonpolar	4.4	96.0	17.0
	Dimethoate	organophosphate	Polar	0.8	93.0	9.0
	dimoxystrobin	strobilurin	nonpolar	3.6	95.0	11.0
	diuron	Urea	nonpolar	2.9	89.0	12.0
	dodemorph	Morphine	nonpolar	4.6	96.0	13.0
	epoxiconazole	triazole	nonpolar	3.3	117.0	22.0
	Ethiofencarb sulfone	methylcarbamate	-	-	73.0	11.0
	ethiofencarb sulfoxide	methylcarbamate	-	-	94.0	17.0
	ethoprophos	organophosphate	nonpolar	3.0	89.0	16.0
	fembuconazole	triazole	nonpolar	3.8	73.0	15.0
	Fempropatrin	pyrethroid	nonpolar	6.0	105.0	18.0
	phenpropimorph	Morphine	nonpolar	4.5	80.0	14.0
	Fluazifop-p-butyl	Aryloxyphenoxypropionic acid	nonpolar	4.5	89.0	12.0
	flusilazole	triazole	nonpolar	3.9	96.0	17.0
	flutolanil	carboxamide	nonpolar	3.2	101.0	14.0
	flutriafol	triazole	nonpolar	2.3	78.0	14.0
	furathiocarb	carbamate	nonpolar	4.6	99.0	16.0
	imazalil	imidazole	nonpolar	2.6	74.0	4.0
	iprovalicarb	carbamate	nonpolar	3.2	99.0	16.0
	linuron	Urea	nonpolar	3.0	80.0	15.0

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Table 1. Analytes studied using quEChERS, by different authors (continuation)

Ref.	Analytes	Class[33]	Polarity	log K_{ow} [33]	Recovery (%)	Standard deviation
(27)	Linuron-d6 S	Urea	nonpolar	3.0	86.0	8.0
	Mephospholan	organophosphate	nonpolar	1.0	93.0	10.0
	Metalaxyl-M	phenylamide	nonpolar	1.7	92.0	9.0
	Metobrumuron	Urea	nonpolar	2.5	89.0	9.0
	metolachlor	Chloroacetamide	nonpolar	2.9	83.0	14.0
	mepronil	benzanilide	nonpolar	3.7	82.0	17.0
	metosulam	triazolopyrimidamine	Polar	0.2	95.0	8.0
	Mevinphos	organophosphate	Polar	0.1	76.0	8.0
	monocrotophos	organophosphate	Polar	-0.2	91.0	13.0
	monolinuron	Urea	nonpolar	2.2	84.0	12.0
	omethoate	organophosphate	Polar	-0.9	73.0	10.0
	ethyl paraoxon	organophosphate	-	-	85.0	7.0
	pencicuron	Phenylurea	nonpolar	4.7	93.0	10.0
	penconazole	triazole	nonpolar	3.7	88.0	9.0
	Picoxystrobin	strobilurin	nonpolar	3.6	86.0	15.0
	pyraclostrobin	strobilurin	nonpolar	4.0	97.0	17.0
	Pyrazophos	phosphorothiolate	nonpolar	3.8	79.0	9.0
	pyridafenthion	organophosphate	nonpolar	3.2	84.0	8.0
	pyrifenox	pyridine	nonpolar	3.4	75.0	5.0
	pyrimethanil	anilinopyrimidine	nonpolar	2.84	88.0	4.0
	ethyl pirimiphos	organophosphate	nonpolar	4.8	96.0	15.0
	methyl pyrimiphos	organophosphate	nonpolar	4.2	106.0	16.0
	Profenophos	organophosphate	nonpolar	1.7	94.0	16.0
	propanil	anilide	nonpolar	2.3	108.0	13.0
	propiconazole	triazole	nonpolar	3.7	102.0	9.0
	simazine	triazine	nonpolar	2.1	98.0	15.0
	tebuconazole	triazole	nonpolar	3.7	77.0	13.0
	tebufempyrade	pyrazole	nonpolar	4.9	94.0	15.0
	terbufos	organophosphate	nonpolar	4.5	117.0	2.0
	terbuthylazine	triazine	nonpolar	3.4	95.0	14.0
	tetraconazole	triazole	nonpolar	3.6	95.0	19.0

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Table 1. Analytes studied using quEChERS, by different authors (continuation)

Ref.	Analytes	Class[33]	Polarity	log K_{ow} [33]	Recovery (%)	Standard deviation
(27)	Thiabendazole	benzimidazole	nonpolar	2.4	90.0	5.0
	Thiacloprid	neonicotinoid	nonpolar	1.3	85.0	4.0
	Thiamethoxam	neonicotinoid	Polar	-0.1	99.0	12.0
	Thiobencarb	Thiocarbamate	nonpolar	4.2	99.0	15.0
	methyl thiophanate	benzimidazole	nonpolar	1.4	50.0	0.5
	Triazophos	organophosphate	nonpolar	3.6	103.0	18.0
	trifloxystrobin	strobilurin	nonpolar	4.5	99.0	12.0
	Triflumizole	imidazole	nonpolar	4.8	108.0	14.0
(18)	Acephate	organophosphate	Polar	-0.8	It did not show recovery values.	It did not present standard deviation values.
	azoxystrobin	strobilurin	nonpolar	2.5		
	carbaryl	carbamate	nonpolar	2.4		
	carbendazim	benzimidazole	nonpolar	1.5		
	carbofuran	carbamate	nonpolar	1.8		
	Clomazone	isoxazolidinone	nonpolar	2.6		
	Imidacloprid	neonicotinoid	Polar	0.6		
	methamidophos	organophosphate	Polar	-0.8		
	methiocarb	carbamate	nonpolar	3.2		
	Methomyl	carbamate	Polar	0.1		
	Thiabendazole	benzimidazole	nonpolar	2.4		
	Thiacloprid	neonicotinoid	nonpolar	1.3		
	Thiamethoxam	neonicotinoid	Polar	-0.1		
(28)	2.4-DDD	organochlorine	nonpolar	6.0	112.0	8.0
	2.4-DDE	-	-	-	122.0	7.0
	2,4-DDT	-	-	-	70.0	7.0
	2,4-Methoxychlor	-	-	-	97.0	7.0
	4,4,-methoxychloro olefin	-	-	-	124.0	6.0
	4.4-DDD	-	-	-	63.0	2.0
	4.4-DDE	-	-	-	117.0	8.0
	4,4-DDT	-	-	-	118.0	14.0
	4,4-Dichlorobenzophenone	-	-	-	109.0	2.0
	Aldrin	organochlorine	nonpolar	6.5	98.0	6.0

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Table 1. Analytes studied using quEChERS, by different authors (continuation)

Ref.	Analytes	Class[33]	Polarity	log K_{ow}[33]	Recovery (%)	Standard deviation
(28)	Alpha- BHC	-	-	-	110.0	6.6
	alpha-endosulfan	organochlorine	nonpolar	4.7	101.0	10.0
	azinphos-ethyl	organophosphate	nonpolar	3.2	NR	NR
	azinphos-methyl	organophosphate	nonpolar	3.0	NR	NR
	Beta-BHC	-	-	-	115.0	6.0
	beta-endosulfan	organochlorine	nonpolar	3.8	70.0	8.0
	bromfenvinphos	organophosphate	-	-	NR	NR
	bromfenvinphos-methyl	-	-	-	NR	NR
	bromophosethyl	organophosphate	nonpolar	6.2	86.0	4.0
	bromophos-methyl	organophosphate	nonpolar	5.2	96.0	5.0
	piperonyl butoxide	-	-	-	126.0	5.0
	carbophenion	organophosphate	nonpolar	4.8	74.0	4.0
	cis-chlordane	organochlorine	nonpolar	2.8	76.0	8.0
	cis-nanochlor	-	-	-	102.0	4.0
	chlorbenside	organochlorine	nonpolar	5.6	110.0	7.0
	chlorfenson	bridged diphenyl	nonpolar	4.2	66.0	2.0
	chlorfenvinphos	organophosphate	nonpolar	3.8	83.0	6.0
	chloroneb	substituted benzene	nonpolar	3.6	56.0	5.0
	Chlorpyrifos	organophosphate	nonpolar	4.7	102.0	3.0
	chlorpyrifos-methyl	organophosphate	nonpolar	4.0	107.0	5.0
	Chlorthiophos	-	-	-	102.0	3.0
	Coumaphos	-	-	-	152.0	15.0
	diazinon	organophosphate	nonpolar	3.7	120.0	4.0
	dieldrin	chlorinated hydrocarbon	nonpolar	3.7	120.0	6.0
	disulfoton	organophosphate	nonpolar	4.0	119.0	6.0
	Edifenphos	organophosphate	nonpolar	3.8	NR	NR
	endrin aldehyde	-	-	-	NR	NR
	endrin ketone	-	-	-	NR	NR
	endrina	organochlorine	nonpolar	3.2	78.0	5.0
	EPN	organophosphate	nonpolar	5.0	86.0	4.0
	heptachlor epoxide	-	-	-	57.0	4.0

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Table 1. Analytes studied using quEChERS, by different authors (continuation)

Ref.	Analytes	Class[33]	Polarity	log K_{ow}[33]	Recovery (%)	Standard deviation
(28)	endosulfan ether	organochlorine	nonpolar	4.8	81.0	13.0
	ethion	organophosphate	nonpolar	5.1	84.0	5.0
	ethyl	-	-	-	NR	NR
	Phenamiphos	organophosphate	nonpolar	3.3	NR	NR
	Fenchlorphos	organophosphate	nonpolar	4.9	104.0	4.0
	Phenitrothion	organophosphate	nonpolar	3.3	108.0	11.0
	Fenson	organochlorine	nonpolar	3.6	107.0	4.0
	fenthion	organophosphate	nonpolar	4.8	70.0	2.0
	Phonofos	organophosphate	nonpolar	3.9	120.0	5.0
	phorate	organophosphate	nonpolar	3.86	111.0	4.0
	fosalone	organophosphate	nonpolar	4.0	NR	NR
	Gamma-BHC	-	-	-	117.0	10.0
	heptachlor	organochlorine	nonpolar	5.4	94.0	6.0
	hexachlorobenzene	chlorinated hydrocarbon	nonpolar	3.9	107.0	8.0
	iodofenphos	organophosphate	nonpolar	5.5	105.0	15.0
	isazophos	organophosphate	nonpolar	3.1	110.0	10.0
	isodrin	cyclodiene	nonpolar	6.8	112.0	6.0
	leptophos	organophosphate	nonpolar	6.3	NR	NR
	Lindane	organochlorine	nonpolar	3.5	118.0	11.0
	malathion	organophosphate	nonpolar	2.8	110.0	11.0
	Metacrypha	organophosphate	nonpolar	1.5	101.0	8.0
	Mevinphos	organophosphate	Polar	0.1	110.0	9.0
	mirex	organochlorine	nonpolar	5.3	106.0	6.0
	parathion	organophosphate	-	-	92.0	8.0
	Parathion-Methyl	organophosphate	nonpolar	3.0	109.0	14.0
	pentachloroanisole	-	-	-	119.0	5.0
	pentachlorobenzene	-	-	-	75.0	1.0
	pentachlorothioanisole	-	-	-	78.0	2.0
	pyraclophos	organophosphate	nonpolar	3.8	NR	NR
	Pyrazophos	phosphorothiolate	nonpolar	3.8	129.0	5.0
	pyridafenthion	organophosphate	nonpolar	3.2	110.0	10.0

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Table 1. Analytes studied using quEChERS, by different authors (continuation)

Ref.	Analytes	Class[33]	Polarity	log K_{ow}[33]	Recovery (%)	Standard deviation
(28)	pyrimiphos-ethyl	organophosphate	nonpolar	4.8	113.0	4.0
	pyrimiphos-methyl	organophosphate	nonpolar	4.2	90.0	4.0
	Profenophos	organophosphate	nonpolar	1.7	NR	NR
	prothiophos	organophosphate	nonpolar	5.7	NR	NR
	Quinalphos	organophosphate	nonpolar	4.4	79.0	3.0
	endosulfan sulfate	organochlorine	-	-	111.0	3.0
	sulfotepp	organophosphate	nonpolar	4.0	108.0	2.0
	sulprophos	organophosphate	nonpolar	5.5	108.0	4.0
	terbufos	organophosphate	nonpolar	4.5	117.0	2.0
	tetrachlorvinphos	organophosphate	nonpolar	3.5	148.0	27
	Tetradiphon	organochlorine	nonpolar	4.6	108.0	4.0
	Tolclofos-methyl	organophosphate	nonpolar	3.8	71.0	3.0
	trans-chlordane	organochlorine	nonpolar	2.8	84.0	7.0
	trans-nanachlor	organochlorine		--	147.0	21.0
	Triazophos	organophosphate	nonpolar	3.6	60.0	4.0
(29)	broflanilide	Diamide	nonpolar	5.2	It did not show recovery values.	It did not show recovery values.
	DM-8007	-	-	-		
	S (PFH-OH)-8007	-	-	-		
(30)	baycarb	-	-	-	102.0	2.0
	carbaryl	carbamate	nonpolar	2.4	98.0	3.0
	diazinon	organophosphate	nonpolar	3.7	105.0	4.0
	Durban	organophosphate	-	-	108.0	6.0
	Metalaxyl	phenylamide	nonpolar	1.8	101.0	10.0
	propamocarb	carbamate	Polar	0.8	92.0	5.0
	Thiamethoxam	neonicotinoid	Polar	-0.1	91.0	4.0
	Thiobencarb	Thiocarbamate	nonpolar	4.2	91.0	3.0
(31)	fluopyram	benzamide	nonpolar	3.3	80.3	7.3
	Fluopyram benzamide	-	-	-	83.5	7.7
	tebuconazole	triazole	nonpolar	3.7	78.5	6.5

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Table 1. Analytes studied using quEChERS, by different authors (continuation)

Ref.	Analytes	Class[33]	Polarity	log K_{ow} [33]	Recovery (%)	Standard deviation
(32)	de-iodine fluobendiamide	-	-	-	97.2	6.4
	flubendiamide	Diamide	nonpolar	4.1	96.4	7.5
(21)	azinphos-methyl	organophosphate	nonpolar	3.0	111.5	10.0
	Coumaphos	organophosphate	-	-	106.0	8.1
	Demeton-S-methyl sulfone	organophosphate	-	-	118.0	10.1
	diazinon	organophosphate	nonpolar	3.7	102.2	7.9
	dichlorvos	organophosphate	nonpolar	1.9	92.7	0.7
	ethoprophs	organophosphate	nonpolar	3.0	87.2	11.4
	Fensulfotion	organophosphate	nonpolar	2.2	94.2	7.7
	fenthion	organophosphate	nonpolar	4.8	94.5	12.0
	Mevinphos	organophosphate	Polar	0.1	98.3	6.9
	sulprophos	organophosphate	nonpolar	5.5	101.8	12.7
	tetrachlorvinphos	organophosphate	nonpolar	3.5	99.5	3.4

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ARTICLE

Chemometric Evaluation of a Biodegradable Tannic Acid-Polyurethane System for the Removal of Pb(II) and Hg(II) Ions from Water

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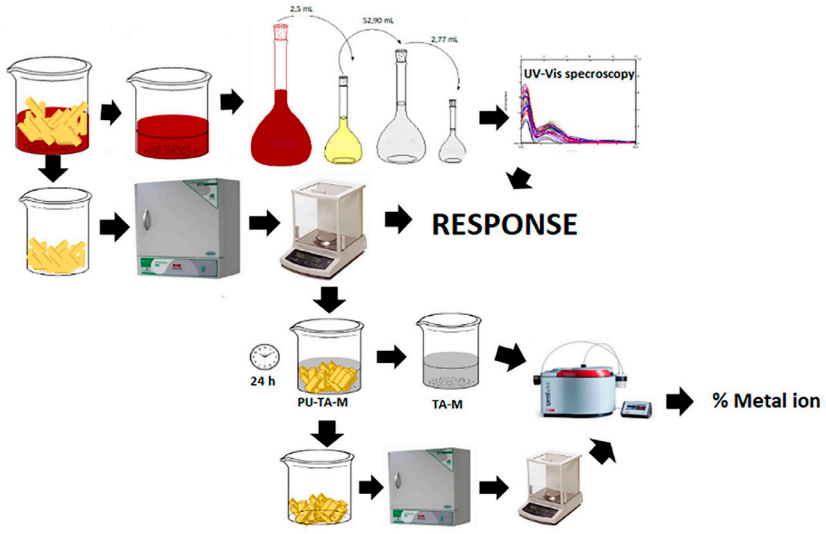

The industrialization has brought advances that have enabled a better quality of life for people and improved production stages and business models. However, some impacts still need to be resolved, such as water pollution. Industrial pollutants containing potentially toxic metal ions are expensive for the industry, so studying new materials and new processes has helped solve this problem. Adsorption processes using biodegradable adsorbent materials have been presented as relevant alternatives for reusing metallic ions from water bodies and sewage networks. In this perspective, tannic acid (TA) immobilization in biodegradable polyurethane (PU) foams based on vegetable oil (castor oil) was used to remove metal ions Hg(II) and Pb(II) from water. The preliminary study was carried out in TA's immobilization in PUs, with a 2^k order factorial experimental design. The responses were obtained and evaluated by gravimetry and UV-Vis spectroscopy using Central Composite Design (CCD). The molar concentration of 0.1000 mol L^{-1} by TA solution at pH equal to 7 and 19 hours of contact time was defined as optimal conditions for TA adsorption in the PU. The optimized PU-TA system was evaluated for removing

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Pb(II) and Hg(II) ions in aqueous solutions, and the tests showed that 59.93% and 51.48% were removed from water, respectively. The use of the PU-TA adsorbent system for removing metals in water can be widely valuable in industrial plants that need water treatment.

Keywords: tannic-polyurethane, Pb(II), Hg(II), adsorption, chemometrics.

INTRODUCTION

The growth of industrialization has brought benefits to humanity, but it has witnessed several environmental issues resolved. Numerous environmental tragedies are caused by changes in the physical, chemical, and biological properties of the environment. The contamination of ecosystems can come from the disposal of industrial and domestic effluents that threatens water resources integrity since they are constituted by metals considered toxic.[1–4]

The allocation of industrial effluents in water bodies and sewage network without previous treatment can cause health and environmental problems.[1] Therefore, it is considered essential that the industries carry out a pre-treatment of the effluents before their discharge into sewers and rivers to remove the toxic metals.[2] Several techniques are used for physical removal of metallic ions, from which the most studied is the adsorption with adsorbent of natural origin like zeolites, clay, peat moss and agricultural wastes and modified materials like halloysite/chitosan and activated carbon.[5–8] These methods are based on the immobilization of metallic ions, where they involve intermolecular interactions between the analyte and the adsorbent material.[5] The adsorption with activated carbon, though, becomes often economically unviable for small industries, since it requires complexing agents, increasing treatment costs. In this way, alternative solutions have been studied for production of adsorbent systems, preferably of natural origin.[1,7]

Some studies have evidenced the functionality of polyurethane (PU) foam with sulfonic acid groups for Pb(II) ions removal. This study indicated that the concentration of sulfonic acid and the reaction time are significant variables in removing Pb(II) ions.[6] The great interest for studying the adsorbent system of PU is that, besides their petrochemical origin, they can also be obtained by polyols extracted from renewable sources, mainly polyols of natural oils. The advantage of PU foams of plant origin is related to the relative ease of biodegradation, fully integrating with nature.[7,8]

Numerous methods in the literature investigate the behavior of PU foams to remove metal ions. However, solely the PU foam of castor origin is not efficient for ions removal. Thus, a complexing agent is required to increase this immobilization efficiency. Researchers have carried out a study comparing the use of activated carbon with and without Tannic Acid (TA) for adsorption of Cu(II), Cd(II), Zn(II), Mn(II) and Fe(III), varying different parameters, which evidenced that both activated carbons interact with metallic ions.[9] However, the metals are adsorbed with a better efficacy on activated carbon with Tannic Acid. The TA is considered a natural antioxidant, besides possessing other properties, such as the capacity of interaction with proteins, polymers, complexing of metal ions Pb(II), Cu(II), Cd(II) e Zn(II) and ion reducer for the formation of metallic nanoparticles.[9–17] The degree of the potentiality of the reducing characteristics and complexing by TA are dependent on some parameters such as pH variance, temperature, TA concentration, and others.[13,18,19] The ability to interact with macromolecules and metal ions develops a toxicity factor. This factor may be linked to the higher weight and concentration of the molecule in the body.[19]

Chemometric tools are widely used to evaluate several variables that influence a given system.[10,20,21] A comparative study using different chemometric tools for Pb(II) ions adsorption onto tree leaves was realized. Firstly, a complete factorial design was carried out to identify the most significant variables and then the application of models for obtaining the response surfaces, such as the CCD (Central Compound Design), BBP (Box - Behnken Planning) and DM (Doehlert Matrix), which allowed analysis to optimize the adsorption process. From software help, numerous tests were used to adapt the mathematical model proposed, such as residue analysis, variance analysis (ANOVA), and coefficient of determination (R^2).[22]

The present study has the objective to determine optimal parameters for immobilization of TA in PU and then in-depth evaluation with the addition of a central point and a star point to estimate the optimum region;

also, to perform a comparative study of the analytical methods used to evaluate which one is efficient to quantify TA in PU. The chemometric tools used make it possible to study the possibility of removing Hg(II) and Pb(II) ions from industrial effluents.

MATERIALS AND METHODS

Reagents

The tannic acid solution (TA) was obtained by direct dissolution (Sigma-Aldrich, purity of 99%) in deionized water.

The aqueous buffer solutions used were prepared in the laboratory: acetic acid solution (QHEMIS) / sodium acetate (Synth 99%) 1 mol L^{-1} (pH = 5); ammonium hydroxide (Vetec 99%) / ammonium hydrogen carbonate 1 mol L^{-1} (pH = 8); citric acid (Ecibra 99%) / citrated sodium (Ecibra 99%) 1 mol L^{-1} (pH 4); hydrogen phosphate (Synth 98%, of purity) / dihydrogen sodium phosphate 1 mol L^{-1} (pH = 6.5), ammonium chloride (Vetec 99%) / ammonium hydroxide 1 mol L^{-1} (pH = 9). Aqueous solutions of NaOH (Synth 97%) in concentrations of 1 and 2 mol L^{-1} were also used. Polyol of vegetable origin (castor oil) and prepolymer (diphenylmethane-4,4-diisocyanate) were used for the preparation of polyurethane (PU).

Preparation of polyurethanes

PU were prepared by addition of polyol (PO) in the prepolymer (PP), following the bulk ratio of 1.0(PO):1.7(PP) (m/m), under manual agitation until homogenization of the materials. After formation, the PU's were cut into the standardized dimensions: length of 4.0 cm, width of 1.0 cm and thickness of 0.5 cm.[23] (Figure 1)

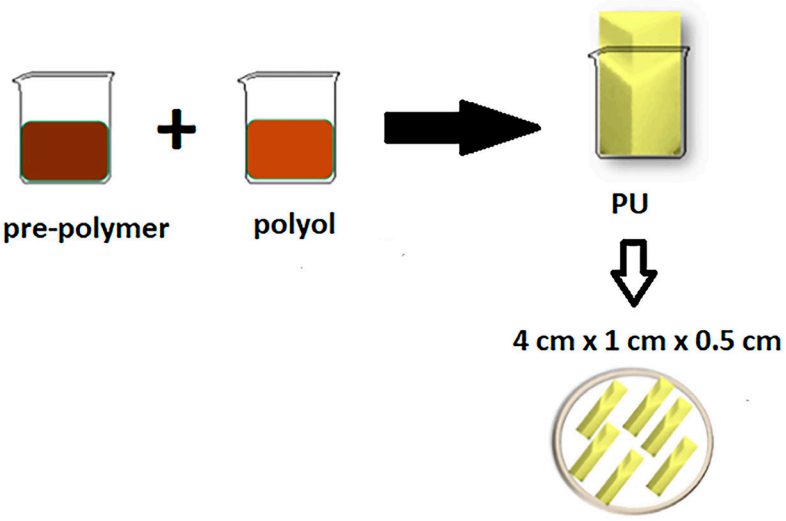

Figure 1. Scheme of preparation of cut PU foams.

Experimental design

The factorial design was performed using preliminary tests (see Table I). Firstly, to analyze the individual effects, as well as the interactions between the independent variables, the temperature (V1) of the TA immobilization process, the molar concentration of TA (V2), the pH value of TA solution (V3), the contact time of the TA with PU (V4) and the amount of PU (V5) were studied by applying a factorial design (2^k). The experimental range for (V2) was defined as of high concentrations from preliminary tests, by observing the mass varying of the floated product with experiments from low (0.0100 mol L^{-1}) to high concentrations (0.1000 mol L^{-1}). Although small, the change in mass only occurred for high concentrations.

The entire experimental data set was globally analyzed by multiple regression, measuring those variable effects, and fitting the data to an empirical model. The parameters with a significance level higher than 0.05 were considered insignificant.

Table I. Experimental variables used for factorial design 2^5

Factors	Parameters	Higher level (+)	Lower level (-)
V1	Temperature / °C	60	30
V2	Molar concentration /mol L^{-1}	0.1000	0.0100
V3	pH	8	5
V4	Contact time / hours	26	12
V5	Amount of PU / units	20	10

A second stage involved tests studied by applying a central composite design (CCD) with two center replicates, and with α equal to 1,68. To maintain rotatability, The CCD was obtained by fixing two variables (V4 and V5). The value of α was calculated by number of experimental runs in the factorial portion of the central composite design ($\alpha = [2^k]^{1/4}$). The experimental data obtained from the central composite design were statistically analyzed (Statistica® software) by measuring the effects of those variables.

The immobilization results were determined by gravimetry and UV-Vis spectroscopy. The immobilization of each test was calculated using Equation 1.

$$\%QTA = \frac{M_i - M_f}{M_i} x100 \qquad \text{Equation 1}$$

where M_i represents the initial mass of tannic acid, M_f is the remaining tannic acid mass and Q is the amount of immobilized tannic acid.

Gravimetry and UV-Vis Spectroscopy obtained the responses of the experiments. Gravimetry method: the mass measures were realized using a Shimadzu model AX 200 analytical balance. The PU units cut into standardized dimensions (length of 4.0 cm, width of 1.0 cm and thickness of 0.5 cm) were grouped according to the experimental planning, and their masses were measured and recorded. After weighing, the PU's were placed in beakers containing 100.0 mL of the respective aqueous solutions of TA, according to experimental design. After the contact times, the PU's were removed and dried in an oven at 25 °C for 24 hours and kept in a desiccator until constant mass.

The maximum wavelength observed in the UV-Vis spectra was investigated by a Varian Cary 50 Scan Spectrophotometer. The spectra were obtained in a quartz cell with a 1 cm pathlength and the maximum wavelength was established at 374 nm. The analytical curves were performed using TA concentrations between 2.0 and 11.0 μg mL^{-1}. The immobilization of TA in the PU's were obtained by absorbing TA's aqueous solutions before and after the PU's immersions.

Pb(II) and Hg(II) removal experiment in water

The 16 samples of PU-AT were used and determined by a central composite design (CCD). For the experiment, 52.0 mg L^{-1} and 309.0 mg L^{-1} of water solutions of Pb(II) and Hg(II) chlorides were prepared, respectively. The PU-TA system was immersed in the respective metal ion solutions, Hg(II) and Pb(II), for 24 h. The M-PU-TA (metal-polyurethane-TA) systems were dried, and their masses were measured and recorded.

The digestion of the M-PU-TA system was performed using an aqueous solution of HNO_3 (65%) and HCl (35%) in a microwave digester. Microwave treatment was performed using a sample system digester, Speedwave 4 model (Berghof), using 60 mL Teflon flasks. The irradiation power was set at 800 watts and the pressure was set at 40 bar.

After opening the samples, the systems were diluted, and the metal ions were analyzed using ICP-AES technique. The ICP-AES Spectroscopy was performed using an Atomic Emission Spectrometer of the iCAP7400 model, Duo Thermo Scientific. Detector operation: axial was used for quantitative analysis of the respective metal ions.

RESULTS AND DISCUSSION

Experimental design

In the preliminary stage of the experimental trials, the results were analyzed by mass measurement and electronic absorption. For both techniques, variables that significantly influence TA into PU's adsorption were evaluated at a level of confidence of 95% (α = 0.05). To better evaluate the significance of the estimated effects and verify the possibility of a difference between the responses, null (Ho) and alternative (H1) hypothesis tests were used, comparing the p-value of the statistical test of the samples[24] with a significance level of α = 0.05. Therefore, we considered the hypotheses H0: μ1= μ2= μij= 0, so that interaction effects will not be significant when p > 0.05; and H1: μij ≠ 0, so that interaction effects will not be significant when p < 0.05.

The error was estimated with the exclusion of the effects of a high order. The effects of third and fourth order were presented closely to the error variability, indicating not to influence significantly. The 32 experiments were randomly performed.

Experimental design using UV-Vis spectrometry

The maximum wavelength obtained the responses at 374 nm observed in the UV-Vis spectra UV-Vis spectroscopy (Supplementary material) showed that in the experiments 17–26 and 29–32, the TA's immobilization was at least 92%. As indicated in Figure 2a, the variables that showed significant effects using the UV-Vis spectroscopic technique are indicated as (I) main interactions: amount of adsorbent (V5), pH (V3); and (II) second-order interactions (V3)*(V4); (V2)*(V4). The high order of interaction of the UV-Vis response variables belongs to a region close to the error variability, making its effects not significant.

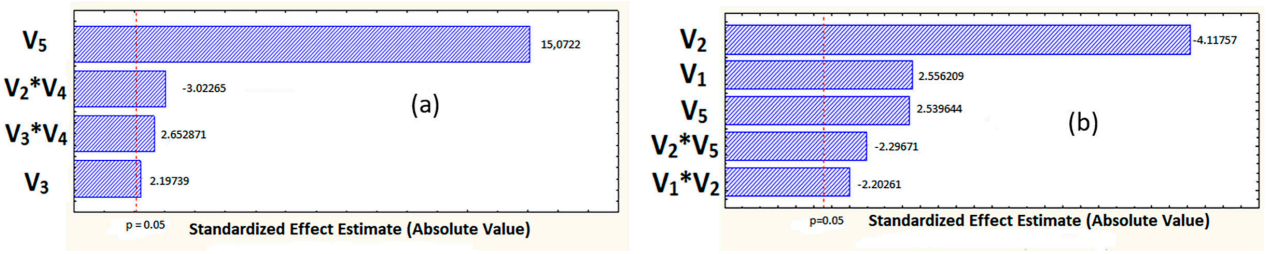

Figure 2. Pareto graph of significant responses (a) UV-Vis spectroscopy (MS Residual = 151.3785) and (b) Gravimetry (MS Residual = 110.1887).

The main interaction of the variable, pH value, may indicate direct proportionality to TA's immobilization in PU. The system using a pH value equal to 8 showed immobilization of 55.42% TA in PU. This effect was probably favored by the lower interaction of phenolic groups present in the TA with water. The V5 effect became more pronounced when associated with the contact time effect (V4). Probably, in low contact times, the physicochemical equilibrium of the phenomena has not yet been carried out.

Some discrepancies can be explained. For example, when TA immobilization in PU was evaluated at a pH value of 5 and a contact time of 26 hours, TA adsorption was disadvantageous, probably due

to the microbial degradation of tannic acid, favored by the pH value. This observation was confirmed by visualization of fungi in the TA solutions after one week of storage.

The TA interaction effect with contact time showed a negative influence in the immobilization, and this effect is similar, both when associated with a high contact time and lower [TA], and vice versa (see Figure 2a).

The main interaction effect (E), adsorbent quantity (V5), significantly influenced the TA immobilization process in PU. We can observe in the complementary material (Table S1) that when the V5 level was increased, there was a more significant number of active sites in the PU.

The table of ANOVA (Table II) made it possible to evaluate the variance between the experimental data and if the determined effects have differences between them. F and p tests were considered.

The generated mathematical model can be observed in Equation 2. The correlation coefficient of the model presented value equal to 90.19%, which is considered acceptable.

$$Y = 56.431_{(\pm 2.047)} + 4.779\ v_{3(\pm 2.174)} + 32.781\ v_{5(\pm 2.174)} - 6.574\ v_2\ v_{4(\pm 2.174)} + 56.143\ v_3\ v_{4(\pm 2.174)} \qquad \text{Equation 2}$$

Table II. Analysis of Variance (ANOVA) for UV-Vis spectroscopy and gravimetry

ANOVA							
		SS[a]	Df[b]	MS[c]	F	p	R^2 (%)
UV-Vis Spectroscopy	V3	730.94	1	730.94	4.83	0.03675	90.19
	V5	34388.80	1	34388.80	227.17	0.00000	
	V2 * V4	1383.06	1	1383.06	9.14	0.0053	
	V3 * V4	1065.36	1	1065.36	7.04	0.0132	
	Error	4087.22	27	151.38		0.0367	
	Total SS	41655.38	31				
		SS	Df	MS	F	p	R^2 (%)
Gravimetry	V1	719.995	1	719.995	6.5340	0.0168	78.19
	V2	1868.180	1	1868.180	16.9547	0.0003	
	V5	710.694	1	710.694	6.4497	0.0170	
	V1 * V 2	534.580	1	534.580	4.8550	0.0367	
	V2 * V 5	581.231	1	581.231	5.2749	0.0290	
	Error	2864.907	26	110.189			
	Total SS	7279.587	31				

[a]SS represents Sum of squares; [b]Df represents Degrees of freedom; [c]MS represents Mean of squares.

Experimental design using gravimetry

In the responses obtained by gravimetry, the amount of adsorbent (V5), TA concentration (V2) and Temperature (V1) can be observed as variables that showed significant effects and second-order interactions (V1)*(V2); (V2)*(V5). However, the immobilization values did not exceed 60%, in addition to presenting low adsorption values.

V2 was the most significant (Figure 2b). This phenomenon can be explained by water interaction in PU, already reported in the literature.[23] The amount of adsorbent and temperature did not show as significant effects as [TA].

The interaction between variables (V1) and (V5) did not show a significant effect, but in the interaction of each of them, respectively, with [TA] effect, it can be observed that these second-order interactions are significant. These evaluations make [TA] effect a preponderant effect on the response obtained by gravimetry. Comparing the results of the effects determined by both techniques, it can be observed that the estimation of the most significant variables in the study region was different (Figure 2).

According to the results indicated by the experimental planning 2^5, it was possible to determine by the two methods which variables could be considered significant, as indicated in the previous section. The mathematical model generated by gravimetric techniques can be observed in Equation 3. The correlation coefficient presented a value equal to 60.45%, showing a correlation inferior to that observed by spectroscopic technique.

$$Y = 9.237_{(\pm 1.85)} + 4.734V_{1(\pm 1.85)} - 7.607V_{2(\pm 1.85)} + 4.717V_{5(\pm 1.85)} - 4.087V_1V_{2(\pm 1.85)} - 4.219V_2V_{5(\pm 1.85)} \quad \text{Equation 3}$$

Despite the quality of the linear gravimetric model, it was possible to estimate that the variables, temperature, [TA], and amount of adsorbent are significant.

Central Composite Designs

In UV-Vis spectroscopic technique, a significant variation rate was observed, explained by the indicated mathematical model. In this way, central and axial points were added to the significant variables (Figure 2) with these new points to obtain new experimental planning to carry out an in-depth study on the variables estimating the existence of a maximum condition in the region investigated.

The V4 and V5 were fixed during the planning to predict the surface response. Thus, the independent variables (Table III) were evaluated by central compound planning with axial points, at the temperature of 30 °C, with ten units of adsorbent and 50.00 mL of TA solution.

Table III. Experimental conditions of CCD, at constant temperature of 30 ºC

Conditions: CCD Matrix					
	Levels				
Variables	-α (-1,681)	(-1)	0	(+1)	+α (+1,681)
(V1) pH	3.97	5	6.5	8	9
(V2) [AT]/mol L^{-1}	0.0100	0.0300	0.0600	0.0800	0.1000
(V3) Contact time/h	17	12	19	26	30

Central and star points were added in the independent variables determined as significant to estimate the optimal region of AT immobilization. In this way the new conditions were determined from the experimental coding, see Equations 4, 5 and 6.

$$V_1 = \frac{[AT] - 0.055}{0.025} \quad \text{Equation 4}$$

$$V_2 = \frac{pH - 6.5}{1.5} \quad \text{Equation 5}$$

$$V_3 = \frac{Contact\ time - 19}{7} \quad \text{Equation 6}$$

The experimental matrix, completed with 16 experiments (Table S2), was performed using two analytical techniques. Two types of responses were again obtained, and their correlation coefficients and optimal conditions regions were determined. The errors were estimated by replicates at the central point.

The results of the analysis of variance for TA immobilization response, determined by the UV-vis spectroscopy, were described in Table S3. The results which presented percentages of explained variation are considered satisfactory (86.47%). The result obtained can be confirmed by the regression value of $F_{calculated}$ (6.572; 5%), because the value was higher than $F_{tabulated(7,8)}$(3.505%), so that we can reject the null hypothesis (H_0), that is, the adjusted model can represent the experimental data and suggest inferences in the sample population. Based on the results, the CCD was found to be statistically significant for the responses.[24,25]

The quadratic model with the highest rate of variation explained by the mathematical model was obtained by UV-Vis spectroscopic technique. Thus, the studied region of the adsorbent system can be represented by the mathematical expression (Equation 7). Residual analysis in regression showed an independent distribution and like the normal distribution, see Equation 7.

$$Y = 91.0982_{(\pm 1.569)} + 24.7343V_{1(\pm 0.602)} - 13.364V_1^1{}_{(\pm 0.7314)} + 11.7895V_{2(\pm 0.7314)} - 23.2812V_2^2{}_{(\pm 0.7314)} - 11.6459V_3^3{}_{(\pm 0.7314)} - 13.1407V_1V_{3(\pm 0.787)} + 11.2589V_2V_{3(\pm 0.787)} \quad \text{Equation 7}$$

The response surface corresponding to this mathematical expression is shown in Figure 3. The model generated, and the construction of the response surface permitted to obtain the optimal coded points. They could be determined for the variables V1, V2 and V3, and are of (1.073092), (0.224872) and (-0.272495), respectively. Then, the values were decoded, estimating optimal conditions of 0.1000 mol L^{-1} by TA solution, at the pH of 7 and for 19 hours of contact time to obtain the maximum TA adsorption in the PU region of study.

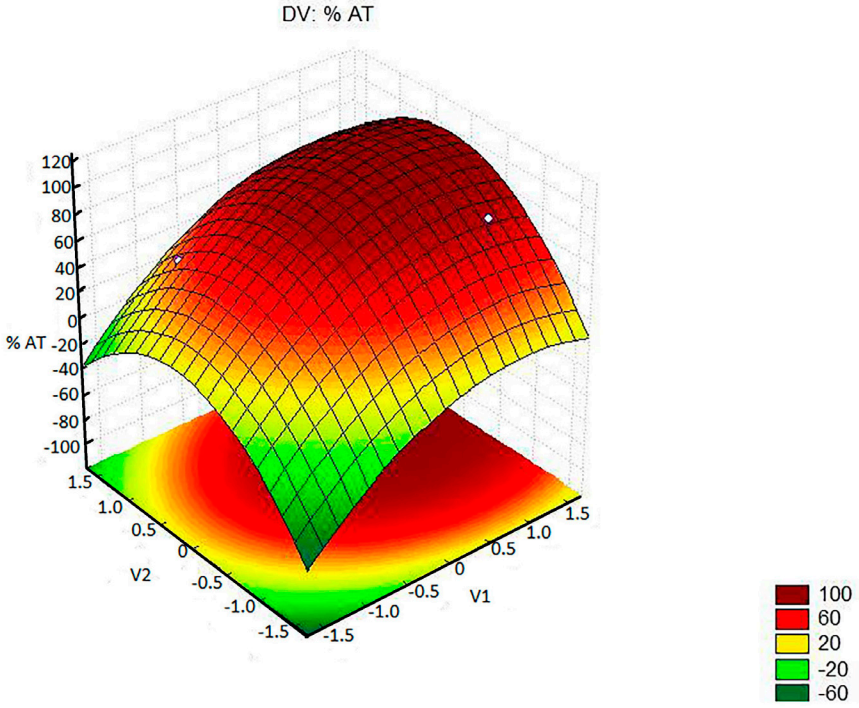

Figure 3. Response surface design for TA adsorption on PU.

The TA percentage (%) determined by the technique using the analytical balance, showed a low explained variation of the experimental data (Table S4), unable to determine the lack of adjustment. Therefore, this model was unsatisfactory to infer the population sample in the region studied, an inappropriate model for predictive purposes. The gravimetric technique was unsatisfactory, probably due to the higher affinity of water with the PU`s. The drying process of the PU-TA system may have influenced the evaluation of this technique.

CCD in metal ion removal

The instrumental technique ICP OES obtained the response of the experimental matrix. Therefore, the quantification of metal ions was performed by two types of the data treatment: the intensity of the analytical signal and recovery (%) of metal ions.

Metal ions were quantified as a function of the sum of the element intensity ratios by their maximum diagnosed intensity at a specific wavelength, which can be represented by Equation 8.

$$RM = (I_{Pb}/I_{max.Pb}) + (I_{Hg}/I_{max.Hg}) \qquad \text{Equation 8}$$

The significance of the experimental data indicated through ANOVA and applying the hypothesis test, was considered as follows:

I) $H_0 = \mu_1 = \mu_2 = \mu_{ij} = 0$: the interaction effects are not significant when $p > 0.05$; the model is inadequate to represent the experimental data;

II) $H_1 = \mu_{ij} \neq 0$, the interaction effects are not significant when $p < 0.05$). The mathematical model is adequate to represent the experimental data.

The validation of the hypotheses can be determined by Fisher's test, which assesses the reason for the variation within or between the variables analyzed by the experimental design. This test evaluates the difference in the samples, depending on the size of the variation within each sample, making it possible to estimate from the comparison of tabulated values with the data obtained experimentally.

The null hypothesis (H_0) was considered to analyze the responses obtained by the digester since the $F_{calculated}$ values were lower than $f_{tabulated(6.9)}5\% = 3.37$, as it can be seen in the ANOVA table (Table S5). Considering that the variation explained is low, the regression model generated was inadequate for representing the data sets.

The values obtained for the response of the remaining solution are represented in the normal probability graph, see Figure 4, observing that they are close to the error variability. These values do not represent any physical significance and may represent random errors during the process. Thus, the effects of the variables determined by the remaining solution's response do not statistically influence the adsorption process of metals.

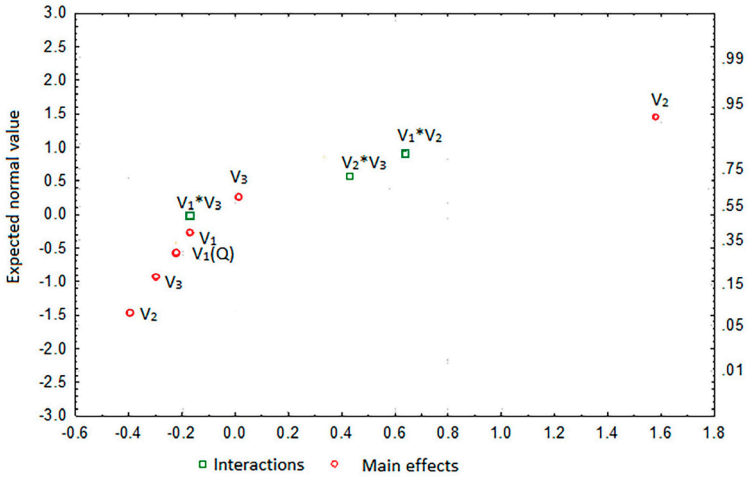

Figure 4. Plot of normal probability to metal adsorption by remaining TA solution.

With the recovery response of the metal by the adsorbent, the significant effects and significance of the model obtained by the Analysis of Variance (ANOVA) were determined, considering the same hypotheses described above. The variance of the experimental data analyzed by the ANOVA table of "recovery /%" (Table S6) obtained a variation of 55.17%, considered a low variation. Also, the lack of adjustment showed significance, that is, the value of $f_{calculated}$ = 331.334, was higher than the $f_{tabulated(1.1)}$5% = 161.4, showing that the linear model does not describe the experimental data well.

The variable response of the remaining solution for metal recovery showed effects that are not significant for the metal adsorption process, as the results tend to be linear around zero, where the error variability is located (Figure 5). The evaluation of the amount of adsorbed metal by analyzing the remaining solution containing M-PU-TA and the digestion method, evaluated by different types of responses, presented unsatisfactory models to represent the experimental data. The variation in the metal adsorption data showed a low quadratic correlation coefficient. Thus, it was not possible to estimate the optimized condition for the metal's behavior with statistical reliability. Despite the inexistence of the optimum point in the region investigated by the experimental planning, it is possible to carry out a qualitative assessment of the analyzes. Thus, the amount of Pb(II) and Hg(II) removed from water by the adsorbent system was investigated and compared proportionally with the amount of metal detected by analytical techniques. The values of the analysis were summarized and evaluated graphically. (Figures 6 and 7)

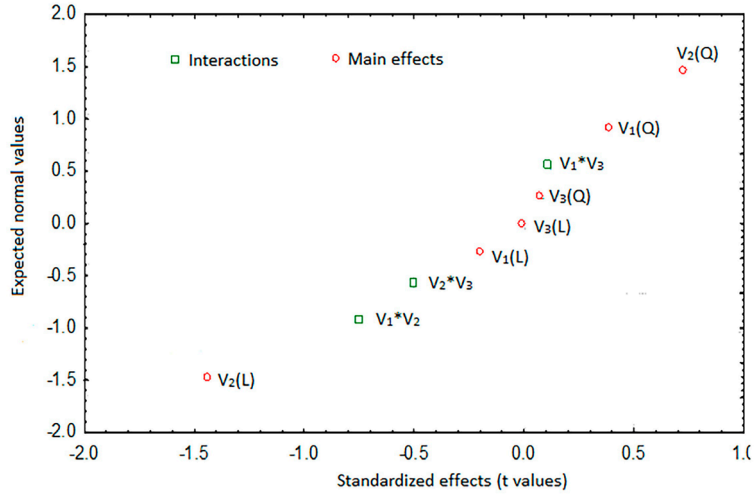

Figure 5. Plot of normal probability to metal adsorption after the M-TA system goes through microwave digester.

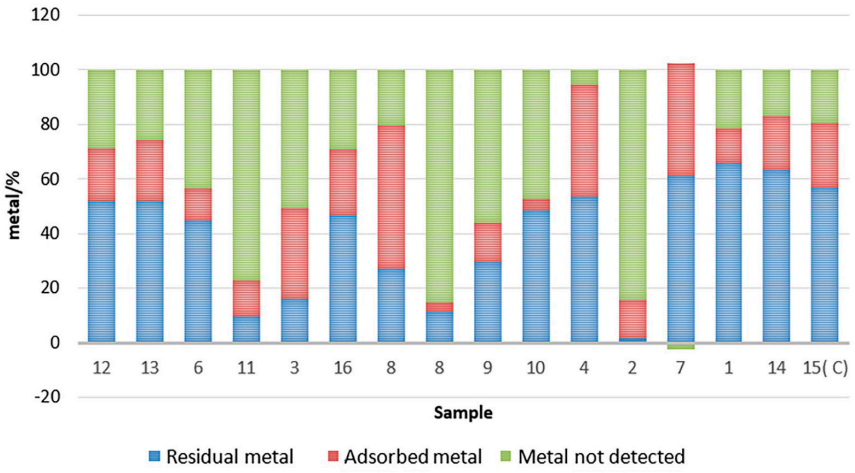

Figure 6. Evaluation of the Hg(II) ion content in the adsorption study.

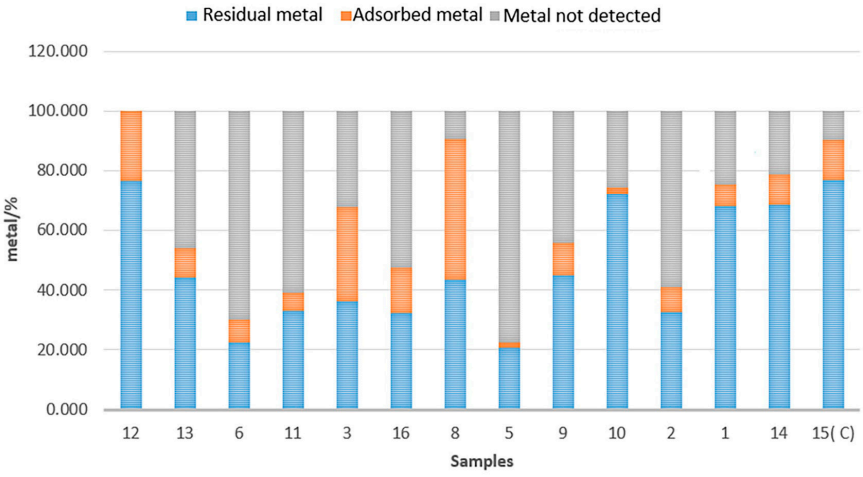

Figure 7. Evaluation of the Pb(II) ion content in the adsorption study.

The values obtained for the remaining solution response is represented in the normal probability graph, see Figure 3, observing that they are close to the error variability. These values do not represent any physical significance and may represent random errors during the process 21. Thus, the effects of the variables determined by the remaining solution response do not statistically influence the adsorption process of metals.

When analyzing experimental condition 13, were observed 52.07% of Hg(II) and 44.30% Pb(II) in the remaining solution. Theoretically, the rest of the metals that were not evaluated in the remaining solution should be adsorbed on the adsorbent 47.94% and 55.70%, respectively. When evaluated by the digestion process, 21.98% and 9.797% of Hg(II) and Pb(II) metals were, respectively determined, showing that the digestion process was not completely efficient, as the material was probably deposited in the digestion block during the process, so that the complete evaluation was not allowed. With the absence of reliability of the digestion method, the amount of metals in the remaining solution becomes more reliable, indicating that the adsorbent system adsorbed on average 59.93% and 51.48% of Hg(II) and Pb(II) metals, respectively, making it an efficient adsorbent system for ions removal.

The PU-AT system is formed by natural materials, cheap, and showed to be a promising adsorbent. In addition, it is metal absorbing capacity indicated to be greater than other toxic metal adsorbents.[26,27]

CONCLUSIONS

For the quantification of TA, the method that used UV-Vis spectroscopy proved to be more suitable for both estimation of the significant variables, as well as to obtain the response surface and the generation of optimal points. Thus, the experimental planning contributed to analyze the influences of several variables in the adsorbent system, in an agile way, contributing to future analyzes, as well as to understand the behavior of the adsorbent system. The molar concentration 0.1000 mol L^{-1} of the AT solution, pH value equal to 7, and contact time equal to 19 hours were determined as the optimal condition for the adsorption of AT on PU.

For the process of determining Hg(II) and Pb(II) metals, the most appropriate was the analysis of the remaining solution, which was more reliable than the determination by digestion. Despite the results of the metals, an optimal region was not found, and significant variables, the PU-AT system evaluated showed to be a good metal ion scavenger, even in the coexistence of different ions and in different amounts. The optimized PU-TA system removed 59.93% of Pb(II) and 51.48% Hg(II) ions in aqueous solutions.

Therefore, the maximum control of external influences of the system is ideal, making the analytical instrumental techniques adequate and efficient for detecting toxic ions, enabling better conclusions about the behavior of metals, and assisting in real future studies of metal retention present in industrial systems.

Conflicts of interest

The authors declare that there is no conflict of interest/competing interest (financial or not) for this study.

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SUPLEMMENTARY MATERIAL

Table S1. Experimental matrix 2^5 and the respective responses using spectroscopic absorption UV-Vis and gravimetry

Experiment	V_1	V_2	V_3	V_4	V_5	% AT UV-Vis	% AT Gravimetry
1	-1	-1	-1	-1	-1	9.506_3	0.697_9
2	1	-1	-1	-1	-1	32.871_7	6.809_7
3	-1	1	-1	-1	-1	40.545_3	0.367_2
4	1	1	-1	-1	-1	25.417_6	0.347_2
5	-1	-1	1	-1	-1	10.760_2	0.542_7
6	1	-1	1	-1	-1	19.834_8	15.902_1
7	-1	1	1	-1	-1	10.674_4	0.662_9
8	1	1	1	-1	-1	46.214_6	2.316_8
9	-1	-1	-1	1	-1	10.201_7	1.645_5
10	1	-1	-1	1	-1	21.616_7	25.865_3
11	-1	1	-1	1	-1	8.9700_0	0.260_1
12	1	1	-1	1	-1	14.799_0	3.198_1
13	-1	-1	1	1	-1	36.755_4	7.721_6
14	1	-1	1	1	-1	55.036_0	4.0415_4
15	-1	1	1	1	-1	24.536_6	1.261_6
16	1	1	1	1	-1	10.368_2	0.7507_2
17	-1	-1	-1	-1	1	92.8500_4	10.268_8
18	1	-1	-1	-1	1	95.3302_0	39.747_3
19	-1	1	-1	-1	1	93.560_0	0.508_4
20	1	1	-1	-1	1	95.013_8	6.618_8
21	-1	-1	1	-1	1	95.661_7	13.241_8
22	1	-1	1	-1	1	95.476_3	60.769_0
23	-1	1	1	-1	1	94.683_5	1.691_1

(continues on the next page)

Table S1. Experimental matrix 2^5 and the respective responses using spectroscopic absorption UV-Vis and gravimetry (Continuation)

Experiment	V_1	V_2	V_3	V_4	V_5	% AT UV-Vis	% AT Gravimetry
24	1	1	1	-1	1	95.938_7	0.575_7
25	-1	-1	-1	1	1	95.112_6	0.371_2
26	1	-1	-1	1	1	94.825_0	3.303_4
27	-1	1	-1	1	1	41.233_1	0.419_6
28	1	1	-1	1	1	54.296_5	1.598_9
29	-1	-1	1	1	1	96.170_5	29.887_9
30	1	-1	1	1	1	96.040_3	49.229_3
31	-1	1	1	1	1	94.502_8	2.350_9
32	1	1	1	1	1	96.433_1	2.614_1

Table S2. Full CCD Experimental Matrix

2**(3) CCD. nc=8 ns=6 n0=2 Runs=16				Replies	
Samples	V_1	V_2	V_3	% AT (UV-Vis)	% AT Gravimetry
12	0	1.68179	0	27.6235	22.2419
13	0	0	-1.68179	13.3489	8.7266
6	1	-1	1	12.8734	8.6495
11	0	-1.68179	0	11.9208	6.1108
3	-1	1	-1	28.4276	5.5913
16 (C)	0	0	0	93.6122	2.5356
8	1	1	1	86.6779	12.7864
5	1	-1	-1	91.2938	7.00568
9	-1.68179	0	0	4.140998	22.5061
10	1.68179	0	0	91.5031	16.2081
4	-1	1	1	47.6055	17.0565
2	-1	-1	1	9.0748	7.0353
7	1	1	-1	91.3084	9.2204
1	-1	-1	-1	6.1781	5.9338
14	0	0	1.68179	92.0140	0.31838
15 (C)	0	0	0	90.4636	17.2019

Table S3. Analysis of variance for CCD using UV-Vis spectroscopy

ANOVA					
	SS	**Df**	**MS**	**F**	**p**
Regression	19566.95	7	3947.333	6.572	0.032504
Waste	4804.75	8	600.594		
Lack of adjustment	4799.79	7	685.685	138.326	0.065378
Error	4.96	1	4.957		
Total SS	22625.42	15			

Variation explained 86.47%
Explainable maximum variation: 99.98%

Table S4. Analysis of variance for CCD using Gravimetry

ANOVA					
Variables	**SS**	**df**	**MS**	**F**	**p**
(2)Var2 (L)	136.3969	1	136.3969	4.827875	0.046734
Var3 (Q)	167.7112	1	167.7112	5.936271	0.029966
Error	367.2753	13	28.2519		
Total SS	671.3834	15			

Variation explained 45.30%

Table S5. Analysis of the signal intensity obtained from ICP OES by ANOVA

ANOVA				
	SS	**Df**	**MS**	**F**
Regression analysis	2.5157	9	0.2795	1.433
Residual error	1.077	6	0.1795	
Lack-of-fit	0.9525	5	0.1905	1.5
Error	0.1245	1	0.1245	
Total SS	3.6219	15		

Variation explained 69.458%

Table S6. ANOVA for adsorption of metals using microwave digester

ANOVA				
	SS	**Df**	**MS**	**F**
Regression analysis	3.104	3	1.034	0.7950
Residual error	2.601	2	1.3005	
Lack-of-fit	2.601	1	2.601	331.334
Error	0.000785	1	0.000785	
Total SS	5.803511	15		

Variation explained 55.17%
Explainable maximum variation 99.99%

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ARTICLE

Carbon and Oxygen Isotope Profile in Brazilian Blueberries (*Vaccinium spp.*)

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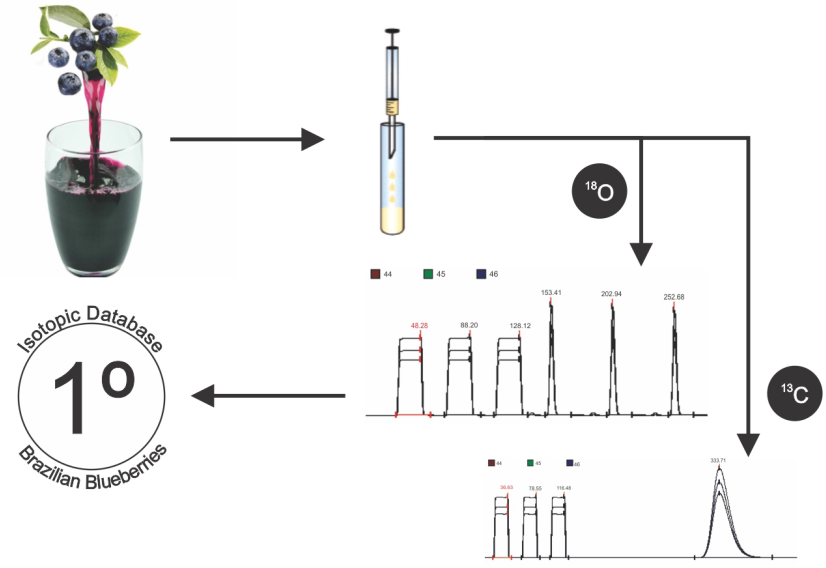

The Brazilian blueberry (*Vaccinium spp.*) production has made remarkable progress in developing quality and quantity. The price of the fruit for fresh and processing purposes is linked to its quality and origin. An effective method for authenticity control and traceability is the stable isotopes method. Carbon (^{13}C) and oxygen (^{18}O) of Brazilian blueberries have never been extensively explored. In this work, the results of ^{13}C and ^{18}O (performed by IRMS) of thirty blueberry samples were presented and discussed, being eleven different cultivars produced in the Southern Brazilian region from mountain and high-altitude. The blueberry showed the typical range of carbon isotopes for C_3 plants, with significant differences between Rabbiteye and Southern Highbush. The $\delta^{13}C$ and $\delta^{18}O$ values showed a significant difference between a mountain and a high-altitude region. This study represents the first isotopic database for Brazilian blueberries, and it can be incorporated into a traceability system. In addition, these results can be used to verify the authenticity of the fruit composition declared on the label and as an effective tool for identifying the geographical origin.

Keywords: isotopic composition, quality control, mass spectrometry, food and beverage analysis, geographical discrimination

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INTRODUCTION

Blueberry (*Vaccinium spp*.) production and consumption have increased in the last decade and become more popular because this fruit shows excellent sensory properties. Also, it has aroused particular interest of people due to its beneficial properties for human health. Blueberries can be consumed naturally or in products, like juice. However, the benefits will only be achieved if the products from blueberry are genuine, without adulteration with cheaper raw, like sugar cane or apple juice.[1] Three blueberry cultivars are responsible for the worldwide production: Highbush (*Vaccinium corymbosum* L.), Lowbush (*V. angustifolium* Ait.) and Rabbiteye (*Vaccinum virgatum* Ait.).[2]

The gastronomic versatility and the growing demand for blueberry stimulated a significant increase in global production, which more than doubled between 2010 and 2019, rising from 439,000 metric tons to nearly 1.0 million metric tons. The most significant production is in the United States, followed by Canada, Chile, and Peru.[3] In Brazil, the production is still relatively low. It was introduced in the 80's in the South region, motivated by the growing world demand and the attractive fresh fruit prices on the European market. Furthermore, Brazil has some advantages such as the possibility of early production in the Northern Hemisphere off-season and the availability of water and land suitable for cultivation.[4] The main cultivars produced are Rabbiteye (Aliceblue, Bluebelle, Bluegem, Briteblue, Climax, Delite, Powderblue and Woodard) and Southern highbush (Misty, O'Neal and Georgiagem). New varieties of Southern highbush cultivars have been introduced, such as Star, Jewel, Emerald, Millenia, Primadonna and Snowchaser.

Several studies have shown the benefits of blueberry, such as effects on cardiovascular disease, type 2 diabetes mellitus, neurological decline, ophthalmologic disorders, bone protection, decrease blood pressure and cholesterol, anti-diabetic properties, cytotoxic activity, for example. In addition, anti-inflammatory action associated with blueberry intake is supported by clinical, animal and in vitro studies.[5] The bioactivity of blueberry extract is mainly due to phenolics compounds, such as anthocyanins, phenolic acids and proanthocyanidins, making it one of the most desirable and nutritious fruits.[6]

Accordingly, the isotope ratio $^{13}C/^{12}C$ expressed in $\delta^{13}C$, C_3 plants such as blueberry, grape and orange, show $\delta^{13}C$ values ranging from -33 to -22‰, and C_4 plants such as corn, sugar cane and sorghum range from -16 to -10‰. Therefore, knowing the isotopic profile is an important way to control adulteration, and preserve the authenticity and the benefits of products from blueberry fruits.[7,8]

Natural variation in carbon isotope ratio ($^{13}C/^{12}C$) has been immensely investigated. Isotopic composition such as $^{13}C/^{12}C$ is used to reduce the database's variability and to detect relatively small amounts of sugar added to fruit products, even when the isotopic values of investigated samples are close to the natural variation range of the fruit.[7] Plants have lower levels of ^{13}C to ^{12}C, these two carbon isotopes occur naturally in nature, due to the fractionations during the photosynthetic cycle. The photosynthetic cycle is responsible for the most significant difference between the values of ^{13}C in C_3 (Calvin cycle) and C_4 (Hatch-Slack cycle) plants. The CO_2 fixed in the photosynthesis will reflect the botanical origin of carbon in plants, as the metabolic pathway discriminates differently against the heavier isotope ^{13}C present in atmospheric CO_2, for plants C_3 and C_4.[9]

Recent research confirmed a variation in chemical composition in small fruits due to the influence of cultivar, genetic background, climate, growing conditions, maturity and post-harvest handling techniques.[10] So, the factors cited, plant type and agriculture practices reflect the isotopic composition of $^{13}C/^{12}C$ and $^{18}O/^{16}O$.[11] For example, the mild water stress increase berry sugar, early or late.[12] In the same way water stress affects stable isotopes, because changes the gradient between atmospheric CO_2 and intercellular CO_2 concentration during the photosynthesis process in the plant.[13]

In the literature, there are several references for isotopic characterization from different fruits,[14,15] but few studies about different blueberry cultivars. Furthermore, there are no studies for stable isotopes in Brazilian blueberries. Therefore, the study aimed to build the first database for stable isotopes of blueberries, including eleven cultivars: Northern Highbush (Bluecrop, Elliot and Duke), Southern Highbush (Misty and O'Neal) and Rabbiteye (Bluegen, Briteblue, Climax, Delite, Florida, and Power blue), characterizing the range of carbon ($^{13}C/^{12}C$) and oxygen ($^{18}O/^{16}O$) values in different blueberry cultivars. In addition, to assess

the effectiveness of the stable isotopes of carbon and oxygen to determine the geographical origin of blueberries.

MATERIALS AND METHODS

Samples

The blueberries were collected during the 2017 and 2018 vegetation seasons from 30 sampling sites in the Southern Brazilian region of Rio Grande do Sul. Samples of different cultivars belonged to three varieties: Northern Highbush (Bluecrop, Elliot and Duke), Southern Highbush (Misty and O'Neal), and Rabbiteye (Bluegen, Briteblue, Climax, Delite, Florida, and Power blue).

Sampling site description

Samples were collected in different private fields located in 7 cities of Rio Grande do Sul. Five from the mountain region: Bento Gonçalves (1 sample Rabbiteye: Bluegen), Caxias do Sul (11 samples: being 10 Rabbiteye: Florida: 3, Briteblue:1, Climax: 3, Bluegen: 2 and Powder Blue:1 and 1 Southern Highbush: O'Neal: 1), Flores da Cunha (3 samples: being Powder Blue:1, Bluegen: 1 and Climax: 1) , Nova Petrópolis (1 sample Florida) and São Marcos (5 samples: Delite: 1, Bluegen: 1, Bluecrop: 1, Climax: 1 and Florida: 1) and two from high-altitude region: Vacaria (8 samples: O'Neal: 2, Climax: 1, Bluecrop: 1, Elliot: 1, Duke: 1, Misty: 1 and Bluegen: 1) and Campestre da Serra (1 sample Florida).

The two regions are about 110 km apart, and the mountain region has an altitude of 750 meters, whereas the high-altitude region has 971 meters. The climatic and environmental data variables, such as the precipitation, the mean annual temperature and the mean annual chill hours, were obtained from National Institute of Meteorology (INMET). Furthermore, information about soil was obtained from the Soil Museum of Rio Grande do Sul (MSRS). The mountain region is marked by a soil well-drained, shallow, developed from volcanic rocks, rocky and sloping. In contrast, the high-altitude region presents the soil poorly drained, deep, developed from basalt, hydromorphic and rocky. Based on the published literature, these variables were the most likely to have significant effects on carbon and oxygen isotope ratio, and the data are relatively accurate for each location. All the samples were collected from irrigated field.

Sample preparation

The juice was extracted from the fruit using a juice maker (Skimsen, Brusque, Brazil), and the samples were manually crushed and after centrifuged at 1750 rpm to separate and remove the seeds and peels from the fruit. The juice was filtered by a membrane with a pore size of 0.8 µm (Millipore Merck, Germany). All samples were subjected to the analyses of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ in triplicate.

Stable isotope ratio ($^{13}C/^{12}C$) analysis

The carbon isotopic ratio ($^{13}C/^{12}C$) of each sample was determined in triplicate using an isotope ratio mass spectrometer (Delta Plus XP, Thermo Fisher Scientific, Bremen, Germany) following total combustion in an elemental analyzer (Flash EA 1112, Thermo Fisher Scientific). Juice from blueberry (1 µL) was injected directly into the reactor; the sample was converted into CO_2 gas by combustion in a quartz reactor with copper oxide, and silvered cobaltous-cobaltic oxide. Under a continuous flow of ultra-pure helium (flow rate at 100 mL min^{-1}) to form CO_2 and H_2O. The gases passed through a reduction column at 680 ºC containing reduced copper whilst water was trapped with magnesium perchlorate for the gases to be separated on a Porapak Q chromatographic column at 45 ºC. The resulting gas (CO_2) was then transferred to the isotope ratio mass spectrometer via a universal continuous flow interface (Conflo III, Thermo Fisher Scientific) to determine the $^{13}C/^{12}C$.[16]

The values of $^{13}C/^{12}C$ are denoted in delta related to the intentional V-PDB (Vienna-Pee Dee Belemnite) and the $\delta^{13}C$ values were calculated against the international reference material Sucrose IAEA-CH-6. (RM 8542, from International Atomic Energy Agency, Viena, Austria). A reference gas CO_2 4.8 (Air Liquide, Paris, France) was used to calibrate against the reference material to ensure the results trueness. The analytical instrumental error was lower than ± 0.20‰.

Determination of $\delta^{18}O$ of water by equilibration (GasBench-IRMS)

Sample preparation was based on the CO_2 equilibrium obtained through the isotopic exchange reaction between CO_2 (g) and water in the sample (L). Samples (500 µL) were transferred into the vial using a pipette, and then the vial was flushed with a mixture of 0.3% CO_2 in helium at a flow rate of 100 mL min^{-1} for 5 min. The complete exchange between the CO_2 from the sample and the helium-CO_2 gas was reached after 24 h of equilibration at 25 ºC. The headspace formed was injected into the peripheral device (Gasbench II, Thermo Fisher Scientific). The helium flow gently moves the headspace of the vial into the fused silica capillary through the diffusion traps (Nafion®) to remove the water and then to a Valco® loop for injection into the gas chromatograph.

The loop injection set up 100 µL aliquots of the sample gas into the gas chromatography column at 70 ºC, with a helium flow of 20 mL min^{-1} at 10-12 psi. The spectrometer then measured the isotope species with mass 44, 45 and 46. The analytical error was lower than 0.20‰, and the samples were analyzed in triplicate.

Method validation

The method was validated, and the following parameters were evaluated: selectivity, linearity, working range, detection limit, quantification limit, accuracy, recovery and calculation of measurement uncertainty. For selectivity and accuracy, tests were carried out by adding cane sugar to blueberry juice. A solution (100% C_4) with cane sugar in ultra-pure water (80 g L^{-1}) was added to blueberry juice at the following concentrations: 1, 2, 5, 10, 20, 30, 50 and 80%. The accuracy values were evaluated by the AOAC Association of Official Agricultural Chemists criteria. A curve reading seven concentrations in seven replicates was prepared for linearity and working range. The detection and quantification limits were calculated by testing a sample containing the matrix. Finally, the calculation of measurement uncertainty of the methods was performed using GUM Workbench 2.4 software.

Statistical analysis

All statistical analyses were performed by SPSS software, version 22.0 (IBM Corporation, New York, USA) at 5% significance level. The normal distribution of the residuals was evaluated through the Kolmogorov Smirnov. Data were analyzed by ANOVA, and if a statistically significant effect was verified, multiple pairwise comparisons were performed using Tukey's test.

RESULTS AND DISCUSSION

Carbon isotopic composition ($^{13}C/^{12}C$)

The $\delta^{13}C$ values for blueberries cultivars are summarized in Table I.

Table I. Isotope composition $\delta^{13}C$ (‰) of pulp from eleven different blueberry cultivars, expressed by maximum, minimum, mean and standard deviation (SD)

Cultivar	n	Variety	$\delta^{13}C$ Mean ± SD* (‰)	$\delta^{13}C$ Minimum (‰)	$\delta^{13}C$ Maximum (‰)
Bluecrop	2	Northern highbush	-24.69 ± 0.47	-25.26	-24.18
Bluegen	6	Rabbiteye	-25.86 ± 0.93	-27.38	-24.93
Briteblue	1	Rabbiteye	-25.80 ± 0.07	-25.86	-25.73
Climax	6	Rabbiteye	-26.06 ± 1.12	-27.41	-24.02
Delite	1	Rabbiteye	-24.97 ± 0.08	-25.06	-24.92
Duke	1	Northern highbush	-24.53 ± 0.05	-24.61	-24.44

continues on the next page

Table I. Isotope composition $\delta^{13}C$ (‰) of pulp from eleven different blueberry cultivars, expressed by maximum, minimum, mean and standard deviation (SD) (continuation)

Cultivar	n	Variety	$\delta^{13}C$ Mean ± SD* (‰)	$\delta^{13}C$ Minimum (‰)	$\delta^{13}C$ Maximum (‰)
Elliot	1	Northern highbush	-24.56 ± 0.04	-24.60	-24.52
Florida	6	Rabbiteye	-25.55 ± 0.70	-26.34	-24.15
Misty	1	Southern highbush	-23.21 ± 0.05	-23.26	-23.15
O'Neal	3	Southern highbush	-24.72 ± 1.13	-26.16	-23.44
Powder Blue	2	Rabbiteye	-25.83 ± 0.99	-26.78	-24.86

*SD = Standard Deviation, significance level of 5%.

Blueberries' $\delta^{13}C$ values ranged from -27.37 to -23.21‰, nearly identical to those found by other authors in C_3 plants, as expected for plants with the same metabolic pathway.[17] The $\delta^{13}C$ values for the Northern Highbush variety in this study were similar to the results found in blueberry produced in the Northern Italian region (-24.20 ± 1.30‰) and the Rabbiteye close to found in the Eastern European (-26.20 ± 1.40‰).[7] However, the isotopic ranges of sugar from blueberry pulp classified by varieties showed a significant difference between the varieties Rabbiteye and Southern Highbush, with lower $\delta^{13}C$ values for Rabbiteye. Previous studies have also indicated that genetic diversity can influence the $\delta^{13}C$ values of plant biomass.[18]

The difference is not statistically significant because blueberry cultivars present a natural variation and $\delta^{13}C$ values specific for each cultivar. Furthermore, other influences in $\delta^{13}C$ values are important, such as plant physiology, plant resistance to climatic factors and cultivation practices adopted.[11] Southern Highbush blueberries are hybrids from *Vaccinium corymbosum* (Highbush, tetraploid). This variety shows characteristics like the need for upland soils, short fruit development periods without organic amendment and plants that do not tolerate winter temperatures below freezing.[19]

The lowest $\delta^{13}C$ values were found for Bluegen cultivar and the highest for Misty. In a study developed with samples from Italy, Poland and Romanian, the values of $\delta^{13}C$ for pulp ranged between -28.60 to -22.10‰ comparing different cultivars; the Brigitta Blue showed the highest $\delta^{13}C$ values and Bluecrop showed the lowest $\delta^{13}C$ value.[20] In our study, the cultivar Climax showed the lowest value. Previous studies with apples showed $\delta^{13}C$ and $\delta^{15}N$ values were statistically influenced by the cultivar and geographical origin, and their interactions. Furthermore, they confirmed the existence of a great effect of the geographical origin and a limited influence due to the variety.[15]

The lower standard deviation found for Briteblue, Delite, Duke, Elliot and Misty happened because there is just one producer for those cultivars in the South.

Oxygen isotopic composition ($^{18}O/^{16}O$)

The $\delta^{18}O$ values for blueberries cultivars are summarized in Table II.

Table II. Isotope composition $\delta^{18}O$ (‰) of pulp from eleven different blueberry cultivars, expressed by maximum, minimum, mean and standard deviation (SD)

Cultivar	n	Variety	$\delta^{18}O$ Mean ± SD* (‰)	$\delta^{18}O$ Minimum (‰)	$\delta^{18}O$ Maximum (‰)
Bluecrop	2	Northern highbush	-0.62 ± 0.48	-1.18	-0.08
Bluegen	6	Rabbiteye	-0.27 ± 1.24	-2.62	1.78
Briteblue	1	Rabbiteye	-0.52 ± 0.09	-0.64	-0.37

continues on the next page

Table II. Isotope composition $\delta^{18}O$ (‰) of pulp from eleven different blueberry cultivars, expressed by maximum, minimum, mean and standard deviation (SD) (continuation)

Cultivar	n	Variety	$\delta^{18}O$ Mean ± SD* (‰)	$\delta^{18}O$ Minimum (‰)	$\delta^{18}O$ Maximum (‰)
Climax	6	Rabbiteye	-0.83 ± 1.99	-3.01	2.62
Delite	1	Rabbiteye	-0.53 ± 0.09	-0.69	-0.45
Duke	1	Northern highbush	-0.10 ± 0.06	-0.17	-0.01
Elliot	1	Northern highbush	+0.14 ± 0.09	-0.07	+0.23
Florida	6	Rabbiteye	-1.65 ± 1.04	-3.04	+0.09
Misty	1	Southern highbush	+0.86 ± 0.08	+0.73	+1.00
O'Neal	3	Southern highbush	-1.05 ± 1.61	-3.12	+1.01
Powder Blue	2	Rabbiteye	-2.00 ± 0.67	-2.79	-1.20

*SD = Standard Deviation, significance level of 5%.

Blueberries $\delta^{18}O$ values ranged from -3.12 to +2.62‰. These results were very similar to those found by Camin et al.[20] in highbush blueberry collected in Italy, Poland and Romania, where the researcher found values ranged from -2.2 to +4.3‰. A variation of $\delta^{18}O$ values also was observed by Klavins et al.,[21] working with blueberries from different countries. They observed a pattern based on the ^{18}O water cycle. Duet to the natural water cycle and precipitations, the heavy oxygen isotope (^{18}O) tends to concentrate more in regions close to the ocean, and lower $\delta^{18}O$ values indicate a greater distance from the ocean. Considering these factors $\delta^{18}O$ values are more characteristic to the geographical location.

Geographical origin

The samples were separated from two regions: mountain and high altitude. The difference between the regions was highly significant at the 95% confidence level for the $\delta^{13}C$ and $\delta^{18}O$ values. The results can be seen graphically in Figure 1, where the $\delta^{13}C$ are plotted concerning the $\delta^{18}O$, differentiating them by region.

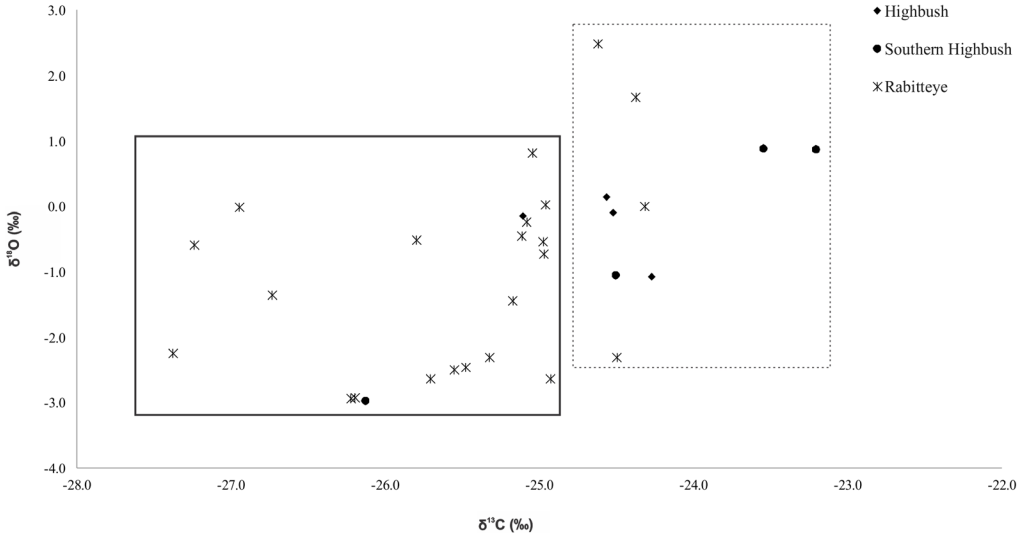

Figure 1. $\delta^{13}C$ versus $\delta^{18}O$ values of all blueberry samples by region.

Variation in the $\delta^{13}C$ values is usually connected to the cultivar, due to the influence of CO_2 fixation during photosynthesis. However, differences in geographical origin considering $\delta^{13}C$ were also found in products, such as olive oil. For example, the $\delta^{13}C$ allowed to differentiate olive oil from Sicily to other regions of Italy. The influence was assigned to the geographical position, considering the latitude of the olive grove.[22] Significant differences related to $\delta^{13}C$ were also found in mangoes cultivated in Mexico from those cultivated in the Ivory Coast, Senegal, and Spain. Meanwhile, $\delta^{18}O$ values in the same study allowed to differentiate mangoes produced in Brazil, Equatorial Guinea, Ivory Coast and Spain from those produced in Mexico, Peru, and Senegal.[23]

In this study, the $\delta^{18}O$ for samples from high-altitude region (0.42 ± 1.18‰) were significantly higher than the values found for samples from mountain (-1.37 ± 1.21‰). Considering that the mountain region has an altitude of 750 meters whereas the high-altitude region has 971 meters. The results showed an increase in the values with increasing altitudes. Taous et al.[24] also found an influence of geographical position in Argan oil in $\delta^{13}C$ and $\delta^{18}O$, with an increased value with increasing altitudes and a negative tendency with latitude, suggesting a continental effect on the coastal oils showing more enriched isotopic values.

Geographical influence, such as latitude, altitude, elevation, and in addition climatic factors like precipitation, hydric stress and light exposure, affect the efficiency of CO_2 fixation. The reason is that $\delta^{13}C$ and $\delta^{18}O$ of plants are influenced by the availability of water, relative humidity, and environmental temperature.[25] These factors influence the plant's stomata, in which regulate leaf diffusive conductance and influence water loss and carbon gain.[26]

The two regions considered in this study showed different precipitation volumes in 2017, in which mountain region showed 1864 mm during this year, whereas in the high-altitude region, the precipitation volume was 1715 mm in the same year.[27] The difference is reflected in the $\delta^{18}O$ values; lower values was found for the mountain region, where the precipitation volume was higher than in the high-alitude region. The volume of precipitation directly influences the oxygen because the heavy oxygen isotope tends to concentrate more in the oceans due to the influence of the natural water cycle and precipitation. As a result, the lower $\delta^{18}O$ values are characteristic of regions distant from the ocean.[21] On the other hand, the temperature for the mountain region was higher, showing an annual mean of 18.30 ºC, while in the high-altitude region 16.10 ºC.[27]

Concerning the geographical position, differences in soil type between the mountain and the high-altitude region can also influence the $\delta^{13}C$ and $\delta^{18}O$ values due to water availability. The soil in the mountain is well-drained, while it is poorly drained in the high-altitude region.[28]

The blueberries can provide excellent nutrients and nutraceutical benefits to human health. However, the region's characteristic and environmental influences are important reasons for the differences found in the $\delta^{13}C$ and $\delta^{18}O$ values in this study. Furthermore, global warming and associated precipitation change impact many agricultural ecosystems.

Results of validation method

The analytical procedure has been adapted to the case of blueberry juice from a method proposed for other fruit juice. All parameters analyzed for the method validating were considered satisfactory (Table III).

Table III. Results of validation parameters of the method

Parameter	Results
Linear range (%)	0 – 100
R^2	0.99
LOD (‰)	0.27
LOQ (‰)	0.87

continues on the next page

Table III. Results of validation parameters of the method (continuation)

Parameter	Results
Z-score (%)	-1.41
Accuracy (%)	0.31
Repeatability (‰)	0.22
Intermediate accuracy (‰)	0.07
Recovery (%)	100.7
Measurement uncertainty (‰)	0.31

The standard deviation used to calculate the repeatability was 0.09‰. It was very similar to the standard deviation that Jamin et al.[29] found and according to the repeatability recommended by European Committee for Standardization (CEN) for orange and pineapple juice, beet and cane sugar (CEN - ENV12140) particularly on the question whether the ENV can be converted into an European Standard (EN).[16]

The isotopic recovery has been determined using low, medium and high concentrations of a test mixture of sucrose solution in a blueberry juice with known isotopic content. The recovery reached was between 97-103% according to the recommendation by Codex Alimentarius Commission.[30] It shows that the method employed effectivelly applies to blueberry juice and is free of isotopic effects.

CONCLUSION

In conclusion, this study represents the first isotope database for Brazilian blueberries that covers eleven cultivars from three different varieties. The range of $\delta^{13}C$ and $\delta^{18}O$ were characterized and the database could be incorporate into a traceability system, helping future studies about the food industry and authenticity products. In addition, the results showed satisfactory classification performances to discriminate blueberries of the mountain from those of the high-altitude region. The results are rather explorative, and they can be excellent support for the existence of a potential indication of geographical origin for Brazilian blueberries.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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ARTICLE

Use of Hydrodistillation to Obtain and Fractionate Essential Oils Simultaneously

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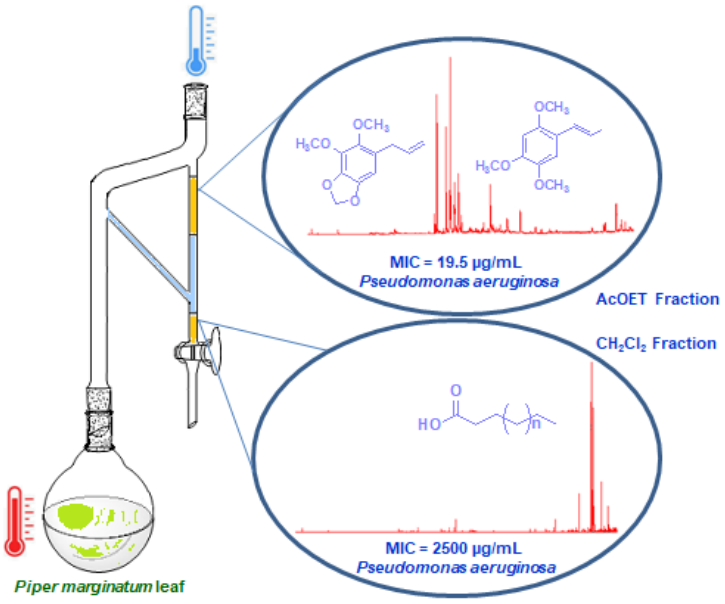

A simple method was developed to obtain and fractionate essential oil simultaneously by hydrodistillation. With this method it was possible to obtain essential oils from the leaves of *Piper arboreum* with the cadinol content ranging from 17.7 to 57.9%. In the essential oils from the leaves of *P. aduncum* was identified dillapiole with content ranging from 4.6 to 96.9%; and further, essential oil from *P. marginatum* with the presence of phenylpropanoids as minor compounds. The essential oils of the three *Piper* species varied in antimicrobial activity when fractionated, with *P. marginatum* oil exhibiting the lowest minimum inhibitory concentration at 19.5 µg mL^{-1} for the *Pseudomonas aeruginosa* bacterium. The method was efficient for the separation and concentration of chemical constituents of essential oils from the same plant, able to distinguish the different chemical profiles, both qualitatively and quantitatively.

Keywords: hydrodistillation, liquid-liquid extraction, essential oil, *Piper*, Piperaceae, antimicrobial

INTRODUCTION

Essential oils obtained from plants are generally constituted of complex mixtures of volatile compounds, mainly terpenes and their oxygenated derivatives such as alcohols, aldehydes, ketones, carboxylic acids, phenols, ethers, and esters as well as phenylpropanoids.[1,2] Essential oils are obtained by various types of analytical methods, most often by hydrodistillation in the research laboratory.[3] Care with the essential oil purification steps is important, due to the oil's high volatility, complex mixtures, chemical instability and low yield. The fractionation of essential oils is generally carried out by supercritical fluid extraction using

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CO_2, liquid–liquid extraction and vacuum fractionating column.[4-6] Orange essential oil was fractioned by liquid–liquid extraction using a perforated rotating disc contactor. This is an efficient extraction method but complex apparatus is needed to control the many variables.[7] Most essential oil fractionation is not performed during the extraction process, as in the case of the fractionation of acid lime essential oil containing different systems of solvent.[8] The simultaneous obtainment and fractionation of essential oils has been the subject of some studies with positive results, but requires specific equipment, which is not commercialization available.[9-10] Simultaneous distillation-extraction of *Artemisia argyi* and *Xylopia aromatica* essential oils were carried out with a specific apparatus.[11-12]

The present study was directed to the simultaneous obtainment and fractionation of essential oils from plants via hydrodistillation without the distillation of organic solvents, using only the Clevenger apparatus, which is widely available and inexpensive. The species of *Piper aduncum, Piper marginatum* and *Piper arboreum* were chosen because they belong to the Piperaceae family, which was the target of our systematic study of plant bioactive compounds. A further reason was because their essential oils present well-differentiated chemical profiles. The essential oil of *P. aduncum* is basically constituted of one major compound. Essential oil of *P. arboreum* consists of several chemical constituents; and the essential oil of *P. marginatum* is predominantly from one chemical class, the phenylpropanoids. The essential oil of *P. aduncum* is a natural source of phenylpropanoid dilapiol and has several biological activities, mainly as an insecticide, given its high content of dilapiol.[13] Essential oil of *P. arboreum* showed antimicrobial activity and sesquiterpene curcumene was identified as the major compound.[14] *P. marginatum* is a medicinal plant commonly used in the form of tea against diseases of the gastrointestinal tract; the phenylpropanoids *E*-asarone and *Z*-asarone are considered one of the chemical markers of their essential oil.[15] Additionally, the antimicrobial activity oils and their organic fractions obtained were evaluated against the microorganisms *Enterococcus faecalis, Bacillus subtilis, Staphylococcus aureus, Klesiella pneumoniae, Pseudomonas aeruginosa* and *Microsporum gypseum.*

MATERIALS AND METHODS

Botanical material

For the proposed study, were selected three *Piper* species: *P. arboreum, P. marginatum and P. aduncum*. Leaves from the *Piper* species were collected from a fragment of forest located in the city of Recife, state of Pernambuco, Brazil. These species were identified by Dr. Margareth F. de Sales of the Department of Biology of the Federal Rural University of Pernambuco. A voucher of each species had been previously deposited in the Vasconcelos Sobrinho Herbarium of UFRPE with the numbers 18179, 48210 and 49250.

Obtaining of essential oils

To obtain each oil sample, it was used 500 g of fresh leaves from *P. arboreum, P. marginatum* and *P. aduncum*. Leaves were crushed and submitted to hydrodistillation in a modified Clevenger apparatus for 2 hours with addition of 5 mL of each of the following organic solvents: hexane, chloroform, ethyl acetate, and ethyl ether in Clevenger apparatus as illustrated in Figure 1. The samples of essential oils were treated with anhydrous sodium sulfate and stored at around 8 °C for further analysis. The organic fractions collected were dried under vacuum at 40 ºC.

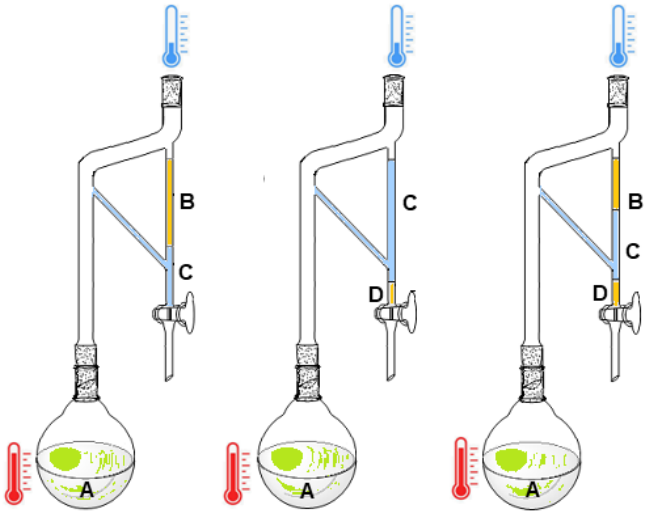

Figure 1. Design of the modified Clevenger device used to obtain and fractionate essential oils simultaneously. Water decoction (A); hexane or ethyl acetate or ethyl ether (B); hydrolate (C); chloroform or dichloromethane (D).

GC Analysis

Oil samples were analyzed using a Hewlett–Packard 5890 Series II GC apparatus equipped with a flame ionization detector (FID) and a J & W Scientific DB-5 fused silica capillary column (30 m × 0.25 mm i.d.), with a programmed temperature from 60 to 246 ºC at 3 ºC/min. The injector and detector temperatures were 260 and 280 ºC, respectively. Hydrogen was used as the carrier gas at a flow rate of 1.0 mL/min; injection was in split mode (1:30) and the injection volume was 1.0 µL.

Chemical analysis

Oil samples were analyzed using a Varian GC/MS (GC: Varian 431/GC-MS: Varian 220-MS, ionic trap detector) system operating in the EI mode at 70 eV, equipped with a J & W Scientific DB-5 fused silica capillary column (30 m × 0.25 mm i.d.), with programmed temperatures from 60 to 246 ºC by 3 ºC/min. The injector and detector temperatures were 260 and 280 ºC, respectively. The carrier gas was helium, 1 mL/min flow rate, split mode (1:30), with an injected volume of 1.0 µL of a solution containing 3 mg mL^{-1} of oil in hexane. The initial identification of the separated components of the essential oil was carried out by comparison with previously reported values of retention indices, obtained by co-injection of oil samples and C11–C24 linear hydrocarbons and calculated using the Van den Dool & Kratz equation.[16] Subsequently, the MS acquired for each component was matched with those stored in the Wiley/NBS mass spectral library of the GC–MS system and with other published mass spectral data.[17] All the analyses were carried out in triplicate.

Antimicrobial activity

Essential oil samples were used to evaluate the antimicrobial activity for the bacteria *Staphylococcus aureus* (02), *Enterococcus faecalis* (138), *Bacillus subtilis* (86), *Klesiella pneumoniae* (396), *Pseudomonas aeruginosa* (416) and *Microsporum gypseum.* Saubouraud liquid culture media were used for fungi and a Mueller Hinton liquid medium for bacteria. The microplates were grown at 37 ºC for 24 h for bacteria and at 30 ºC for 72 h for fungi. The microplates were developed with the addition of 10 µL of a 0.01% resazurin solution and incubated for 3 h. The minimum inhibitory concentration (MIC) values of each sample were determined according to the previously reported protocol.[18] Metronidazole and Fluconazole were used as the positive control and organic solvents as the negative control.

RESULTS AND DISCUSSION

With the proposed method, essential oils were obtained from the leaves of *P. aduncum* with different chemical profiles without performing additional steps to obtain the oil. Simultaneous extraction and fractionation allowed obtaining the essential oil from *P. aduncum* leaves with only two chemical constituents: dillapiole (96.9%) and myristicin (0.9%) when hexane (AdHex) was used for fractionation (Table I). Essential oil obtained by fractionation with chloroform (AdChl) showed, in addition to the dillapiole, the compounds δ-cadinene (1.3%), α-amorphene (4.0%), myristicin (1.2%), germacrene B (0.8%) and 9-epi-(E)-caryophyllene (2.2%). A surprising result was observed for the oil fractionated with ethyl ether (AdEte), where oleic acid was identified as the major compound (64.4%) and the dillapiole content only 4.6%. The essential oil that presented the highest number of compounds had been fractionated with ethyl acetate (AdOEt). In this fraction were identified nine chemical constituents and dillapiole (68.2%) was obtained as the major compound. Previous studies with the essential oil of *P. aduncum* leaves revealed the presence of dillapiole to a maximum content of 90%.[19] Variation from 4.6 to 96.9% in dillapiole content in essential oils of *P. aduncum* species collected at the same location has not been reported, not even in studies of seasonality and circadian rhythm.[20, 21]

The chemical profiles of essential oils from *P. arboreum* leaves fractionated with the solvent chloroform (ArChl) and hexane (ArHex) were qualitatively and quantitatively different when compared with the unfractionated oil (Figure 2). Cadinol sesquiterpenoid was identified as the major compound in the ArChl and ArHex oils in amounts of 57.9% and 55.0%, respectively (Table I). Cadinol content was identified in the unfractionated oil at 17.7%; the major compound was γ-cadinene, with 23.4%. In previous studies with essential oil from *P. arboreum* leaves, the major compounds identified were the terpenes curcumene, *δ*-cadinene, *ß*-caryophyllene, germacrene D and bicyclogermacrene, with cadinol identified only as a minor compound.[22,23]

Table I. Chemical constituents of essential oils fractionated and unfractionated from *P. arboreum*, *P. aduncum* and *P. marginatum* leaves

Compounds	IR[a]	IR[b]	*P. arboreum oils*			*P. aduncum oils*					*P. marginatum oils*					
			Ar	ArChl	ArHex	Ad	AdChl	AdHex	AdEte	AdOEt	Ma	MaHex	MaDic1	MaEte	MaOEt	MaDic
α -Pinene	932	925	-	-	-	-	-	-	-	-	3.4	-	-	-	-	-
β-Pinene	974	975	-	-	-	-	-	-	-	-	3.8	-	-	-	-	-
δ-3-Carene	1008	1008	-	7.4	-	-	-	-	-	-	-	-	-	-	-	-
1.4-Cineole	1014	1014	-	5.8	-	-	-	-	-	-	-	-	-	-	-	-
β-Phellandrene	1025	1029	-	-	-	-	-	-	-	-	6.8	-	-	-	-	-
E-ocimene	1044	1049	-	-	-	-	-	-	-	-	2.6	-	-	-	-	-
β-*Z*-ocimene	1226	1226	-	-	-	-	-	-	-	0.9	-	-	-	-	-	-
Carvona	1239	1238	-	-	-	-	-	-	-	4.3	-	-	-	-	-	-
β-*E*-ocimene	1235	1235	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-
Phenylacetaldehyde	1036	1036	-	-	-	-	-	-	-	2.4	-	-	-	-	-	-
δ-Elemene	1335	1338	-	-	-	-	-	-	-		1	-	-	-	-	-
isoledene	1374	1374	4.2	-	-	-	-	-	-		-	-	-	-	-	-
α-Copaene	1374	1375	3.1	-	-	-	-	-	-		-	-	-	-	-	-
Z-Caryophyllene	1408	1409	9.3	-	-	-	-	-	-	2.3	-	-	-	-	-	-
Aromadendrene	1439	1439	-	-	-	-	-	-	-		2.1	13.1	7	1	1.2	0.7
cis-Prenyl limonene	1443	1445	-	-	-	-	-	-	-	0.8		-	-	-	-	-
α-Himachalene;	1449	1450	-	4.17	-	-	-	-	-		2.1	-	-	-	-	-
α-Humulene	1452	1452	4.6	-	-	-	-	-	-	-		-	-	-	-	-
Croweacin	1457	1458	-	-	-	-	-	-	-	-	3.5	17.5	-	-	2.6	1.2
9-*epi-(E)*-Caryophyllene	1464	1465	5.6	-	-	-	2.2	-	-	-	-	0.8	7.8	3.6	-	-

(continues on the next page)

Table I. Chemical constituents of essential oils fractionated and unfractionated from *P. arboreum*, *P. aduncum* and *P. marginatum* leaves (cont.)

Compounds	IR[a]	IR[b]	*P. arboreum* oils			*P. aduncum* oils					*P. marginatum* oils					
			Ar	ArChl	ArHex	Ad	AdChl	AdHex	AdEte	AdOEt	Ma	MaHex	MaDic1	MaEte	MaOEt	MaDic
γ-Muurolene	1478	1479	1.3	-	-	-	-	-	-	-	-	-	-	-	-	-
γ-Himachalene	1481	1482	-	3.1	5.9	-		-	-	-	-	-	-	-	-	-
α-Amophene	1483	1487	-	-	-	-	4	-	-	-	7.3	7.6	2.1			
Methyl isoeugenol	1491	1493	-	-	-	-	-	-	-	-	1.1	0.3	0.6	0.8	-	-
cis-cadina-1.4-diene	1495	1495	-	-	-	-	-	-	-	5.4	-	-	-	0.7	-	-
Valencene	1496	1497	4.9	-	8.19	-	-	-	-	-	-	-	-	-	-	-
Viridiflorene	1496	1496	9.6	-	1.98	-	-	-	-	-	-	-	-	-	-	-
Epizonarene	1501	1502		-	-	-	4.0	-	-	5.9	-	-	7.7	-	-	-
γ-Cadinene	1513	1513	23.4	0.5	2.0		-	-	-	-	-	-	-	-	-	-
Myristicin	1517	1518	-	-	-	1.0	1.2	0.9	-	-	-	-	-	-	-	-
δ-Cadinene	1522	1522	-	-	-	1.2	1.3	-	-	-	-	-	-	-	-	-
Nerolidol	1561	1561	-	-	-	-	-	-	-	-	4.7		1.8	1.2	20.1	
Spathulenol	1577	1579	-	8.0	14.0	-	-	-	-	-			-	2.2	-	0.3
Germacrene B	1559	1560	-	-	-	-	0.8	-	-	-		-	-	-	-	
Carotol	1594	1597	-	-	-	-	-	-	-	-	1.9	-	-	-	-	
Guaiol	1600	1601	-	-	-	-	-	-	-	-	0.5	-	-	-	10.0	1.7
Z-asarone	1616	1616	-	-	-	-	-	-	-	-	18.2	19.3	25.4	22.4	26.4	1.2
Dillapiole	1620	1622	7.3	4.1	-	91.0	76.7	96.9	4.6	68.2	13	14.8	19.5	17.3	9.1	0.6
Cadinol	1652	1651	17.7	57.9	55.0	-	-	-	-	-	-	-	-	-	-	-
E-asarone	1675	1676	-	-	-	-	-	-	-	-	4.7	4.7	11.5	10.3	7.5	0.5

(continues on the next page)

Table I. Chemical constituents of essential oils fractionated and unfractionated from *P. arboreum*, *P. aduncum* and *P. marginatum* leaves (cont.)

Compounds	IR[a]	IR[b]	*P. arboreum* oils			*P. aduncum* oils					*P. marginatum* oils					
			Ar	ArChl	ArHex	Ad	AdChl	AdHex	AdEte	AdOEt	Ma	MaHex	MaDic1	MaEte	MaOEt	MaDic
Geranyl linalol	1960	1963	-	-	-	-	-	-	0.7	-	-	-	-	-	-	-
Octadecanol	2077	2074	-	-	-	-	-	-	0.4	-	-	-	-	-	-	-
Methyl linoleate	2095	2094	-	-	-	-	-	-	0.6	-	-	-	-	-	-	-
Linoleic acid	2132	2130	-	-	-	-	-	-	64.4	-	-	-	-	-	-	-
Oleic acid	2141	2144	-	-	-	-	-	-	9.1	-	-	-	-	-	-	-
Tetracosane	2400	2404	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-
Pentacosane	2500	2509	-	-	-	-	-	-	11.4	-	-	-	-	11.1	-	-
Fatty acids	-	-	-	-	-	-	-	-	-	-	-	-	1.9	-	3.8	65.7
Total (%)			91.1	91.0	87.1	93.2	90.2	97.8	93.7	90.4	76.7	78.1	85.3	70.6	80.7	71.9
Oil yields (%)			0.02	0.01	0.03	0.20	0.09	0.11	0.12	0.12	0.33	0.23	0.11	0.31	0.14	0.08

[a]Retention indices from the literature; [b]Retention indices calculated; *P. arboreum* essential oil unfractionated, (Ar) chloroform (ArChl), hexane (ArHex) fractions; *P. aduncum* essential oil unfractionated (Ad), chloroform (AdChl), hexane (AdHex), ethyl (AdEte), ethyl acetate (AdOEt) fractions; *P. marginatum* essential oil unfractionated (Ma), dichloromethane (MaDic1) hexane (MaHex), ehyl (MaEte), ethyl acetate-dichloromethane (MaOEt-MaDic) fractions.

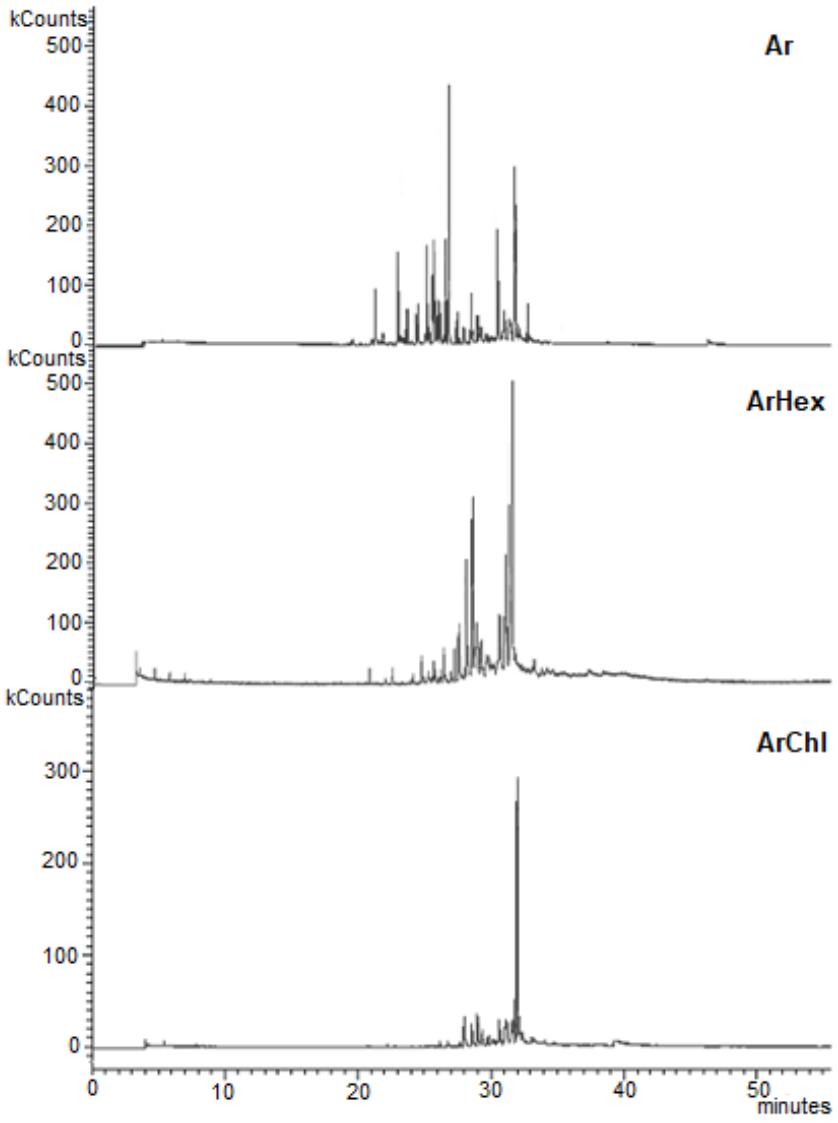

Figure 2. Chromatogram of essential oils unfractionated (Ar), chloroform fraction (ArChl) and hexane fraction (ArHex) from *P. arboreum* leaves.

Obtaining and fractioning the essential oil from *P. marginatum* leaves was carried out with a three-phase solvent system composed of dichloromethane-water-ethyl acetate, with two chemical profiles of the oil obtained simultaneously (Figure 3). In the ethyl acetate fraction were identified four major compounds: phenylpropanoids *Z*-asarone (26.4%) and dillapiole (9.1%) as well as sesquiterpenes nerolidol (20.1%) and guaiol (10.0%). The dichloromethane fraction showed major peaks related to hydrocarbons and long chain fatty acids. The fractionation of the essential oil from the leaves of *P. marginatum* using ethyl ether, hexane or dichloromethane separately were also obtained, enabling identification, in addition to the phenylpropanoids Z-asarone, dillapiole, croweacin and *E*-asarone, the sesquiterpenes aromadendrene and himachalene as the major constituents. The chemistry of *P. marginatum* essential oil is well known and more than seven different chemotypes have been reported for the plant.[24] Previous studies with essential oil obtained from the leaves of allopatric species of *P. marginatum* have revealed a great variation in chemical composition: anethole (45.9%), isosafrole (37.3%), *E*-asarone (32.6%), *Z*-asarone (30.4%), anisaldehyde (22.0%) and notosirnol (22.7%).[25-27]

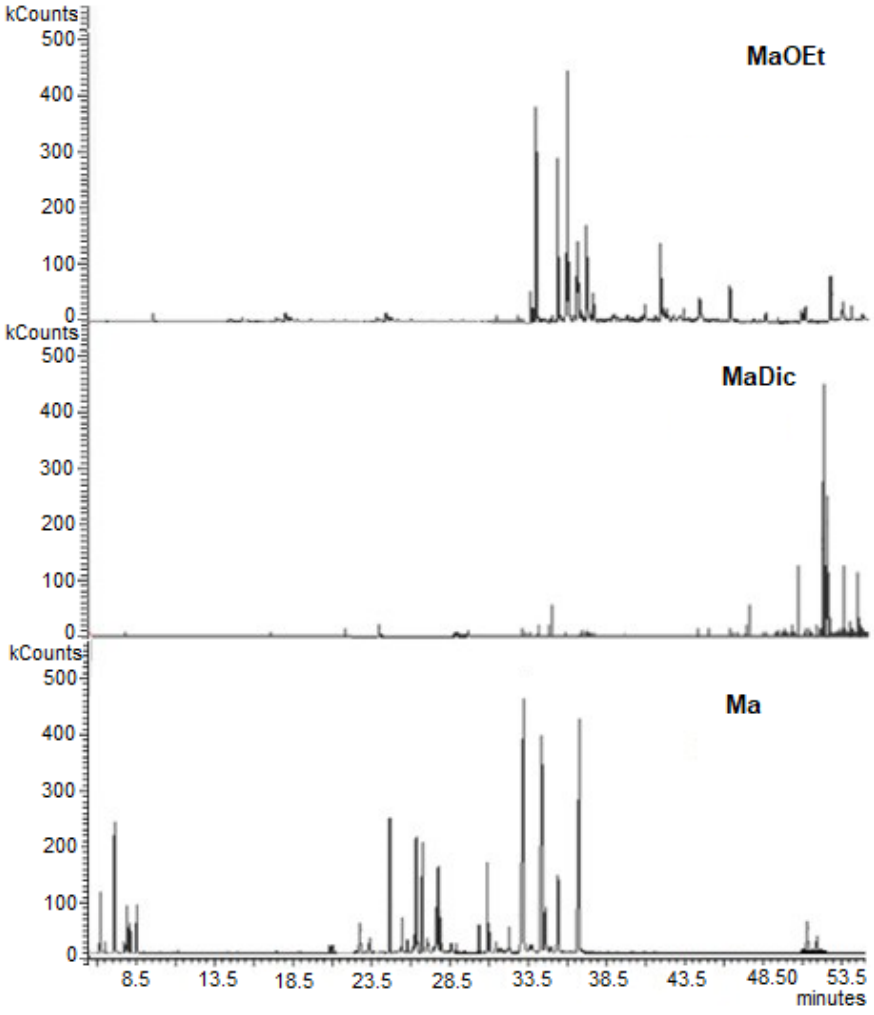

Figure 3. Chromatogram of essential oils unfractionated (Ma) and ethyl acetate-dichloromethane (MaOEt-MaDic) fraction from *P. marginatum* leaves.

In general, the method allowed obtaining different chemical profiles of the essential oils of the leaves of *P. aduncum*, *P. arboreum*, and *P. marginatum*, using only the Clevenger apparatus. This is the main difference between our method and those previously reported: organic solvents were added to the Clevenger apparatus as the stationary phase of a chromatographic column and condensed water together with the plant volatiles, as the mobile phase. Additionally, all fractionated and unfractionated essential oils showed antimicrobial activity against the six microorganisms tested with MIC values ranging from 19.5 to 2500 µg mL^{-1} (Table II). The best results were observed for *P. marginatum* oils fractionated with ethyl acetate (MaOEt) and ethyl ether (MaEte) which presented MIC of 19.5 and 78.1 µg mL^{-1} against *P. aeruginosa* bacteria. However, *P. marginatum* oil fractionated with dichloromethane exhibited an MIC of 2500 µg mL^{-1}; the phenylpropanoids present in MaOEt and MaEte oils were identified in the dichloromethane fraction as minor compounds. The essential oils of *P. aduncum* were the least active against the microorganisms tested. Essential oil containing dillapiole (68.4%) exhibited lower MIC of 625, 625 and 312.5 µg mL^{-1} against the microorganism *E. faecalis, K. pneumoniae* and *M. gypseum*, respectively, indicating the influence of dillapiole content on the antimicrobial activity of the oil. The antimicrobial activity of the essential oil of *P. arboreum* also varied when the oil was fractionated. ArChl oil had the highest cadinol content, being the least active when compared to the Ar and ArHex oils, except for the bacteria *S. aureus* where ArChl oil exhibited a MIC of 312.5 µg mL^{-1}.

Table II. Antimicrobial activity with MIC values in μg mL^{-1} of fractionated and unfractionated essential oils from leaves of *P. arboreum*, *P. aduncum* and *P. marginatum*

Oil Essential Samples	Bacteria					Fungi
	E. faecalis	***K. pneumoniae***	***S. aureus***	***B. subtilis***	***P. aeruginosa***	***M. gypseum***
Ma	1250	1250	1250	2500	19.5	1250
MaOEt	312.5	312.5	2500	1250	19.5	625
MaDic	1250	1250	2500	> 2500	2500	2500
MaHex	312.5	312.5	625	1250	321.5	312.5
MaEte	1250	1250	2500	2500	78.1	2500
MaDic1	625	1259	1250	1250	312.5	1250
Ad	2500	2500	2500	2500	2500	2500
AdOEt	625	625	625	1250	1250	312.5
AdEte	2500	2500	1250	2500	2500	2500
AdHex	2500	2500	1250	2500	1250	na
AdChl	2500	2500	250	1250	2500	na
Ar	625	312.5	625	625	1250	na
ArChl	2500	2500	312.5	2500	1250	na
ArHex	625	1250	625	625	1250	na
Metronidazole[a]	156.3	19.5	78.1	2500	19.5	na
Fluconazole[a]	na[b]	na	na	na	na	19.5

[a]Positive controller; [b]not evaluated

CONCLUSIONS

The model developed made it possible to obtain essential oils with unidentified chemical constituents in unfractionated oils, with a significant variation in the contents of the major compounds. Most of the time, because essential oils consist of a complex mixture of volatile compounds, these are difficult to distinguish.

It is not always possible to carry out a liquid-liquid fractionation of essential oils, after obtaining them by hydrodistillation, due to the low concentration of the constituents in the hydrolate and the immiscibility of most of the oil in an aqueous medium. In the present study, it was possible to obtain and fractionate the essential oils simultaneously, without adding a fractionation step, using a small amount of solvent, in a quick and simple way.

The method is applicable to biomonitored chemical studies of essential oils with identification of minor compounds and for association of different chemical profiles with the biological activity of the oil.

Conflicts of interest

No potential conflict of interest was reported by the authors.

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ARTICLE

Voltammetric Assessment of 8-Oxoguanine at a Nano-Structured Carbon Materials Based Modified Glassy Carbon Electrode

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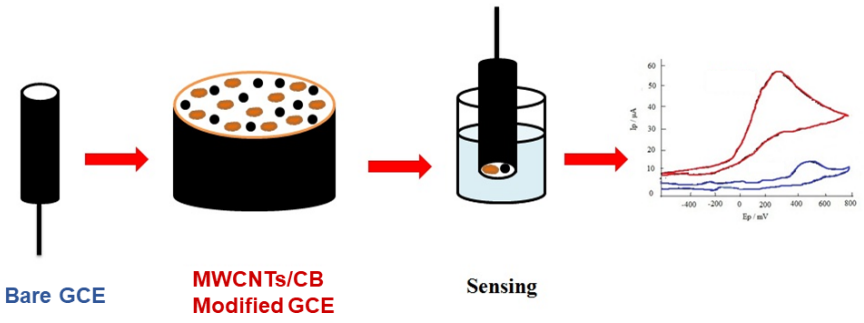

In this study, we have investigated the voltammetric behavior of 8-Oxoguaine on a Multi walled carbon nanotubes / Carbon black modified glassy carbon electrode. The voltammetric oxidation of 8-Oxoguaine on modified electrode was electro-catalytic in nature. The voltammetric oxidation of the substrate was a pH dependent reversible process. Using cyclic voltammetry, a method for the determination of 8-Oxoguaine was developed. At a pH of 7.4 in phosphate buffer solution, the concentration of 8-Oxoguanine was linear from 30 to 100 µM. The detection limit and quantification limit of 8.38 µM and 27.95 µM were obtained from calibration graph, respectively. The modified electrode shows good recovery and reproducibility with relative standard deviation of 2.12%. The inter-day and intra-day assays were also studied.

Keywords: DNA damage, 8-Oxoguanine, voltammetry, modified electrode, nanomaterials

INTRODUCTION

Some of the genetic diseases like tumors were caused by the damage of DNA. One has to investigate the mechanism with which the diseases occur once the damage of DNA happens that causes genetic information change, so that we can find the root cause and prevention measures of these diseases. Therefore, it is very much needed to develop methods for the analysis of damaged DNA molecules which will act as tools for these types of investigations. The main challenge is that, in presence of large quantities of normal bases, it is very difficult to analyze adducts which are present in very low amounts. Very high amount of 8-Oxoguanine were detected in the tissues of urine and lungs of smokers,[1] also in patients body fluid suffering from cancer, chronic hepatitis, diabetes, and deceases related to aging.[2] Therefore, the method developed to detect them should be highly sensitive and selective in nature.

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One of the major common DNA lesions obtained from reactive oxygen species is 8-Oxoguanine whose structural formula is shown in Figure 1. There were many reports on the detection of 8-Oxoguanine using conventional methods such as high performance liquid chromatography with electrochemical detection[3,4,5] and gas chromatography with mass spectroscopy.[6,7]

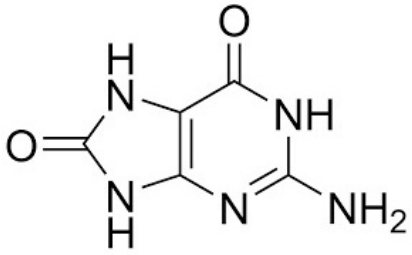

Figure 1. Structure of 8-Oxoguanine.

Electrochemical sensors are becoming a very attractive method to monitor concentration of many drug molecules in analytical field. The remarkable significance of nanomaterials and nanoparticles in recent years has attracted many researchers in the area of developing sensors.[8,9,10] Especially, nanomaterials synthesized from carbon have attracted many research scientists after their discovery. Among them, carbon nanotubes, fullerenes, graphene and carbon dots have gained much attention in the field of development of electrochemical sensors for detection of variety of molecules. Carbon nanotubes are one-dimensional nanomaterials of hollow tubular shape consisting of carbon atoms in hexagonal shape.[11] Based on arrangements of the graphene cylinders in them, carbon nanotubes were classified into single walled carbon nanotubes (SWCNTs) and multi walled carbon nanotubes (MWCNTs). Even though both show electrochemical properties like conductivity,[12] catalytic activity,[13] stability[14] and biocapatibility,[15] multi walled carbon nanotubes are extremely active than the single walled carbon nanotubes since earlier one contains hole-like defects during its formation. Carbon black (CB), a nanomaterial having low cost shows excellent electrochemical properties. Because of the presence of defective sites, it can also be a good choice for the development of electrochemical sensors.[16] Because of all these properties, we have tried to develop a voltammetric sensor for the detection of 8-Oxoguaine.

There are few reports on electrochemical oxidation and determination of 8-Oxoguaine. In the year 2000, there was an attempt to study the electrochemical oxidation on a glassy carbon electrode.[17] There was a method described for detection of 8-Oxoguaine along with uric acid.[18] The differential pulse voltammetry was used in a wide pH range. The gold electrode was used by Ferapontova to study electrochemistry of guanine and 8-Oxoguaine.[19] They used cyclic voltammetry for the kinetic analysis of oxidation and found that it was a quasi-reversible in nature. There were use of pretreated boron doped diamond electrodes,[20] screen printed electrodes,[21] edge plane pyrolitic graphite electrode modified with SWCNTs,[22] edge plane pyrolitic graphite electrode modified with SWCNTs and AuNPs,[23] SWCNTs modified GCE,[24] MWCNTs modified GCE,[25] rGO-MWCNTs modified GCE,[26] sulphur doped rGO modified GCE,[27] PtNF-GO modified GCE,[28] and screen-printed carbon electrodes modified with carboxy-functionalized multi walled carbon nanotubes[29] were also used for the voltammetric study. Recently there was a comprehensive review on sensing of 8-Oxoguanine.[30] But the voltammetric behavior of 8-Oxoguanine was not reported yet on a glassy carbon electrode modified with multi walled carbon nano tubes / carbon black. Therefore, we have undertaken the title study since there is a scope for construction of new nano-composite electrodes and use them to determine 8-Oxoguaine.

MATERIALS AND METHODS

Chemicals

All chemicals used are of analytical grade. Multi-walled carbon nanotubes (O.D. x L of 7-15 nm x 0.5-10 μm), 8-Oxoguaine (in amber glass vial) and Carbon black (CB) were obtained from Sigma-Aldrich. The required amount of the substrate was weighed accurately to prepare stock solution and nitrogen gas was flushed gently to remove any oxygen so that premature oxidation of 8-Oxoguaine can be avoided. 0.1 M monobasic sodium phosphate and 0.1 M dibasic sodium phosphate trihydrate were employed to obtain the phosphate buffer solutions (PBS). Doubly distilled water was used to prepare all the solutions which are used in this study.

Instrumentation

Voltammetric runs were recorded on a MARUTEK Potentiostat (Bangalore, India), which has a usual three-electrode cell. Three electrodes were used in the cell: a glassy carbon electrode (unmodified and modified) acting as a working electrode, a Pt wire acting as a counter and a Ag/AgCl serving as a reference electrode. All the potentials in this paper were recorded and given against the Ag/AgCl reference electrode (3 M KCl). The pH of the solutions employed were determined using a pH meter.

MWCNTs/CB modified electrode preparation

2 mg of MWCNTs and CB each was dispersed in 10 mL acetonitrile and agitated in an ultrasonicator to get a stable suspension of MWCNTs/CB. On a polishing cloth, the GCE (unmodified and modified) was polished carefully using α-alumina slurry, and subsequently washed thoroughly with distilled water. Thus, obtained clean GCE was drop coated with 5 μl of MWCNTs/CB suspension already prepared and dried in open atmosphere.

By employing $K_3Fe(CN)_6$ (1.0 mM) as a redox probe at different scan rates, the electroactive surface area of bare and modified GCE were computed. The Randles–Sevcik equation for a reversible process was used:

$$Ip = (2.69 \times 10^5)\, n^{3/2} A D_R^{1/2} Co\, v^{1/2} \qquad \text{Randles–Sevcik equation}$$

where Ip is the anodic peak current, n is the number of electrons transferred, A is the electrode surface area, D_R is diffusion coefficient, v is the scan rate and Co is the concentration of $K_3Fe(CN)_6$.

The values of n and D are 1 and 7.6×10^{-6} cm^2 s^{-1} for 1.0 mM $K_3Fe(CN)_6$ with 0.1 M KCl as electrolyte. Using slope of the graph of Ip against $v^{1/2}$, the electroactive areas of bare and modified electrodes were computed. For the modified electrode, the area was 0.1424 cm^2 and for bare electrode, 0.04326 cm^2. From this, we can conclude that the electroactive area has been increased remarkably after modification.

Analytical procedure

The GCE (unmodified and modified) was swept between -600 and 800 mV till stable voltammograms were obtained in PBS (pH 7.4). Then the electrodes were kept in a cell which contains requisite concentration of the substrate and buffer solution. In open circuit, with accumulation time of 300 s and waiting for 20 s, cyclic voltammograms were recorded at a scan rate of 50 mVs^{-1} by sweeping the potential between -600 and 800 mV. All the determinations were recorded at room temperature of 26 ± 0.2 ºC.

RESULTS AND DISCUSSION

Voltammetric performance of 8-Oxoguanine

Figure 2 shows cyclic voltammograms recorded for 8-Oxoguaine on a bare GCE and GCE modified with MWCNTs/CB. On the bare GCE, oxidation peak was at about 452 mV (Figure 2a), which was a bit weak and broader. On the MWCNTs/CB modified GCE, the oxidation peak was present at about 282 mV

(Figure 2e), which was sharper and stronger than earlier with significant increase in peak current and remarkable decrease in peak potential. The cause for the improved performance of MWCNTs/CB modified GCE may be due to the unique properties of nano-dimensional materials like high aspect ratio, high surface area, and electro-catalytic activity. No oxidation peak was recorded with this modified electrode in buffer solution of pH 7.4, but the background current becomes larger, because the nano-materials used were increasing the surface area.

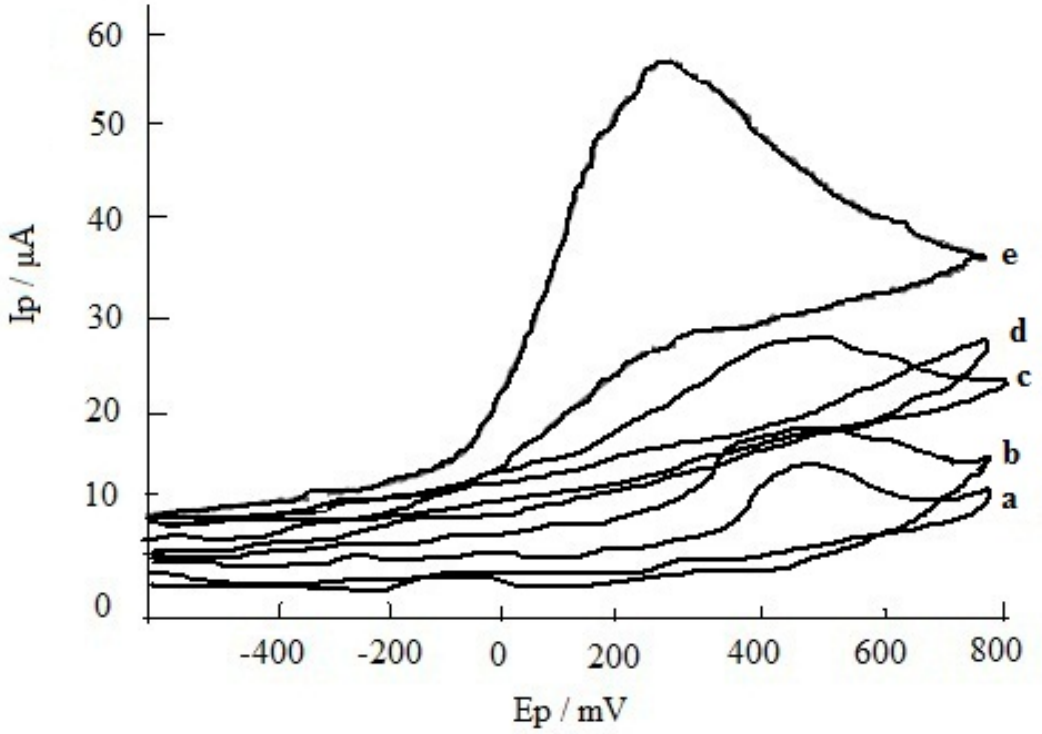

Figure 2. Cyclic voltammogram recorded for 3 x 10^{-4} M of 8-Oxoguaine at pH 7.4 in 0.1 M PBS (a) at bare GCE, (b) at CB modified GCE, (c) at MWCNTs modified GCE, (e) at MWCNTs/CB modified GCE and (d) Blank CV of MWCNTs/CB modified GCE.

Effect of quantity of MWCNTs/CB

The influence of the amount of MWCNTs/CB on oxidation peak current was studied. Highest peak current was obtained when 5 µl of MWCNTs/CB suspension was used for drop coating. But when the amount of suspension is less than 5 µl, there is little decrease in peak current. This may be due to the fewer amounts of nanomaterials present, which results in the less current. But when suspension volume is more than 5 µl, the film becomes thick and the conductivity of this film reduces those results in low peak current. Hence, 5 µl of MWCNTs/CB suspension was employed in the further investigation.

Effect of pH

The pH of the reaction medium plays vital role especially for the reactions which occur on an electrode. The influence of pH on the peak current of the title reaction was studied using CV. Using PBS, the pH of the reaction medium was altered in the range between 5.8–8.0. The study indicated that, the peak potential was moved to lesser positive values as pH of solution increased. The physiological pH of 7.4 was used for further studies.

Effect of scan rate

From the correlation among the peak current and scan rate, some valuable information can be obtained including the mechanism with which the reaction was occurring. Hence, the effect of scan rate on oxidation of 8-Oxoguaine was investigated at dissimilar scan rates from 10 to 100 mVs^{-1} (Figure 3). The peak current increased linearly with scan rate. The following equation was obtained Ip = 10.2 v + 14; R^2 = 0.997 as shown in Figure 4 which is the plot of peak current values against the scan rate. There was a linear relationship observed among log Ip and log v and the plot yields the equation log Ip = 0.505 log v + 1.309; R^2 = 0.985, whose slope value is near to the theoretical value of 0.5, which indicates electro-oxidation was a diffusion-controlled process[17] (Figure 5). As scan rate was raised, the peak potential values moved to more and more positive values. A linear graph was obtained after plotting peak potential against logarithm of scan rate with following equation Ep = 224.8 + 19.2 log v; R^2 = 0.997 (Figure 6).

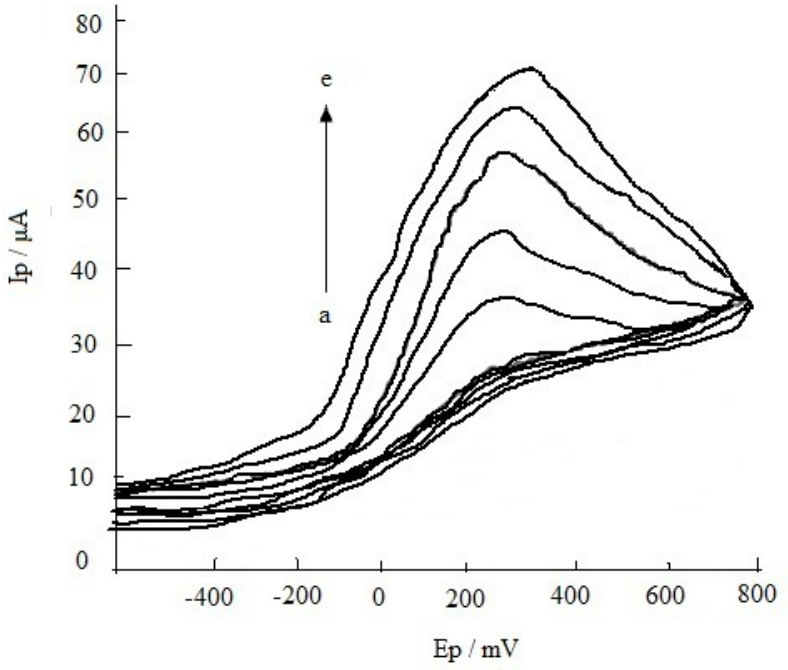

Figure 3. Cyclic voltammograms recorded for 3 x 10^{-4} M of 8-Oxoguaine at pH 7.4 in 0.1 M PBS with scan rates of (a) 10, (b) 20, (c) 50, (d) 70 and (e) 100 mVs^{-1} at MWCNTs/CB modified GCE.

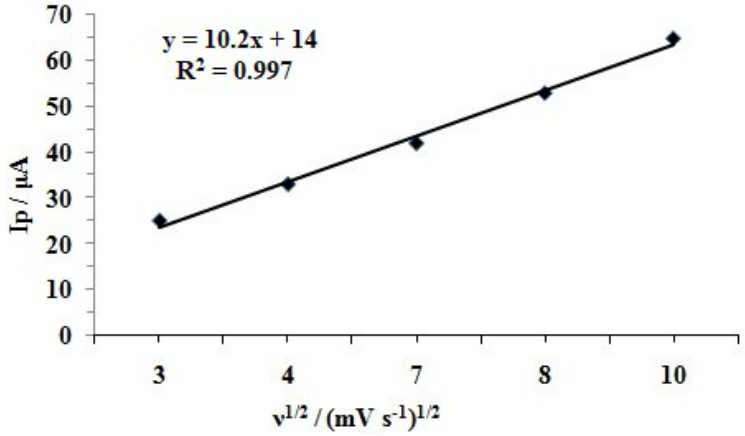

Figure 4. Graph of peak current versus square root of scan rate.

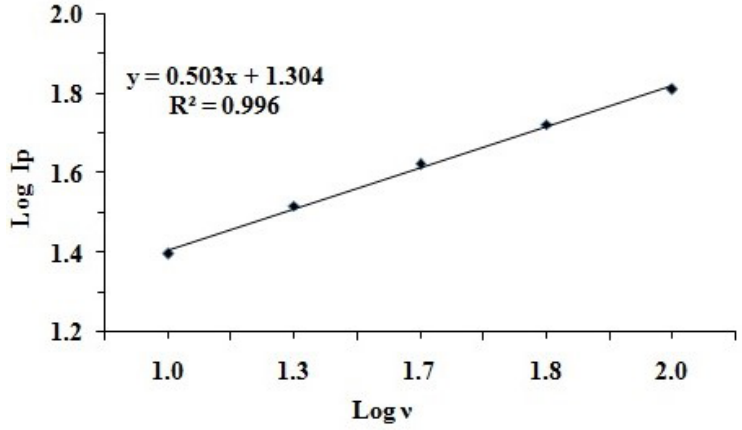

Figure 5. Plot of peak current logarithm to base 10 against scan rate logarithm to base 10.

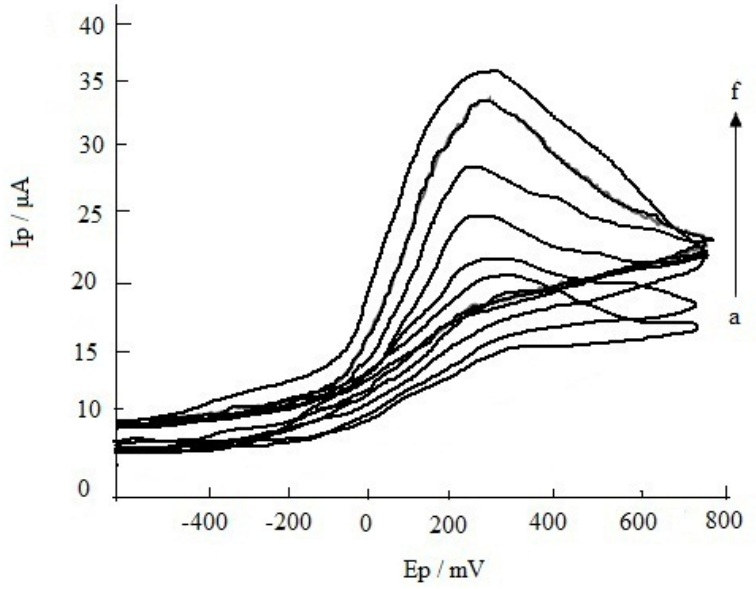

Figure 6. Cyclic voltammograms of 8-Oxoguaine at pH 7.4 in 0.1 M PBS at (a) 30, (b) 40, (c) 50, (d) 70, (e) 85 and (f) 100 µM at MWCNTs/CB modified GCE.

Calibration curve

We tried to develop a voltammetric method for assessment of the drug, for which cyclic voltammetry was used. At a physiological pH of 7.4 in PBS solution, cyclic voltammograms were recorded by increasing the concentration of 8-Oxoguaine. The recorded voltammograms showed that with raise in amount of 8-Oxoguanine, the anodic peak current raised accordingly, which is shown in Figure 6. Using the best possible environment as described above, a calibration graph was obtained for 8-Oxoguaine from 30 to 100 µM (Figure 7). The straight-line equation from calibration graph was Ip (µA) = 0.266 + 4.4 C; R^2 = 0.998 (C is in µM). It was observed that, divergence from linearity for the solution with higher amount of the substrate, owing to the adsorption of substrate or its product after oxidation at the surface of the electrode. The detection limit and quantification limit obtained were 8.38 µM and 27.95 µM, respectively. The detection limit and quantification limit were calculated by the equations: Detection limit = 3s/m and Quantification limit = 10 s/m. The obtained results were compared with those already reported and were given in Table I.

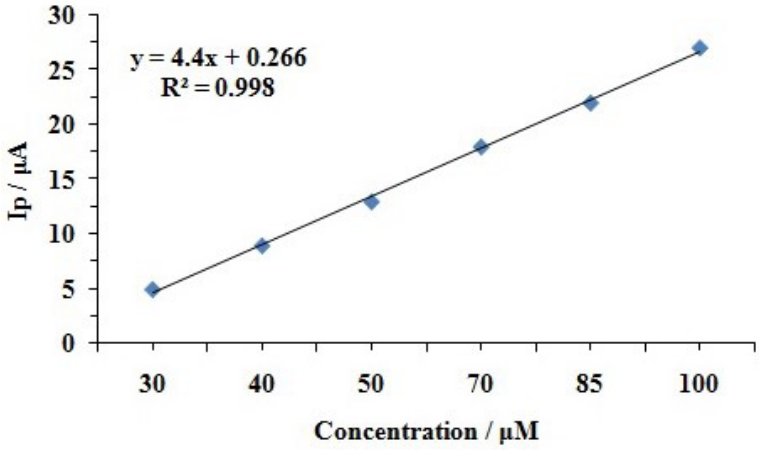

Figure 7. Graph of peak current versus concentration of 8-Oxoguaine.

Table I. Comparison of voltammetric detection of 8-Oxoguanine at carbon electrode modified with various nanomaterials

Electrode	Electrode modifier	Method	Linear range (M)	LOD	Ref.
EPPGE	SWCNTs	SWV	$3x10^{-11}$ – $7.5x10^{-11}$	$1x10^{-11}$	22
EPPGE	SWCNTS/AuNPs	SWV	$1x10^{-11}$ – $1x10^{-8}$	$5x10^{-12}$	23
GCE	SWCNTs	LSV	$2.9x10^{-9}$ – $8.7x10^{-5}$	$1.0x10^{-9}$	24
GCE	MWCNTs	CV	$5.6x10^{-8}$ – $1.6x10^{-5}$	$0.2x10^{-9}$	25
GCE	rGO-MWCNTs	SWV	$3\text{-}75x10^{-6}$	$35x10^{-9}$	26
GCE	Sulphur-doped rGO	DPV	$2x10^{-9}$ – 20	$1x10^{-9}$	27
GCE	PtNF-GO	DPV	$0.7x10^{-9}$ – $2x10^{-6}$	$25x10^{-6}$	28
SPCE	MWCNTs-COOH	DPV	$0.3x10^{-6}$ – $2.4x10^{-4}$	$0.57x10^{-6}$	29
GCE	MWCNTs/CB	CV	$30x10^{-6}$ – $100x10^{-6}$	$8.38x10^{-6}$	This work

EPPGE edge plane pyrolytic graphite electrode, SWCNTs single-walled carbon nanotubes, MWCNTs multi-walled carbon nanotubes, AuNPs gold nanoparticles, rGO reduced graphene oxide, GO graphene oxide, SPCE screen-printed carbon electrode, CB carbon black, SWV square wave voltammetry, LSV linear sweep voltammetry, CV cyclic voltammetry, DPV differential pulse voltammetry.

Reproducibility

The reproducibility of the modified electrode was studied as follows. The voltammograms were recorded for 50 µM 8-Oxoguaine using modified electrode which was renewed every time for every numerous hours within a day, and it was found Relative Standard Deviation (RSD) of 2.12% for the peak current for 10 number of measurements. If the temperature was unchanged, then the reproducibility between days was similar to that of within a day. But the electrode has to be modified again after each reading, due to the adsorption of oxidation product.

Intra-day and Inter-day assay

In order to verify the accuracy and precision of the method, the assay of 8-Oxoguaine was done for inter-day and intra-day. This was done at three concentration levels: at high concentration level of 120 µM, medium concentration level of 60 µM and low concentration of 20 µM of 8-Oxoguaine. Voltammograms were recorded once in a day (n = 5) during four consecutive days for the inter-day assay and under identical conditions at the same concentrations (n = 5) for four times in a day to calculate assay in a day.

The accuracy values obtained for the intra-day assay were 101.68%, 100.23% and 99.84% for high, medium and low concentration levels, respectively (Table II). The average precision values were in the range of 5 to 10% in intra-day as tabulated in Table II. The accuracy values of 100.62%, 99.76% and 101.83% were obtained for high, medium and low concentrations, respectively in a day. In a day, the average precision values were in the range of 4-11% (Table II). The accuracy and precision values obtained were within ±15%. Therefore, the obtained values for intra-day and in a day are within acceptable limits.[31]

Table II. Data obtained for the assay of 8-Oxoguanine for intra-day and in a day

Concentration (µM)	Concentration determined (µM)	Recovery (%)	Accuracy (%)	Precision (RSD%)
Intra-day				
120	120.24±0.31	100.2	101.68	5.43
60	59.87±0.52	99.7	100.23	10.04
20	20.42±0.24	102.1	99.84	7.52
Inter-day				
120	120.31±0.18	100.2	100.62	10.87
60	60.15±0.09	100.3	99.76	4.13
20	19.74±0.65	98.7	101.83	6.57

CONCLUSION

In the present effort, a glassy carbon electrode modified using multi-walled carbon nanotubes/carbon black has been effectively constructed to study voltammetric oxidation of 8-Oxoguaine in PBS. The nanomaterials used have shown electro-catalytic activity during electro-oxidation of 8-Oxoguaine, with improvement in the anodic peak current due to the special properties exhibited by the nanomaterials. For the assessment, the anodic peak at about 282 mV was used and the linear range using calibration graph was also determined. This voltammetric sensor can be employed for voltammetric assessment of 8-Oxoguaine with good reproducibility and recovery. This work adds a new modified electrode to voltammetric field.

Conflicts of interest

The authors declare that there is no conflict of interest.

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ARTICLE

Extraction and Evaluation of Flamboyant Mirim Gum as a Potential Viscosifying Agent for Enhanced Oil Recovery Fluids

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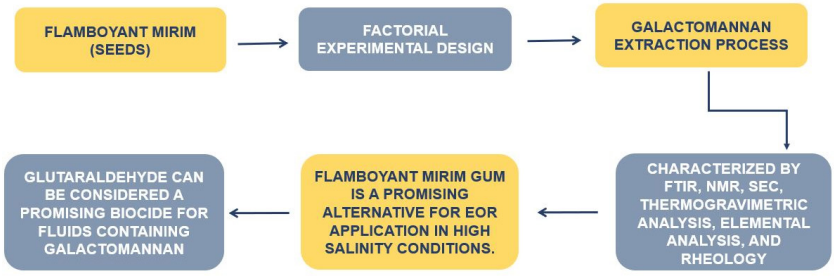

Flamboyant mirim gum is a galactomannan extracted from *Caesalpinia pulcherrima* seeds, a legume family schrub. The main objective of this work was to propose a factorial experimental design to optimize the galactomannan extraction process from its seeds and evaluate its potential as a viscosifying agent for enhanced oil recovery (EOR). The galactomannan was characterized by FTIR, NMR, SEC, thermogravimetric analysis, elemental analysis, and rheology. Structural analyses confirmed the presence of the desired galactomannan content. The best yield obtained was 7.3% for initial seed mass and 24.6% for the endosperm. Rheological analysis showed that Flamboyant mirim gum is a promising alternative for EOR application in high salinity conditions. In addition, stability tests showed that glutaraldehyde can be considered a promising biocide for fluids containing galactomannan since it maintained the viscosity values of the systems for 42 days. Thus, the results confirmed the adequacy of the extraction procedure for obtaining galactomannan.

Keywords: Galactomannan, Flamboyant mirim, *Caesalpinia pulcherrima*, Enhanced oil recovery

INTRODUCTION

Chemical enhanced oil recovery (EOR) is a process in which an oil-immiscible fluid (such as water) is injected together with some chemical additives to increase the oil recovery factor. The application of polymers in EOR fluids increases the oil recovery factor by reducing the mobility ratio, which is a consequence of the increased viscosity the injection fluid. This effect increases the sweep efficiency in the reservoir, and depending on the type of polymer used, the increase in viscosity can also provide a reduction in the permeability of the medium to water.[1]

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The most often used in EOR is partially hydrolyzed polyacrylamide (HPAM). However, under severe reservoir conditions (for example, high salinity and high temperature), HPAM loses the properties necessary to increase sweep efficiency. Other polyacrylamide-based copolymers have been developed to withstand thermal, chemical and mechanical degradation, but are expensive, and depending on the severity of the adverse condition, may have difficulty maintaining the properties necessary to improve oil recovery from reservoir.[2-7]

In this context, biopolymers are possible solutions for oil recovery operations, since they are resistant to high salinity and high temperatures. Some can maintain adequate viscosity under high salinity to improvement the sweep efficiency.[8,9] With the exception of xanthan gum, the biopolymers schizophyllan and scleroglucan are not applied due to the high cost involved in their production, since this requires specific microorganisms, adequate environmental conditions and complex purification processes, and in some cases low yields are obtained. In addition, scleroglucan also has problems of low injectability.[10,11]

In the case of xanthan gum, several articles have mentioned its application as a viscosifying agent for EOR fluids.[8,12-15] However, due to the presence of carboxylate groups in the structure, its application can result in adsorption on the surface of carbonate rocks, due to its positive surface charge.[6] This adsorption leads to loss of viscosity and reduction of sweep efficiency, thus reducing oil recovery. Because of this, galactomannans are a potential solution to the problems mentioned above, since they are nonionic and are inexpensive to produce.[16] In particular, galactomannans can be extracted from the endosperm of seeds of different species and have wide applicability in the industrial sector, especially in the pharmaceutical and food segments, as well as in the production of films.[17-19] In the food industry, they are generally used as stabilizers, thickeners and inedible coatings.[20] However, they have never been evaluated for enhanced oil recovery.

Galactomannans are neutral polysaccharides and compose the second-largest group of reserve polysaccharides available from plants. Besides plants, these polysaccharides can also be obtained from microbial sources, e.g., yeasts and fungi.[21] The structure of galactomannans consists of repeated units of ß-D-mannose, in the main chain, linked together by glycosidic bonds of type 1 → 4 and α-D-galactose units, linked to the main chain by bonds of type 1 → 6. The ratio and distribution of the D-galactose units depend on the origin and legume species, as well as on the extraction techniques.[22,23] The mannose:galactose ratio (man:gal) plays an important role in the solubility of galactomannans, since the higher the proportion of D-galactose, the greater its solubility in water is.[22,24]

The gum selected for analysis was extracted from the species Caesalpinia pulcherrima, a shrub belonging to the legume family (Fabaceae) that can reach 3 to 4 meters in height in adulthood. Its fruits appear in the autumn and are native to the Antilles but is widely cultivated in other countries, including Brazil, in parks and gardens as an ornamental plant. Because Brazilian conditions are extremely favorable for the development of fruits and seeds, they are produced abundantly throughout the year. Since the gum is obtained from a local renewable resource, it can be a good solution to the problems faced in the logistics and application of synthetic polymers for enhanced oil recovery, with the added advantage of low production cost.

The main focus of this study is to evaluate the extraction processes and the obtainment of fluids resistant to high salinity based on Flamboyant mirim gum as a potential viscosifying agent for enhanced oil recovery application. The Introduction should establish the general context and the background of the field the paper is about.

MATERIALS AND METHODS

Materials

Caesalpinia pulcherrima seeds (Flamboyant mirim) were purchased from the company Arbocenter S.A. (São Paulo, SP, Brazil). Ethyl alcohol 95%, used in the extraction of galactomannans, was purchased from Isofar (Rio de Janeiro, RJ, Brazil). The brine used in the preparation of polymeric fluids consisted of sodium chloride PA (NaCl), magnesium chloride hexahydrate PA ($MgCl_2.6H_2O$), pure anhydrous calcium chloride ($CaCl_2$), strontium chloride hexahydrate PA ($SrCl_2.6H_2O$), sodium sulfate (Na_2SO_4) and potassium

chloride PA (KCl), all from Sigma-Aldrich (São Paulo, SP, Brazil). Deuterium oxide (D_2O) was used as NMR solvent for galactomannan characterization, also purchased from Sigma-Aldrich (São Paulo, SP, Brazil).

Determination of galactomannan content in endosperms

According to the literature, the highest concentration of biopolymers with viscosifying power occurs in the endosperm of the seeds. Thus, because the focus here is on the extraction of this biopolymer, knowledge of the endosperm content is important. Although the endosperm is not composed of 100% biopolymer, it is the largest source of these biomolecules, so the polysaccharide extraction yield will always be lower than the endosperm content in the seed.[25,26]

Hence, it was developed a method to determine the endosperm content, in which the Flamboyant mirim seeds were weighed and the respective mass values were recorded. Then, they were placed in an oven at 80 ºC and weighed daily until constant weight. For each determination, the seeds were removed from the oven and placed in a desiccator for 15 minutes and then weighed at room temperature (dry seed mass).

Subsequently, the seeds were subjected to diversified procedures to facilitate the removal of the husks and isolation of their respective endosperms. After isolation, the endosperm material was dried to constant weight following the same procedure described above, to obtain the dry endosperm mass.[27]

The experiment was carried out in duplicate and the determination of the endosperm content in the seeds was determined.

Extraction of biopolymer

The procedure for extracting Flamboyant mirim gum from the seeds was performed as described by the literature,[28] with minor modifications. Initially, 10 g of seeds was boiled at 100 °C for 30 minutes in order to inactivate the enzymes present in the seeds and facilitate the removal of the endosperm. After that, the seeds were washed and placed in a sieve to remove excess water and particles.

Subsequently, the seeds were manually removed and their endosperms were separated from the husks. Then, the endosperms were submitted to extraction with distilled water at a ratio of 1:50 (w/v) in a blender for 10 minutes until obtaining a viscous solution. The solution was centrifuged at 4000 rpm for 15 minutes. The supernatant was precipitated in ethyl alcohol in a proportion of 1:3 (v/v), with subsequent filtration through a standard sieve with 177 µm mesh. Finally, the precipitate was dried in a lyophilizer for 48 hours, macerated, and the yield was determined.

Factorial experimental design of biopolymer extraction from Flamboyant mirim seeds

A 2^3 factorial experimental design was used with 3 central points, totaling 11 experiments. The factorial planning matrix and the extraction process variables (temperature, extraction time and salt concentration) are reported in Table I.

Some extraction methods described in the literature were used as a basis for determining the conditions for construction of the planning matrix. In the case of temperature, the conditions used in the literature obtained maximum extraction under both cold and hot conditions.[22,24] Given the possible favorable influence of the use of high temperature in the extraction, it was chosen 80 °C for hot extraction. On the other hand, it was used 4 °C for cold extraction. Due to the central point planning, the temperature of 42 °C was also evaluated, an intermediate value where there is less energy expenditure to maintain either the hot or cold temperature.

Regarding the addition of sodium chloride, it was reported that using a solution with concentration of 0.1 M of this salt increased the final yield in relation to the use of distilled water without its addition.[29] It was considered that a greater amount of salt could favor the yield even more, so 1.5 M concentration was used as the upper level of this variable.

The extraction time variables were fixed based on the literature, with 4 hours being considered optimal for hot extraction and 12 hours for cold extraction.[30]

From here, all extractions of the Flamboyant mirim gum followed the factorial experimental design described in Table I.

Table I. 2^3 fatorial planning for Flamboyant mirim polymer extraction

	Coded					
Experiment	**NaCl (Molar)**	**Temperature (°C)**	**Time (hours)**	**NaCl (Molar)**	**Temperature (°C)**	**Time (hours)**
1	-1.0	-1.0	-1.0	0.1	4	4
2	1.0	-1.0	-1.0	1.5	4	4
3	-1.0	1.0	-1.0	0.1	80	4
4	1.0	1.0	-1.0	1.5	80	4
5	-1.0	-1.0	1.0	0.1	4	12
6	1.0	-1.0	1.0	1.5	4	12
7	-1.0	1.0	1.0	0.1	80	12
8	1.0	1.0	1.0	1.5	80	12
9	0.0	0.0	0.0	0.8	42	8
10	0.0	0.0	0.0	0.8	42	8
11	0.0	0.0	0.0	0.8	42	8

Initially, the seed husks were removed and the seeds were weighed to form samples of approximately 25 grams, which were boiled in distilled water [1:5 (w/v)] at 100 ºC for 2 hours under stirring, for enzymatic inactivation. They were then kept immersed in water for 18 hours at 25 °C to facilitate husk removal. After this period, the husks were removed manually. However, some seeds remained hard, hindering the husk removal. For these, new heating under stirring was carried out for 2 hours, followed by 18 hours at rest in distilled water, enabling removal of the husks from more seeds. When hard seeds remained, they were weighed and placed in an oven at 40 °C for one day, weighed and then discarded.

After removing the husks, the endosperm material was ground using a blender (50 seconds) with a NaCl solution (1:10 w/v) with the concentration stipulated in the experimental design (Table I), at 25 ºC. Then, the extraction was performed under stirring at 500 rpm, at the temperatures and for the times described in Table I.

After the extraction time, the solution was centrifuged (4000 rpm, 10 minutes), precipitated in 95% ethanol [1:3 (v/v)] and filtered through a 45 µm sieve. The precipitated material was lyophilized, ground, and weighed. The extraction yield was calculated based on the initial seed mass and also the initial dry endosperm mass.

Hydrogen magnetic resonance spectroscopy (^{1}H-NMR)

The Flamboyant mirim gum was characterized by ^{1}H-NMR to measure the mannose/galactose ratio, which is an important characteristic of galactomannans. The samples were solubilized in deuterated water at room temperature. After solubilization, the samples were placed in NMR tubes with diameter of 5 mm. The samples were characterized in a Varian VNMRSYS 500 (500 MHz) spectrometer, with deuterium oxide (D_2O) as solvent. The analyses were performed at room temperature. The analyses were performed using a 5 mm universal probe, spectral width of 4800 Hz, acquisition time of 2.5 s, pulse width calibration of 90 degrees, pulse interval of 10 s and transient number 80.

Fourier-transform infrared spectroscopy (FTIR)

The Flamboyant mirim gum was characterized by FTIR to observe and classify some bands related to functional vibrations. FTIR spectra were obtained using a Perkin-Elmer Frontier FTIR/FIR spectrometer using 20 scans and resolution of 4 cm^{-1}. The main peaks were highlighted using the Perkin-Elmer Spectrum software, version 10.4.2, and each sample was prepared by weighing 5 mg of the dry biopolymer, which was pressed to form KBr pellet (15 mg) and then analyzed at room temperature, using wavelengths from 4000 to 400 cm^{-1}.

Thermogravimetric analysis (TGA) and derivative thermogravimetry (DTG)

The Flamboyant mirim gum was characterized by thermogravimetric analysis (TGA) to evaluate the temperatures of degradation of the samples. Therefore, known masses of gum were subjected to heating using a TA Instruments model Q500 analyzer, nitrogen atmosphere with flow rate of 60 mL/min, applying a heating rate of 10 °C/min to a maximum temperature of 800 °C.

Elemental analysis (CHN)

In this technique, the sample is subjected to high temperatures to convert the carbon present into carbon dioxide, hydrogen into water, and nitrogen into nitrogen gas. Flamboyant mirim gum was characterized by elemental analysis in order to confirm the presence of nitrogen compounds, which can be correlated with the presence or absence of proteins adhered to the extracted biopolymer, since there is no nitrogen in the gum's basic structure.

The equipment used was a Leco QQSC632 analyzer with limit of detection of 0.3%, where about 2 to 3 mg of the sample was burned to measure the percentages by mass of carbon, hydrogen, and nitrogen (CHN) present in the final product.

Size-exclusion chromatography (SEC)

The molar mass determination of the biopolymer was different from other characterizations, since the parameters used were highly dependent on its structure and solubility. To determine the molecular weight of the Flamboyant mirim gum, it was initially necessary to calculate the dn/dc value of each experiment. The dn/dc value is the variation of the refractive index divided by the variation in concentration before injection. This value is highly dependent on the solvent/polymer ratio, since polymers can have different conformations in different solvents, directly influencing the signal observed by the equipment. Thus, the calculation of dn/dc indicates the reliability of the results obtained.

The dn/dc value was determined through direct analysis with a Wyatt Technology Optilab T-rEX refractive index detector, performing sequential injections of the biopolymer solution with different concentration (0.2 to 1.0 mg mL^{-1}), which generated a biased linear curve, which with extrapolation corresponded to the desired value (dn/dc). The operational flow rate was maintained between 0.1-0.5 mL min^{-1}, varying according to the need and viscosity of each sample. From the data obtained, the dn/dc value was calculated and then inserted in the molar mass determination analysis.

For analysis of the biopolymer, an Agilent Technologies 1260 Infinity gel permeation chromatograph was used with Shodex LG-G 6B pre-column and two Shodex LB-806M columns, coupled to a Wyatt Technology DAWN8 light scattering detector and Optilab T-rEX refractive index detector. According to the manufacturer, the error limits for SEC analysis is 10%.

The mobile phase used in the determination of dn/dc and molar mass was based on an aqueous solution of sodium nitrate at a concentration of 0.1 M and sodium azide at a concentration of 250 mg L^{-1}. For injection, the samples were filtered with a 0.22 µm filter before storage in vials. The samples also needed to be within the proper reading range for detection, with a minimum of 0.05 mg mL^{-1} and a maximum of 2.5 mg mL^{-1}.

The conditions for analysis were adjusted to the temperature of 40 °C in the columns and detectors and flow rate of 0.5 mL/min. To guarantee accuracy of the results, the peaks with a low molar mass

poly (ethylene oxide) were normalized and aligned to a standard of the same mobile phase used for the biopolymer. Finally, the sample was injected and the data processed using the Astra 1.7.3 software, which performs the necessary calculations and provides the number-average molecular mass (Mn), weight-average molecular mass (Mw), and polydispersion values for the biopolymer when the dn/dc is supplied to the software.

Rheological analysis

The rheological analyses of the Flamboyant mirim gum were carried out in a TA Instruments Discovery Hybrid rotary rheometer (DHR3), with a 40 mm 2° cone plate accessory of titanium. For all experiments, flow curves were obtained with shear rates varying from 0.1 to 500 s^{-1} and an analysis time of 45 minutes to acquire 5,400 points. These parameters were selected after an extensive previous study to determine the conditions of the rheological analyses necessary for better determination of viscosity values, by giving the necessary time for the polymer molecules to respond to the imposed shear. The analyses were performed at temperatures of 25, 60, and 80 °C. Viscosity values at the oil reservoir shear rate (7,37 s^{-1}) were used to evaluate the biopolymer viscosifying power for enhanced oil recovery application.

All samples were evaluated in injection brine (with compositions similar to those of desulfated brine), called Brine 1, and brine with a higher concentration of salts, simulating water from a typical mixture of injection water and formation water (Brine 2). Brine 1 was composed of NaCl, $MgCl_2$, KCl, $CaCl_2$ and Na_2SO_4, with a total concentration 29711 ppm of total dissolved solids (TDS). Brine 2 was composed by the same salts as Brine 1 with the addition of $SrCl_2$, a salt commonly present in reservoirs, and this brine also presented higher salinity, with a total of 68317 ppm total dissolved solids.

Initially, biopolymer solutions at a concentration of 2000 ppm were prepared in Brines 1 and 2. The solution containing 1000 ppm of biopolymer was obtained from dilution of this solution. To obtain the biopolymer solution with 3000 ppm, only Brine 1 was used as solvent, and only for the biopolymer that presented the best viscosifying power. The time it took the biopolymer to completely dissolve in these brines was about one day, at room temperature.

Stability of biopolymer solutions

This test was carried out to evaluate the stability of the most promising biopolymer (the one that presented the most appropriate viscosity for application as a viscosifying agent for EOR applications).

In this evaluation, the main objective was to verify the need to add a biocide to maintain the viscosity of the biopolymer solution under shelf conditions. The samples were observed regarding color, phase separation, appearance of a precipitate and presence of particulates. Viscosity measurements were performed during 42 days.

Initially, a stock solution of the selected biopolymer was prepared in Brine 1 at a concentration of 3000 ppm. After that, the initial solution was separated into 5 vials containing 20 mL of solution each and then the biocide: glutaraldehyde was added, which has already been applied in the oil industry.[31] The concentrations of the biocide added to the systems were 50, 100, 200, 300, 1000, 2000, and 4000 ppm.

RESULTS AND DISCUSSION

Endosperm content in seeds

The determination of the endosperm content is essential to evaluate the viability of the seed as a source of biopolymer with viscosifying power, because the higher the endosperm content in the seeds is, the greater will be the yield of extractions.[27] The endosperm content in the Flamboyant mirim seeds was 33%.

Extraction yields of biopolymer

The yields of Flamboyant mirim gum were 3% when calculated from the initial seed mass, and 9% when calculated based on the endosperm mass. This seed yield (3%) was low in comparison with the amount of polymer used in enhanced oil recovery operations, making its application economically unfeasible.

Thus, it was concluded that the extraction of galactomannan still needed some adaptations to obtain satisfactory final yield. For this, it was necessary to use an experimental design to ascertain best extraction conditions.

Thermogravimetric analysis (TGA) and derivative thermogravimetry (DTG)

Initially, the thermal stability of the Flamboyant mirim gum was evaluated through thermogravimetric analyses. In addition to thermal stability, other information can be obtained through this technique, such as the percentage of organic and inorganic materials, moisture and presence of a residual solvent, which allow judging the efficiency of the extraction methods used, as well observing the dehydration of the polysaccharide.[32]

The thermogravimetric curves obtained showed similar behavior in all experiments (Table II). All samples showed two mass loss events, as shown in Figure 1, using the thermogravimetric curves obtained in experiment 1.

Table II. Mass losses calculated in the three stages of degradation and the total mass loss of Flamboyant mirim gum extracted through experimental design

Experiment	Volatile solvents (%)	Organic compounds (%)	Inorganic compounds (%)	Total mass loss (%)
1	12	76	12	88
2	8	47	45	54
3	12	79	9	91
4	6	33	61	38
5	14	78	8	92
6	7	39	54	46
7	11	75	14	86
8	9	45	46	53
9	12	70	18	81
10	16	66	18	81
11	14	69	17	83

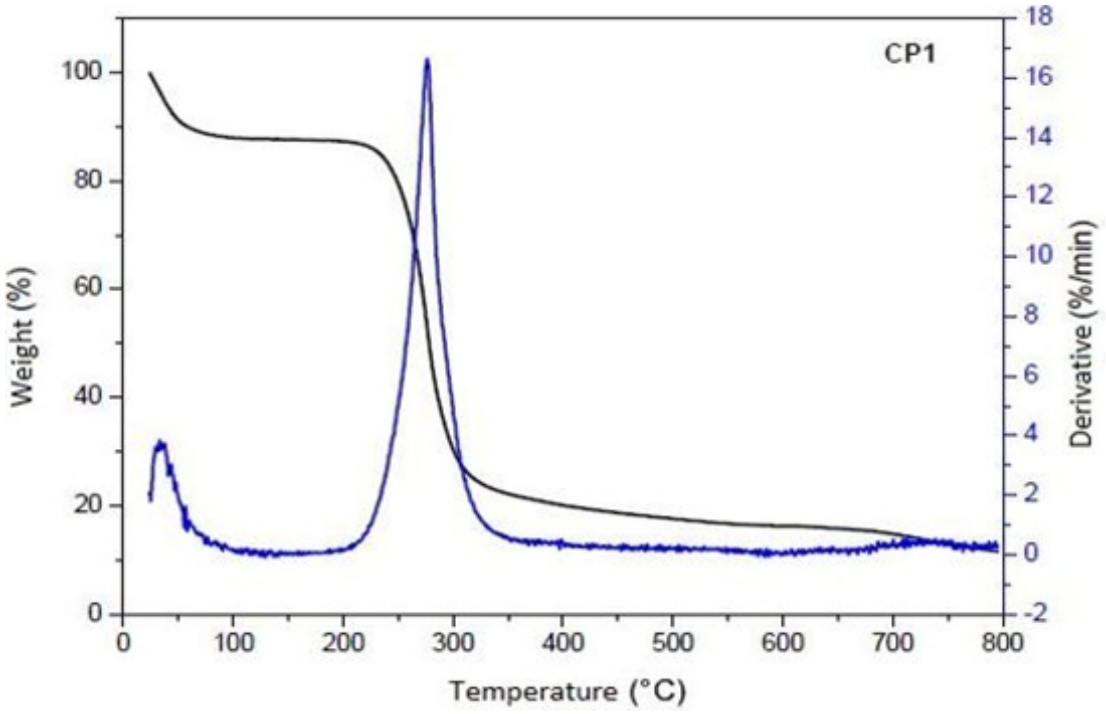


Figure 1. Thermogravimetric curves of the Flamboyant mirim gum (Experiment 1).

The first event is related to the highest volatile output temperatures, which are applied to the mixture of water and ethyl alcohol from the extraction processes. The second event, referring to the degradation of the biopolymer, showed maximum kinetics in the temperature range between 200 and 350 °C, with is the region related to the thermal degradation of polysaccharides.[33] Another important observation from the thermogravimetric curves regarding the extracted galactomannan was the loss of total mass. The residual mass obtained at the end of the analysis, at temperature of 800 ºC, can be correlated with the subproducts of degradation or compounds contained in the raw material used in the extraction process. Therefore, higher mass loss is theoretically associated with greater biopolymer purity.

Based on the total mass loss of the galactomannan samples, the Flamboyant mirim gum extracted in experiment 5 presented the highest degree of purity, as shown in Table II. Still according to Table II, the values of volatile solvents lost from the products obtained through extractions from Flamboyant mirim seeds were between 5 and 16% w/w (first event), while the losses of organic compounds were between 32 and 79% w/w (second event) and of inorganic compounds were from 8 to 61% w/w (residue contents calculated using the percentage of the total mass in the samples).

Table II also shows that experiments 2, 4, 6, and 8 presented the highest levels of residual inorganic matter after the extraction processes. All these experiments were carried out with the addition of 1.5 M NaCl, which probably remained in the final product and was not degraded in the TGA analysis, since it has a boiling point of 1465 °C, higher than the maximum temperature employed in the thermogravimetric analysis.

The contents of inorganic matter and solvents were used to obtain the biopolymer masses for the calculation of extraction yields. It is worth mentioning that the results published in the literature do not consider adjustments in yield. Instead, these results pertain to the final biopolymer products, containing many residues from the extraction processes.[34-38]

Extraction yields obtained through factorial experimental design

The extraction yields for the Flamboyant mirim gum obtained from the factorial experimental design were satisfactory and are listed in Table III. There was an increase in the extraction yield compared to the result obtained by extraction method carried out before experimental design, since the previous maximum yield was 3% in relation to seed mass. After the factorial experimental design, it was possible to reach about 25% in Experiment 4, a yield similar to those found in the literature.[39-41]

Table III. Yields obtained after factorial experimental design for the extraction of flamboyant mirim gum

Experiment[(a)]	Mass[(b)] (g)	Yield in relation to the endosperm (%)	Yield in relation to seed mass (%)	Yield in relation to endosperm mass (%)	Yield in relation to seed mass (%)
		In the presence of residual materials[(c)]		In the absence of residual materials[(c)]	
1	2.3	31	9	23	7
2	4.3	58	17	21	6
3	2.7	36	11	26	8
4	6.2	84	25	25	7
5	2.0	27	8	19	6
6	4.7	63	19	20	6
7	2.1	28	8	20	6
8	2.5	34	10	14	4
9	2.2	30	9	18	5
10	2.7	36	11	21	6
11	2.2	30	9	20	6

(a) Experimental design;
(b) Mass of biopolymer obtained after the lyophilization step;
(c) Residual materials (salt, water and solvent);
Average moisture content present in the seeds = 9.8%; percentage of endosperm calculated (%w/w) = 33%; mass of endosperm used in calculations = 7.5 g; average dry seed mass used in calculations = 22.7 g.

It should be noted that these higher yield values are related to the presence of biopolymer and also residual materials (RM) from biopolymer extraction, such as salt (NaCl), water, and solvent (ethyl alcohol) used in the extraction steps. The presence of these residues was observed in the thermogravimetric analyses. After the entire extraction process, these residues were not completely removed, resulting in an increase in the final mass of the product obtained.

To obtain the greatest accuracy of biopolymer mass measurement, the contents of organic and inorganic materials were used as well as moisture and solvent, obtained utilizing thermogravimetric analyses, to determine the content of salt, water, and residual alcohol in the final product. Through these analyses it was possible to arrive at the corrected yield of biopolymers.

Table III shows the final biopolymer yields related to the total dry seed mass (22.7 g) and the mass of endosperms present in the seeds (7.5 g). The results of yields obtained for seeds clearly show that the adjustments in the procedures used made their production feasible. There was an increase of 22% referring to seed mass.

Results of factorial experimental design

After processing the data generated with the 2^3 factorial design with the Design Ease 10® program, it was possible to obtain information regarding the effects of each experimental variable, as well as the relationships between them. This enabled assessment of which variables and interactions were statistically relevant and influenced the response variable (in this case, final yields of biopolymers obtained from the initial mass of Flamboyant mirim seeds), shown in Table IV, without considering the presence of salt, water, and solvent in this study, since their consideration relates to unlikely values.

Table IV. Estimated effects and significance of each factor and their respective interactions on the final yield response of Flamboyant mirim gum to the initial seed mass

Factor	Effect	Standard error	p-value
Average/interaction	6.09	0.11	0.0
(1) salt	-0.57	0.27	0.2
(2) temperature	0.08	0.27	0.8
(3) time	-1.58	0.27	0.0
1 x 2	-0.48	0.27	0.2
1 x 3	-0.23	0.27	0.5
2 x 3	-0.88	0.27	0.1

The statistical significance of each effect is measured by the p-values. Significant effects are those with p-values less than 0.05 in Table IV. Values less than 0.01 indicate the effect is very significant and values between 0.05 and 0.10 are classified as marginally significant, because although they are not in the stipulated range up to 0.05, they are close. Values greater than 0.10 indicate the effect is not significant.[41]

Based on this observation and the results in Table IV, it was possible to verify that of the three variables studied, only time was significant, with a statistically negative effect. The fact that the production of biopolymer in less time already allowed obtaining a high yield is a positive aspect, since it reduces the cost.

The analysis of variance results can be seen in Table V. The value of $F_{calculated}$ of the regression was not greater (3 to 4 times) than that of F_{tabled},[42] so the model cannot be considered predictive, but it was still possible to verify the effects of variables normally by the p-values. Analysis of variance for experimental design is used to assess whether the model we are suggesting to describe our data is statistically significant or not. For the model to be statistically significant, a hypothesis test must be performed. There are two possibilities:

- The null hypothesis (H0) is the hypothesis that will be tested, and it says that the model is not significant.
- The alternative hypothesis (Ha) says that the model is significant, and will be accepted as long as the null hypothesis is rejected.

To evaluate the hypotheses, the Fischer distribution table F is used. The $F_{calculated}$ is obtained by dividing the mean square of the regression by the mean square of the residuals. Subsequently, this calculated F value is compared with the Fischer distribution F_{tabled}, where the tabulated F (also called $F_{critical}$) is arranged according to the regression degrees of freedom (DF = 6) and the residuals degrees of freedom (DF = 4) (Table IV). For the null hypothesis to be rejected, the $F_{calculated}$ has to be greater than the F_{tabled}. Rejecting the null hypothesis, it can be said that the model is statistically significant.[43]

Table V. Analysis of variance of the central composite rotational design of the Flamboyant mirim study

Source of variation	Quadratic sum	Degrees of freedom	Quadratic average	$F_{calculated}$	F_{tabled}
Regression	7.7	6	1.3	4.6	6.2
Residual	1.1	4	0.3		
Lack of adjustment	0.8	2	0.4	2.9	19
Pure error	0.3	2	0.1		
Total	8.8	10			
R^2	0.9				

The coefficient of determination (R^2) obtained in the experimental design of Flamboyant mirim was 0.87409, which means that a quadratic function explained 87.41% of the total variation of the responses. This shows that the proposed planning can affect the percentages of the experimental data.

However, to consider a model well adjusted, the $F_{calculated}$ value of the regression must be greater than F_{tabled} and that the $F_{calculated}$ of the lack of adjustment is smaller than the F_{tabled}. Besides, the $F_{calculated}$ of the regression is 3 to 4 times greater than the F_{tabled}.[41] Thus, it can be concluded that the model is not well adjusted. Thus, the factorial experimental design still needs adjustments, but the yield values obtained were satisfactory based on those found in the literature.[39-41]

Hydrogen magnetic resonance spectroscopy (^{1}H-NMR)

The chemical structure of galactomannan was determined by ^{1}H NMR analysis of the Flamboyant mirim gum. Based on the gum's chemical shifts, anomeric hydrogen signals were identified of α-D-galactose (5.44 ppm) and β-D-mannose (5.06 ppm) (Figure 2). In addition to the main hydrogens referring to the α-D-galactose and β-D-mannose the hydrogens of the rings of both units with chemical shifts between 3.6 and 4,25 ppm were also identified.[44] All extractions presented very similar NMR spectra (data not shown), regardless of the method used in the process. For this reason, only Experiment 2 is reported, since the others had similar spectra.

As mentioned earlier, the mannose/galactose ratio is an important parameter for the study of galactomannans. The D-mannose/D-galactose ratios found for all the experiments were around 3.1, similar to that found in literature.[38] These ratios were determined through the integration of peaks related to chemical shifts of α-D-galactose and β-D-mannose present in galactomannans. Some studies present different mannose/galactose ratios,[41,45] but these divergences can probably be explained by differences in the galactomannan extraction and purification procedure. Also, the mannose/galactose ratio plays an important role in the solubility of galactomannans, since to have greater solubility in water, a higher D-galactose content is required.[22,24]

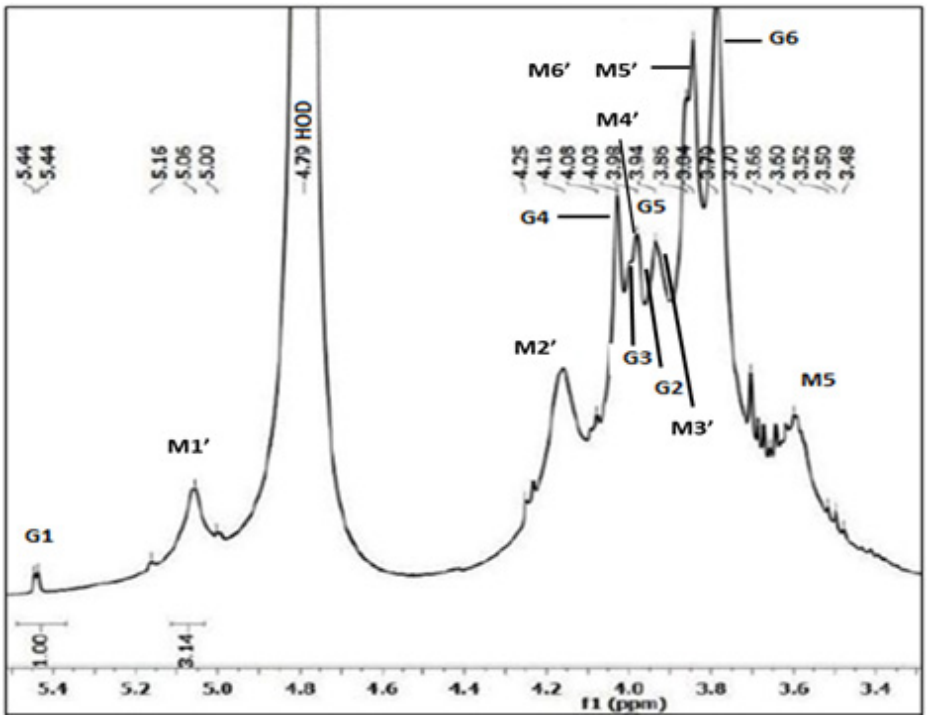
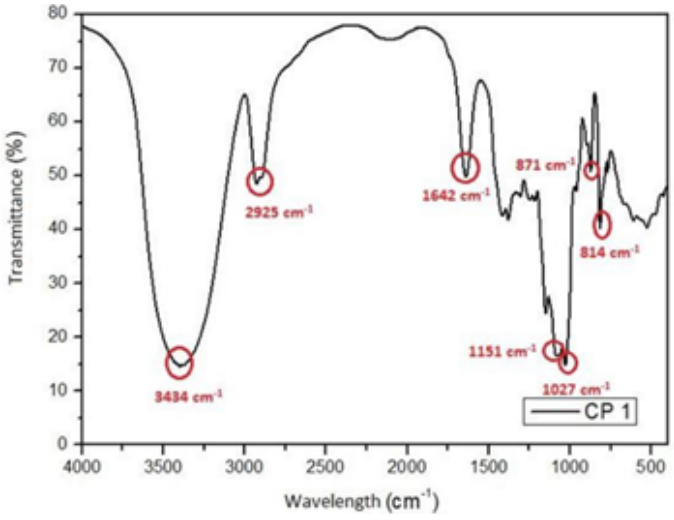


Figure 2. ^{1}H NMR spectrum of the Flamboyant mirim gum (Experiment 2).

Fourier-transform infrared spectroscopy (FTIR)

The analysis by FTIR allows observing and classifying some bands related to the vibrations of the functional groups present in organic compounds. The spectrum obtained and stretching vibrations presented in Figure 3 were similar in all the experiments carried out, as well as to those found in the literature, confirming the structure of galactomannan.[22,33,46-51]



Wavelength (cm^{-1})	Group features
3434	Vibrational stretching of the O-H group
2925	Group stretching -CH2
1642	Stretching of the galactose ring and mannose
1151	Stretching vibrations of glycosidic linkages (C-O-C)
1027	Vibrational CH2 stretch
871	Stretching of the anomeric conformations of polysaccharides (α-D-galactopyranose)
814	Stretching of the anomeric conformations of polysaccharides (β-D-mannopyranose)

Figure 3. FTIR spectrum of the Flamboyant mirim gum and the galactomannan groups identification (Experiment 1).

Elemental analysis (CHN)

To evaluate the efficiency of the purification process, nitrogen microanalysis was also carried out to determine the presence or absence of proteins adhered to the Flamboyant mirim gum, since its structure

does not contain nitrogen, whose presence can hinder the solubilization process.[51,52] According to the manufacturer, the detection limit of the equipment is 0.3%, with concentrations equal to or below this value being considered as absence of a determined chemical compound, and hence absence of proteins. Negligible concentrations of nitrogen were detected, ranging from 0.13 to 0.28% through samples CP 1 to CP 11, demonstrating that the process to separate this polysaccharide and proteins was highly effective. Consequently, the samples could be considered pure concerning the presence of remaining proteins.

Size-exclusion Chromatography (SEC)

The main objective of this analysis is to determine the molar mass of polymers and their distribution. Therefore, for all Flamboyant mirim gum samples the dn/dc values were calculated. These values were close to each other. The variations possibly occurred due to the different conditions for extraction of the biopolymer. However, a small variation in the dn/dc values often equals a large variation in the molar mass values. This determination is important according to the Zimm equation (Equation 1), because the dn/dc value is squared in the equation, making it the factor that most influences light scattering.

$$R_\theta = \left(\frac{\mathrm{dn}}{\mathrm{dc}}\right)^2 . c . M_w \qquad \text{Equation 1}$$

Different mass recovery rates were observed for all tested samples. The reduction in the percentage of recovered mass can occur due to several factors: polymer content of the sample, impurities, insufficient solubilization, and sample loss due to adsorption in the column, among others. This data indicates the actual concentration of biopolymer analysed.[54] The mass recovered varied from each experiment performed, from 18.1% (CP 2) to 100% (CP5). This parameter can be influenced by the purity of the final material, showing that CP 5, one of the samples obtained with the lowest salt concentration salt (0.1M) exhibited greater purity in these analyses. Comparison with the results obtained by TGA (Table II) reveals that in both techniques, the purest biopolymer was obtained in Experiment 5 (CP5).

Regarding the number average molecular mass (Mn) and the weight average molar mass (Mw), Experiment 5 produced the best result, probably due to the low temperature used in the extraction process, which may have favoured less degradation of galactomannan.

The results obtained for all galactomannans extracted from seeds of *Caesalpinia pulcherrima* presented a range of average molar mass around 10^4 - 10^6 g mol^{-1}, similar to the range reported in literature, whereas the smallest molar mass was observed for CP 9 (8.328×104 g mol^{-1}) and CP 5 exhibited the highest molar mass recorded in this work (1.260×10^6). Apart from those, all galactomannans presented molar mass within 10^5 g mol^{-1}.[32]

Rheological analysis

According to the results of the rheological analyses of the galactomannan samples extracted from Flamboyant mirim gum, the samples in general were soluble in water, which may be related to the low ratio between the mannose and galactose groups. The solubility of galactomannan decreased with decreasing galactose content, so the efficiency of aqueous extraction depends on the composition of galactomannan.[22,24]

The brine solutions of galactomannan obtained in Experiments 1(CP1) and 5 (CP5), prepared in Brine 1, showed pseudoplastic behavior and an increase in viscosity values with increasing concentration. Furthermore, Figure 4 shows that the pseudoplastic character declined with increasing concentration of galactomannan in the brine solution, because with the increase in the shear rate there was a lesser reduction in the viscosity values of the solutions. All galactomannan samples obtained in the factorial experimental design showed similar behavior when solubilized in brine.

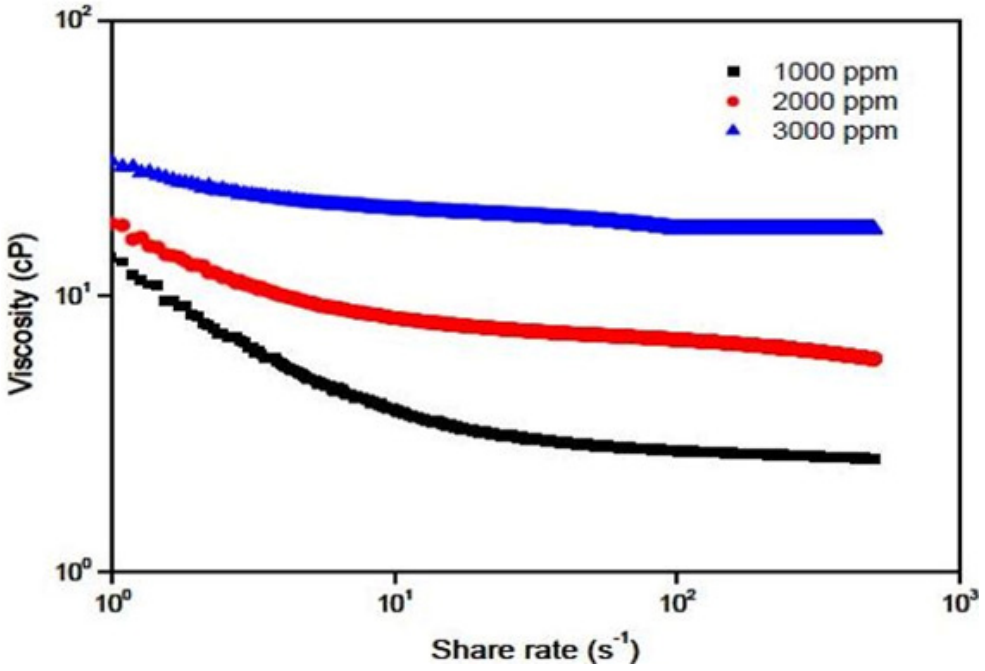


Figure 4. Flow curves of the galactomannan solutions obtained in Experiment 5 (CP5), at concentrations of 1000, 2000, and 3000 ppm in Brine 1, at 25 ºC.

The brine solutions of the samples obtained in the 11 experiments were also analyzed by obtaining flow curves at different temperatures (25, 60, and 80 °C) and at concentrations of 1000 and 2000 ppm, to simulate application conditions closer to reality. These solutions were prepared in both brines used in this study (1 and 2).

For a more detailed analysis, viscosity values of the solutions at shear rate of 7.37 s^{-1} were evaluated, simulating the flow inside an oil reservoir.[55] The values were obtained with polymer concentrations of 1000 and 2000 ppm in the two different synthetics brines. The results are shown in Figure 5. It is possible to notice the brine solution prepared with galactomannan obtained in Experiment 5 presented a higher viscosity value, which was expected since it presented the highest molar mass values and TGA analyses indicated a higher organic content.

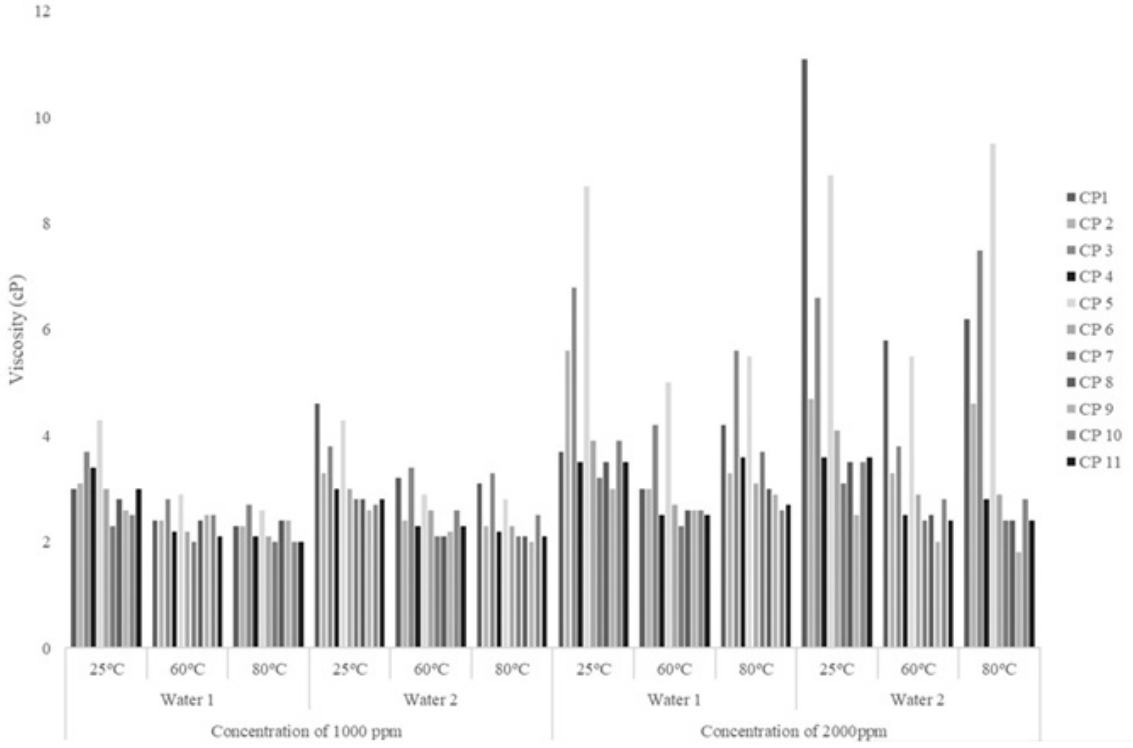


Figure 5. Viscosity values for Flamboyant mirim galactomannan at 7,37 s^{-1} at 1000 ppm and 2000 ppm concentrations in Brines 1 and 2.

Another factor to be considered is that, in general, there were no significant variations in the viscosity values of the solutions prepared with Brine 2 (TDS = 68317 ppm) compared to the values obtained for solutions prepared with Brine 1 (TDS = 29711 ppm). This behavior is satisfactory because even with the increase in the TDS values and with the increase in the concentration of divalent cations in Brine 2, the viscosity values of the solutions did not significantly change. This behavior is not observed for the partially hydrolyzed polyacrylamide synthetic polymer (HPAM), which has been studied and is currently the most applied polymer in EOR fluids. The brine solutions of this polymer in the severe conditions of some oil reservoirs, such as high salinity, have lower viscosity values, caused mainly by the presence of divalent cations, whereas the galactomannan, a non-ionic biopolymer, does not suffer salinity effects.[56,57] This is one more indication of how promising the use of biopolymers really is, when compared to HPAM, obtaining a more or equally viscous material without the use of synthetic polymers.[57,58]

Calculations were also performed to determine the concentration of product extracted in each experiment for preparation of galactomannan brine solutions so that the concentration of this biopolymer in the solutions would be 2000 ppm. These calculations were performed based on the results obtained in the thermogravimetric analyses, with deduction of the percentage of mass of the volatiles (solvents used in the extractions) and the non-degraded material (inorganic matter, like NaCl), to obtain the real values of organic matter, which was considered to be galactomannan in its entirety. Thus, the calculation to obtain the corrected concentration was carried out by applying equation 2. The results are shown in Table VI.

$$\text{Corrected concentration} = \left(\frac{2000}{(\%\ \text{decomposed until 800 °C} - \%\ \text{decomposed in the 1}^{\text{st}}\ \text{event})} \right) . 100 \quad \text{Equation 2}$$

Table VI. Viscosity values at the shear rate of 7.37 s^{-1} of the galactomannan samples obtained in the experiments, solubilized in Brine 1 at 25 ºC

Experiment	Corrected concentration[(a)]	Viscosity (cP)
1	2753	23
2	5405	25
3	2803	12
4	6845	13
5	2839	18
6	6289	19
7	2836	4
8	4879	7
9	3258	6
10	3494	7
11	2926	7

(a) The concentration of the final formulation, to contain 2000 ppm of biopolymer.

The viscosity values obtained in the formulations based on Flamboyant mirim gum were promising for potential application, since they were high for the solutions prepared with the samples obtained through the experiments carried out at low temperature (4 °C) (Experiments 1, 2 5 and 6), as can be seen in Table VI. However, due to the higher salt concentration in Experiments 2 and 6 (1.5 M NaCl), the corrected concentration was very high, not feasible for application. According to the literature, the concentration considered optimal for injection of the final product is up to 3000 ppm,[59] with a viscosity of around 20 cP, at a shear rate of 7.37 s^{-1}. Thus, the samples obtained in Experiments 1 and 5, in addition to presenting the highest molar mass results, also showed better viscosifying power, so they can be considered applicable fluids for enhanced oil recovery.

Stability of biopolymer solutions

The stability of the biopolymer was performed with the product of Experiment 5 (CP 5), which was the one with the most promising properties from all 11 tested in this study. Since it is a green material, i.e., environmentally friendly polymer, it is highly susceptible to biodegregation. So, it is commonly used with biocides to protect them from considered one the most promising biopolymers for EOR application due its good viscosifying power and high stability under high-salinity environments.

First, a solution of 3000 ppm of CP5 was formulated and divided into 5 flasks containing 20 mL each. After that, a 20% glutaraldehyde solution was prepared and added to the respective flasks in concentrations of 50, 100, 200, 300, 1000, 2000, and 4000 ppm.

The viscosity values of the solutions were monitored for 42 days, as reported in Figure 6, to verify the efficiency of the biocide in the prepared solutions. It is possible to observe that the control viscosity already decays by half just 7 days after the beginning of the experiment, followed by another strong decay in 14 days and seems to remain constant for the rest of the experiment. Only the 4000 ppm concentration was able to maintain the solution viscosity after 42 days.

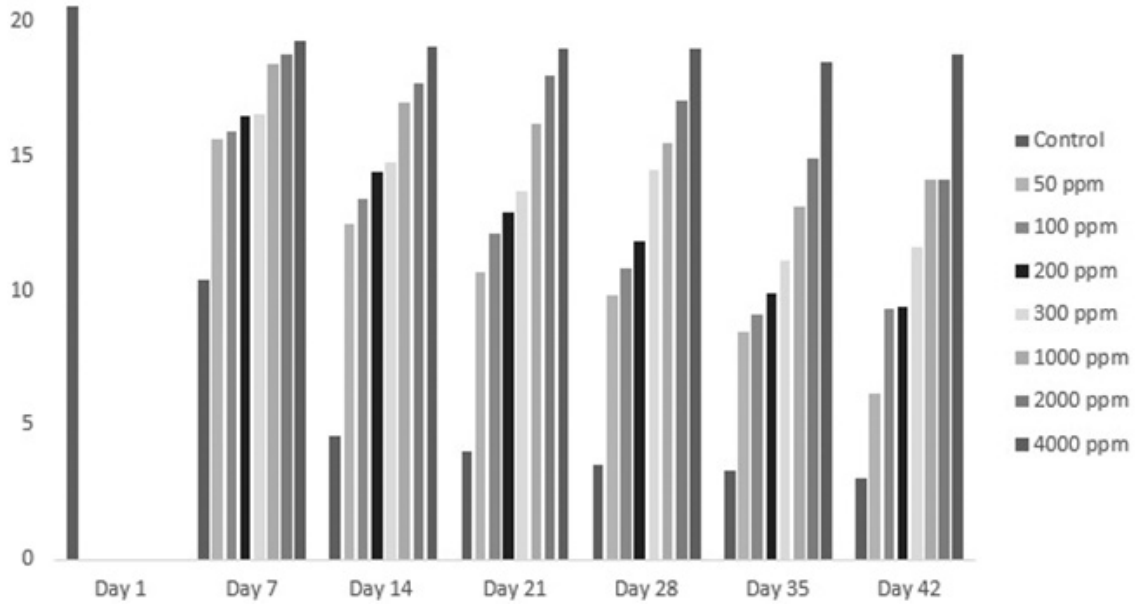


Figure 6. Viscosity of a 3000 ppm galactomannan solution with different biocide concentrations for 42 days.

These results reinforce those found in literature that suggests the optimal polymer: biocide ratio for biopolymers in EOR is 1:1.[60] Thus, as observed in Figure 6, the 4000 ppm concentration was capable of maintain the viscosity of a 3000 ppm biopolymer solution, since it is possible to observe a weekly decay in viscosity for biocides concentrations 50 to 2000 ppm. This result suggests that there is compatibility between the Flamboyant mirim galactomannan and glutaraldehyde, a established biocide in the petroleum industry.

CONCLUSIONS

Galactomannan extracted from Flamboyant mirim seeds can be obtained through simple and low-cost extraction processes, with the addition of inexpensive materials such as NaCl, water and alcohol. The experimental results showed that the most significant factor was time, but this was negative. This behavior suggests that the shortest extraction time was already sufficient to reach the maximum extraction of galactomannan, leading to a lower production cost. The best galactomannan yields obtained, disregarding the residual materials from the extraction processes, were 7.3%, calculated from the initial seed mass, and 24.6%, calculated from the endosperm content.

The galactomannan samples obtained through the experiments showed average molar mass values in the range of 10^4 to 10^6 g mol^{-1}. Also, the absence of protein linked to galactomannan chains was observed. The highest values of molar mass and purity degree of galactomannan were obtained through Experiment 5, which was carried out with the lowest temperature and least NaCl for extraction.

The rheological analysis revealed that the brine solutions of Flamboyant mirim gum with higher molar mass presented viscosity values around 20 cP, at the shear rate of 7.37 s^{-1}, using concentrations up to 3000 ppm in brine 1. These results are promising, considering they are in line with the requirements for fluids for application in EOR.

Finally, the stability tests carried out with the galactomannan solution in Brine 1 showed that the presence of the biocide (glutaraldehyde) resulted in the maintenance of the viscosity of these solutions.

Conflicts of interest

The authors declare no conflict of interest in this work.

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ARTICLE

Tracing the Geographical Origin of Moroccan Saffron by Mid-Infrared Spectroscopy and Multivariate Analysis

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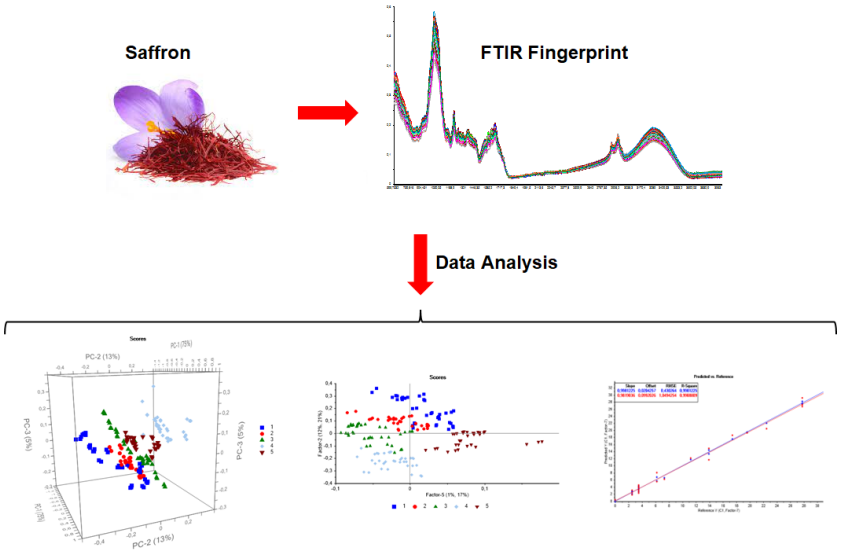

This work aims to investigate the potential of mid-infrared spectroscopy (MIR) and chemometrics algorithms for the determination of geographical origin and detection of adulteration of Moroccan saffron samples. First, the determination of the geographical origin of five saffron varieties was analyzed by linear discriminant analysis (PCA-LDA) and partial least squares discriminant analysis (PLS-DA). As a result, the developed models correctly classified saffron samples in a subset of external validation with 100% predictive ability. Next, partial least squares regression (PLS-R) was conducted to estimate the amount of adulterants (safflower) in the saffron samples. A good performance was found with Coefficient of Determination (R^2) between 0.97 and 0.99. Compared to other techniques, the main advantage of the proposed methods are non-destructive, fast and sensitive which allows to achieve very precise and accurate results.

Keywords: Saffron, Geographical origin, Adulteration, FTIR spectroscopy

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INTRODUCTION

Saffron is the red dried stigma with attached yellowish style of *Crocus sativus* L., family Iridaceae (ISO 3632-1. 2011),[1] and is regarded as one of the most extensively used and expensive spices, as 1 kg of saffron is obtained from about 150 000 flowers, which requires a lot of time and laborious handling and processing.[2] The quality of the spice depends mainly on many factors such as geographical origin and varietal, which also has an important influence on its price.[3]

Saffron is only available in a minority of countries and in limited quantities due to the particular growing conditions of saffron (climate, latitude and longitude), hence its name of red gold.[4] Saffron has been cultivated in Morocco for centuries in the Taliouine area (Taroudant province), and more recently in the Tazenakht area (Ouarzazate province) and in other areas in Azilal. But the saffron of Taliouine is highly reputed at national and international level. It constitutes one of the main supports of the economy of the region.

In response to growing food safety concerns, food authenticity and traceability are becoming a vital issue and a major challenge for consumers and regulatory authorities. Foods with high nutritional and economic value are particularly important for food authenticity. The search for a fast and reliable method of food authentication has therefore become an important need of the food industry.[5] For this reason, food scientists assist this type of research by developing analytical techniques to improve the ability to identify the geographical origin of foods and to detect their adulteration.

Producers and consumers are nowadays increasingly interested in high-quality food products with a clear geographical origin. Therefore, several methods are employed to assess the authenticity and geographical origin of saffron, such as spectroscopic techniques including ultraviolet-visible,[6] mid infrared (MIR),[7,8] nuclear magnetic resonance,[9] and chromatographic techniques such as high and ultra-high-performance liquid chromatography,[10,11] head-space-gas-chromatography,[12] gas chromatography-mass spectrometry and high-performance thin-layer chromatography[13,14] have been applied to ensure the quality control of saffron according to its chemical main components.

Each of the above analytical tools poses its own problems, such as they are costly, usually destructive, demand a high level of skill in technical knowledge of data interpretation, use of solvents and therefore require considerable time. Alternatively, FTIR spectroscopy is a non-destructive, rapid and sensitive tool involving minimal sample preparation.[15] When coupled with multivariate calibrations, FTIR spectroscopy can be utilized as a strong tool for geographic origin determination and adulterant detection in saffron. Currently, coupling FTIR spectroscopy with the user-friendly sampling of attenuated total reflectance (ATR) is a novel technology for saffron analysis due to its potential as a “fingerprinting technique” for quantitative or qualitative analysis. Food analysis using FTIR spectroscopy also can be regarded as “green analytical chemistry” since this technique minimizes the use of chemical reagents that are harmful to human health and the environment.[16,17,18]

The aim of the present work is to develop a rapid analytical approach based on the combination of MIR spectroscopy with multivariate analysis methods enabling to classify and predict Moroccan saffron according to their geographical origin and also, to detect and determine quickly saffron adulteration at levels of practical interest. The construction of a database by this technique constitutes a real novelty presenting considerable advantages in terms of rapidity and simplicity for the monitoring of the quality of Moroccan saffron.

MATERIALS AND METHODS

Sample preparation

A total of 230 saffron samples from five different regions of Morocco (Table I) were collected directly from farmers, guaranteeing their origin and the absence of fraud. All samples were purchased in the 2021 harvest season, packaged in boxes directly after harvest, and stored at room temperature. The samples were then transferred to the laboratory, where the analysis was performed at room temperature (25 °C, 60% relative humidity).

Table I. Geographic origin and number of Moroccan saffron samples employed in this work

Code	Number of samples	Origin	Altitude (m)	Price (€/g)
1	39	AIT-MAZIGH (Azilal province)	1100 - 1300	3
2	33	AIT-OUMDIS (Azilal province)	1100 - 1300	3
3	38	ATRGI (Azilal province)	1100 - 1300	3
4	37	TALIOUINE (Taroudant province)	1900 - 2200	5
5	33	TAZENAKHT (Ouarzazate province)	1450 - 1650	4

Safflower (*Carthamus tinctorius* L) was used as an adulterant of saffron powder due to its similar physical properties, low price, and lack of significant effects on human health.

The totality of samples (saffron and adulterants) was ground into a powder with an agate mortar, then stored in a dark room equipped with a dehumidifier to prevent humidity and ambient light.

To establish the geographical origin of five varieties of saffron, the calibration model was built with a set of 180 samples, and then validated by external validation with new 10 samples of each variety (new set of tests not used in the calibration of the models).

For the assessment of adulteration, a series of 43 samples was prepared by mixing authentic saffron (Taliouine origin) with adulterating safflower at different levels w/w (2.5% to 30%).

$$\%\ \text{Adulteration} = \frac{\text{mass of adulterant in sample}}{\text{total mass of sample}} * 100$$

Spectral Acquisition

For each MIR analysis, spectra were registered on a single reflection diamond ATR (JASCO FTIR 460 PLUS (Pike Technologies, Madison, USA)), by placing the saffron powder sample directly on the ATR cell (diamond crystal). A quantity of powder equivalent to one milligram provides good spectra, recorded from 4000 to 600 cm^{-1}, by taking a total of 60 scans with a spectral resolution of 4 cm^{-1}. After each measurement, the crystalline surface was washed with isopropanol solution and dried with a soft paper. The treatment of the obtained spectra was carried out with the software (Spectra manager) in order to eliminate the effect of moisture (at 3756 cm^{-1}, 3652 cm^{-1}, and 1595 cm^{-1}) and to eliminate the effect of carbon dioxide in three bands (at 2349 cm^{-1}, 1388 cm^{-1}, and 667 cm^{-1}).

Data Analysis

Multivariate statistics of the MIR spectra were performed using Unscrambler X, v10.4 (CAMO Software AS, Oslo, Norway, 2016). An exploratory analysis of the data is usually performed as the first step. For this purpose, PCA is generally used as a common unsupervised recognition method, to visualize the data to search for inconsistencies and aberrant values before performing regression techniques.[19]

The geographical origin of saffron was traced in this study by using two algorithms, linear discriminant analysis (LDA) and partial least squares discriminant analysis (PLS-DA).

LDA is a technique for supervised classification that builds on linear discriminant functions, maximizing the inter-class variance ratio and minimizing the intra-class variance ratio. It analyzes the different variables associated with a particular object and affects the object to a class or group according to the differences and similarities between the variables.[20]

PLS-DA is a linear classification technique that integrates the properties of PLS regression and the discrimination capability of a classification approach. PLS-DA is based on the PLS regression algorithm (PLS1 when dealing with a single dependent Y variable and PLS2 when dealing with several dependent

Y variables); this algorithm looks for latent variables with maximum covariance with the Y variables.[21] The main advantage of PLS-DA is that the pertinent sources of data variability are modeled by the latent variables (LVs), representing the linear combinations of the original variables, and as a result it allows visualization and understanding of the different data patterns and linkages via the LV scores and loadings. The optimal number of LVs is commonly identified by using a cross-validation method to minimize the classification error.[22,23]

The predictive quality of the LDA and PLS-DA model was evaluated by determining the performance inductors, including the sensitivity (number of real positive cases successfully identified), specificity (number of real negative cases successfully identified), the accuracy (proportion of correctly identified cases as true positives (TP) and true negatives (TN)), the precision (number of true positives among all individuals that were predicted to be positive) and the correct classification rate (CCR) which is considered as the overall accuracy.[24,25]

$$Sensitivity(\%) = \frac{TP}{TP + FN} * 100 \qquad Specificity(\%) = \frac{\mathrm{TN}}{\mathrm{TN} + \mathrm{FP}} * 100$$

$$Accuracy(\%) = 1 - \frac{\mathrm{FP} + \mathrm{FN}}{\mathrm{TP} + \mathrm{TN} + \mathrm{FP} + \mathrm{FN}} * 100 \qquad Precision(\%) = \frac{TP}{TP + FP} * 100$$

$$CCR(\%) = \frac{\mathrm{TP} + \mathrm{TN}}{\mathrm{TP} + \mathrm{TN} + \mathrm{FP} + \mathrm{FN}} * 100$$

For quantitative analysis partial least squares regression (PLS-R) was applied to build a calibration model able to predict saffron adulteration, it is recognized as a standard technique for calibration and prediction using the relationship between X and Y matrices. It is widely employed in fluorescence, Raman and infrared spectroscopy for the prediction and quantification of some chemical parameters in agri-food.[26,27,28]

To examine the predictive ability of these models, we considered the Coefficient of Determination (R^2), root mean square error of calibration (RMSEC) and root mean square error of cross-validation (RMSECV). The RMSEC, RMSECV values of a strong model must be low and the R^2 should be high.[29]

$$RMSEC = \sqrt{\frac{\sum \left(\mathrm{Yi} - \hat{\mathrm{Y}}\mathrm{i}\right)^2}{\mathrm{A} - 1}} \qquad RMSECV = \sqrt{\frac{\sum \left(\mathrm{Yi} - \hat{\mathrm{Y}}\mathrm{i}\right)^2}{\mathrm{B} - 1}} \qquad R^2 = 1 - \frac{RSS}{TSS}$$

Where:

- Yi and Ŷi indicate the actual and predicted values, while A and B indicate the number of samples used in the calibration and cross-validation datasets.
- RSS represents the residual sum of squares and TSS represents the total sum of squares.

RESULTS AND DISCUSSION

Spectra Analysis

The MIR spectra of all samples were similar by visual inspection and presented the same profile over the full wavelength range (Figure 1), indicating that there are no significant qualitative differences between the saffron samples. All spectra show common absorption bands as illustrated in Table II. In the spectra, however, small differences are observed between 1800 and 700 cm^{-1} in the relative absorbance intensities of the different cultivars. These minor differences in relative intensity values were exploited in the classification for the determination of the geographical origin of saffron, as discussed below.[7,30,31]

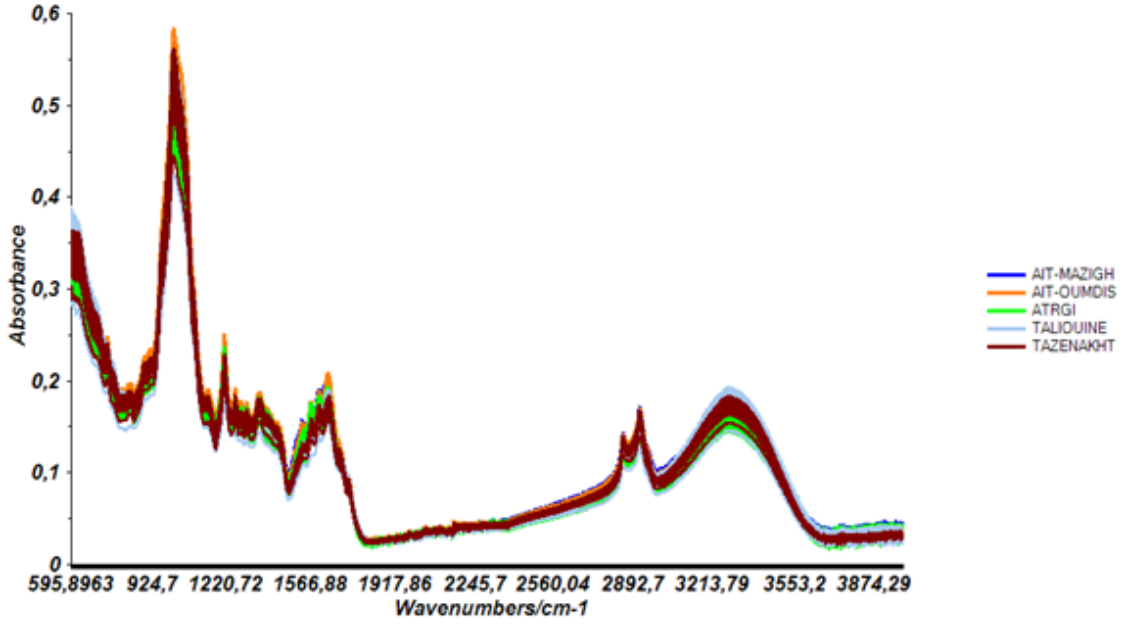

Figure 1. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) raw spectra of five Moroccan varieties of saffron samples.

The multivariate analysis method is nowadays an essential chemometric tool to analyze data coming from many observations performed on numerous variables.[32] The main objective of classification is to find a mathematical relationship between a set of descriptive variables (such as physico-chemical characteristics, spectral measurements, etc.) and a qualitative variable (sample categories). In this study, two classification approaches such as LDA and PLS-DA were employed to construct classification models of saffron origin.

Table II. FTIR wavenumber regions of saffron varieties, correlated with the mode of vibration for specific functional groups, considered as predictor variables for statistical data processing

Location of wave numbers (cm^{-1})	Vibrations	Band assignment of functional group/component
3400 (Broad)	O-H stretch	Hydroxy group
2900 and 3000	C-H asymmetric and symmetric stretching	Aliphatic methylene group
1800 – 1500	C=O stretch	crocetin / aminoacids
1645	C=C and C=O stretch	Picrocrocin / amide I
600 – 1500 (fingerprint region)	$–CH_2–$, $CH_3–$, –OH, C–C, C–O, C–O–C groups	glycosidic linkages of oligo and polysaccharides or in triacylglycerols

Geographical origin discrimination

Principal component analysis

Principal component analysis (PCA) was applied to the MIR spectral results to investigate the structure of the saffron data and also to study the similarity between individuals. The PCA shows that the first three principal components represent 93% of the total variability of the data, the observation of the score plot Figure 2 shows the presence of clusters of saffron samples according to their geographical origin, we can distinguish the presence of five groups indicating that the samples belonging to each of the five groups had similar FT-MIR properties.

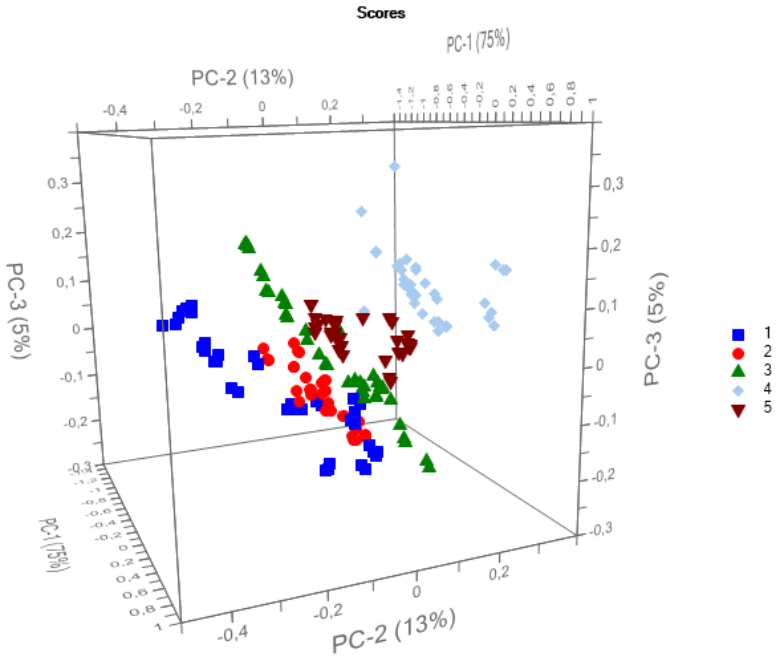

Figure 2. Score plot of the PCA model with three principal components (PC1-PC2-PC3) for the saffron MIR data. The first three PCs can explain more than 90% of the variation.

Linear discriminant analysis (LDA)

The linear discriminant analysis was applied to the MIR spectra of saffron samples, previously processed by principal component analysis (PCA). LDA was applied on the first 10 principal components obtained by PCA. The application of this method shows good discrimination between the 5 groups of saffron coming from different regions of Morocco (Figure 3). This plot shows that groups 1, 2, and 3 are close to each other while groups 4 and 5 are far away, this remark is explained by the distance that separates these regions, the more the distance is great more inter-group variability is high the more the groups are well separated in the discrimination plot obtained by LDA and vice versa.

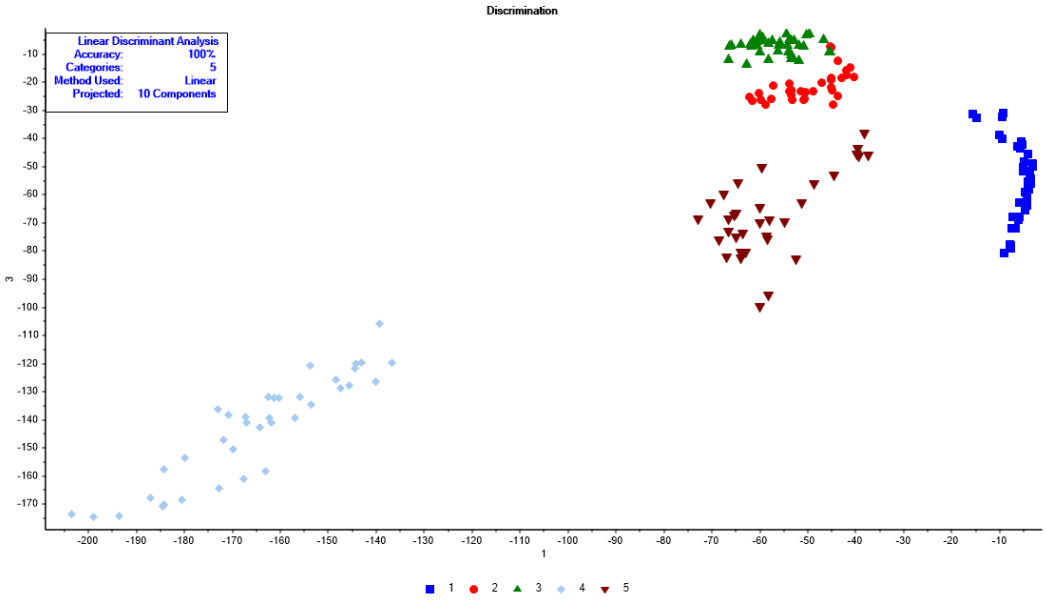

Figure 3. LDA scores plot in the analysis of the FTIR spectra of saffron samples. A strong discrimination of five varieties is displayed by an accuracy of 100%.

The use of the LDA method demonstrates successful results for the classification of different saffron samples according to their geographical origin using MIR spectral data, this discrimination capacity is summarized in Table III in which it can be observed that this model is able to classify the five saffron groups with specificity, sensitivity, and CCR of 100%.

Table III. LDA confusion matrix on the test saffron data set, including the calculation of the calibration performance (sensitivity, precision, specificity, accuracy and CCR)

LDA model (calibration)											
Confusion matrix		**Actual**					**Sensitivity (%)**	**Precision (%)**	**Specificity (%)**	**Accuracy (%)**	**CCR (%)**
		1	**2**	**3**	**4**	**5**					
Predicted	1	39	0	0	0	0	100	100	100	100	100
	2	0	33	0	0	0	100	100	100	100	100
	3	0	0	39	0	0	100	100	100	100	100
	4	0	0	0	37	0	100	100	100	100	100
	5	0	0	0	0	33	100	100	100	100	100

These results reveal that the developed model successfully classify the five groups of saffron according to their geographical origin with an accuracy of 100%. Authentications were based on various discriminant variables including crocins, picrocrocin, triacylglycerols, oligo, and polysaccharides.

Partial Least Square-Discriminate Analysis (PLS-DA)

The PLS-DA model was built considering the FTIR spectra as X variables, while the Y variables were associated with the five different saffron classes (one different Y variable for each group's class, with 1 or 0 depending on whether it belongs or not to the considered data group).

The use of the PLS-DA method (on the first 10 principal components) shows a high capacity for the classification of the saffron groups according to their geographical origin as shown in Figure 4 in which we observe clear and perfect discrimination between the saffron groups following the PC2-PC5 score plot.

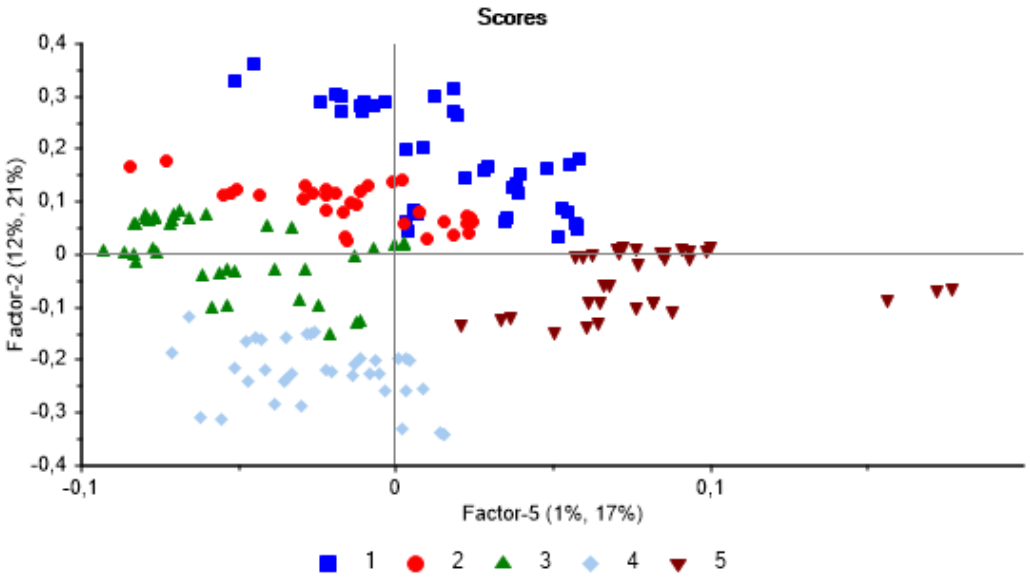

Figure 4. 2D scores plot of PLS-DA in the analysis of the FTIR spectra of saffron samples. Group 1, 4 and 5 are well separated while group 2 and 3 are not far apart on the figure.

This discrimination ability is represented by the high value of the sensitivity, specifcity, and accuracy which reach 100%, indicating that all samples are correctly assigned to their classe (Table IV).

Table IV. Confusion matrix for the classification five types of saffron samples using the PLS-DA method

PLS-DA model (calibration)											
Confusion matrix		**Actual**					**Sensitivity (%)**	**Precision (%)**	**Specificity (%)**	**Accuracy (%)**	**CCR (%)**
		1	**2**	**3**	**4**	**5**					
Predicted	1	39	0	0	0	0	100	100	100	100	100
	2	0	33	0	0	0	100	100	100	100	100
	3	0	0	39	0	0	100	100	100	100	100
	4	0	0	0	37	0	100	100	100	100	100
	5	0	0	0	0	33	100	100	100	100	100

As shown in the loading plot (Figure 5), the most important absorbances are respectively situated between 900 and 1700 cm^{-1} corresponding to the $–CH_2–$, $CH_3–$, –OH, C–C, C–O and C–O–C groups.

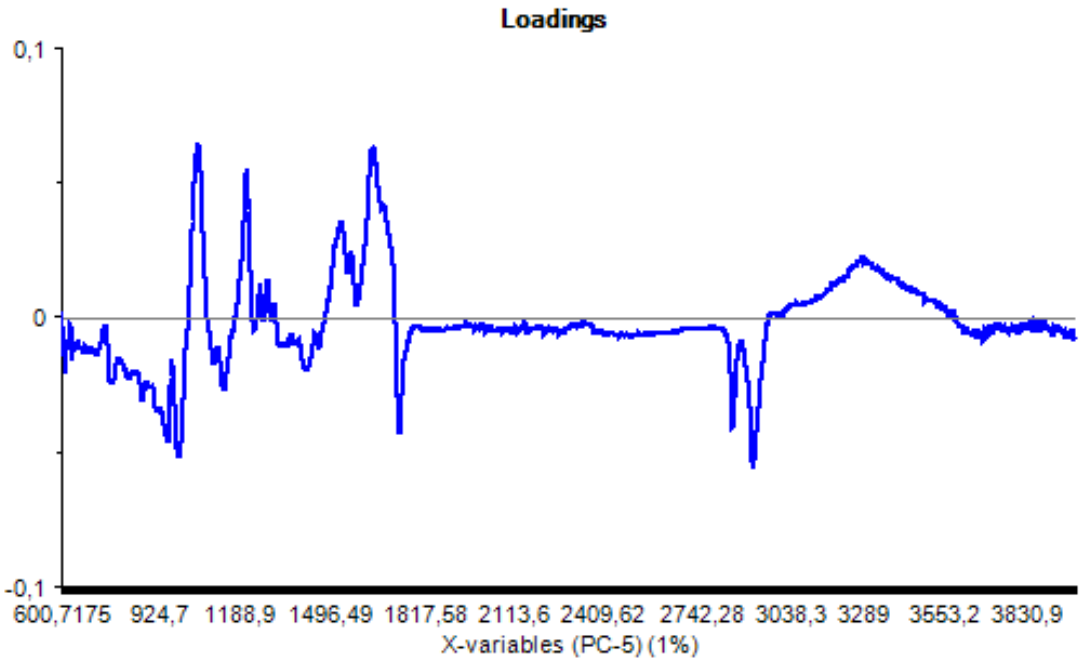

Figure 5. Loading plot from PLS-DA of FTIR spectra data. The 600 to 1500 cm^{-1} region represents the fingerprint region, contains the main functional groups involved in discrimination.

Validation of discriminant Models

The classification and predictive ability of the constructed models was verified by external validation of the models with samples other than those used for models construction (10 samples for each group).

According to the PLS-DA rules when the predicted value of Y is between 0.5 and 1.5 for a class, the sample is regarded as belonging to this class.[33]

The confusion matrix (Table V) achieved by external validation shows that for the validation samples, a 100% correct classification was achieved and all the saffron spectra were correctly matched to the five corresponding classes. These results confirm that the predictive ability of PLS-DA and LDA models was satisfactory good. These results demonstrated the ability of the proposed technique to discriminate between the five different cultivars of saffron samples used in this study.

Table V. Results of the external validation of the PLS-DA and LDA model of the saffron samples

Confusion matrix		Actual set					Sensitivity (%)	Precision (%)	Specificity (%)	Accuracy (%)	CCR (%)
		1	2	3	4	5					
Predicted set (PCA-LDA)	1	10	0	0	0	0	100	100	100	100	100
	2	0	10	0	0	0	100	100	100	100	100
	3	0	0	10	0	0	100	100	100	100	100
	4	0	0	0	10	0	100	100	100	100	100
	5	0	0	0	0	10	100	100	100	100	100
Predicted set (PLS-DA)	1	10	0	0	0	0	100	100	100	100	100
	2	0	10	0	0	0	100	100	100	100	100
	3	0	0	10	0	0	100	100	100	100	100
	4	0	0	0	10	0	100	100	100	100	100
	5	0	0	0	0	10	100	100	100	100	100

According to the obtained results, the geographical origin determination of the five groups of saffron has been well done based on the MIR spectral data, since the application of the PLS-DA and LDA approaches shows a great discrimination performance, which revealed no misclassification in all cases.

Detection of adulteration by Partial Least Square regression (PLS-R)

The development of a quantification model based on PLS regression can therefore extend the potential of this work for the control of saffron falsification using MIR spectroscopy.

The prediction of the added adulterant (safflower) in saffron powder was performed using a PLS-R model. 43 adulterated saffron samples were used for the creation of the calibration model, and 10 new adulterated samples were used for the validation of this model. The linearity of the method was evaluated in the PLS-R calibration model to demonstrate that the predictor variables (band intensity) were proportionally related to the percentage of adulterants.

According to the results obtained by the PLS regression we observed that there is a good fit between the reference values and the prediction values (Figure 6). This ability is displayed by the low value of the root mean square error of calibration (RMSEC=1.59), cross-validation (RMSECV=2.45) and the high value of R-square for the calibration (R^2cal=0.97) and cross-validation (R^2cv=0.92). Based on the results obtained by the cross validation 7 latent variables were selected to build the quantification model.

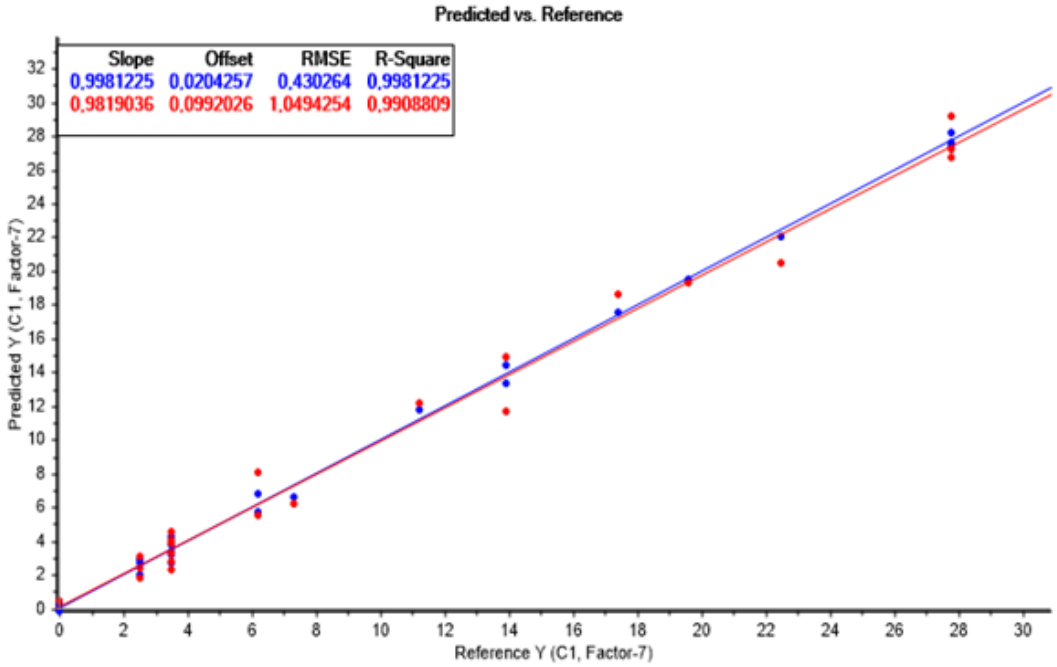

Figure 6. Actual vs. predicted concentrations of adulteration in saffron % (w/w) (blue line corresponds to the calibration and red line to cross-validation).

In order to evaluate the capacity of the model for accurate prediction of the adulteration rate an external validation is performed. The validation results (Table VI) show a strong capacity for the prediction of the adulteration rate. This capacity is represented by the R-square of prediction which reaches 0.95 and the low value of RMSEP=1.75.

Table VI. Prediction of adulterant content (safflower) by external validation of PLS-R model

Reference (w/w)	Predicted (w/w)	Deviation
3.20	1.75	1.94
4.1	3.11	1.72
5.5	2.8	2.53
7.2	6.68	1.75
11.5	13.39	1.95
14	15.85	2.95
16.31	15.97	2.31
20.4	18.27	1.71
21.2	24.20	1.98
31.2	32.32	3.16

In the light of the results obtained, the strategy proposed in this study is not only applicable to determine whether a saffron sample is adulterated or not, but it can also be used to assess the level of adulteration of samples.

In comparing the results of this study with previous research, it is important to underline that the performance criteria (accuracy, specificity and sensitivity) in this study were the highest compared to the other studies.[18,34,35] However, compared to previous work, a higher number of saffron samples in this work were utilized (n=230). Furthermore, it should be noted that it is for the first time that the authentication of the varieties of Moroccan saffron was carried out by this technique, thus this work will make it possible to build a database for the control of the quality of Moroccan saffron.

CONCLUSION

This project proves that the use of FTIR spectroscopy, combined with PLS-DA and LDA algorithms, is able to classify saffron according to its geographical origin. Sample analysis was carried out directly on the saffron powder to provide a fingerprint of the saffron without the preparation of the sample. This approach represents a practical and easy tool to verify the origin of saffron, offering advantages such as speed and ease of use, and liable to represent a good alternative to the method of DNA markers.

The combination of FTIR spectroscopy and PLS-R algorithm reveals to be effective to determine and identify adulteration in saffron, since it is difficult to detect saffron fraud according to ISO 3632 methods, in particular when using identical-looking plants for adulteration and when the spice is marketed as a powder.

As a result, the combined use of MIR spectroscopy and multivariate data analysis was confirmed as a powerful and valid tool to evaluate the authenticity and quality control of saffron by offering unique fingerprint spectra. However, it is necessary to dispose of more robust origin models, able to better detect regional varieties. Specifically, a large dataset representing high variability should be analyzed to create a comprehensive database of Moroccan saffron varieties (geographic origin, harvest year).

Conflicts of interest

The authors have declared no conflicts of interest for this article.

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ARTICLE

Comparison of Ultrasound-assisted Methods for Copper Determination in Bovine and Ovine Liver as Strategies for Food Surveillance and Animal Status Monitoring

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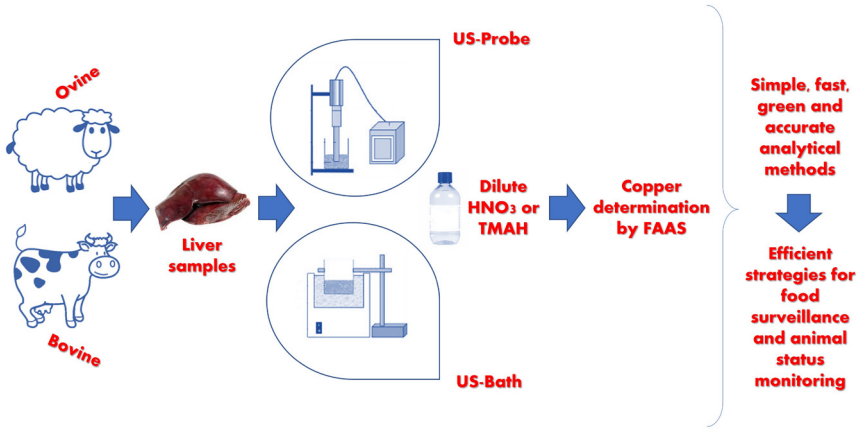

Four methods for the efficient extraction of copper from bovine and ovine liver were optimized. Sample preparation consisted of extractions with dilute nitric acid or dilute tetramethyl ammonium hydroxide, assisted by an ultrasonic bath or an ultrasonic probe. Copper was determined by flame atomic absorption spectrometry. The experimental conditions were optimized using multivariate experiments. All methods were considered adequate for copper extraction, however, the two methods involving the probe turned out to be more efficient and faster, so they were selected for subsequent validation. Trueness was verified after the analysis of a certified reference material and the performance of a microwave-assisted extraction. Results were statistically equivalent, at the 95% significance level, to the values declared on the certificate. Precision (expressed as relative standard deviation) was better than 5% for all methods. Samples obtained from Uruguayan animals were analyzed. Obtained results agreed with previous results from sheep and cattle abroad. The proposed methods are simple alternatives for food surveillance and animal status monitoring, being straightforward and aligned with Green Chemistry principles, as it was demonstrated by performing the analytical Eco-Scale comprehensive approach. A discussion related to the particle size distribution obtained during the multivariate experiments was also included, to give some deeper insight into ultrasound effect on the biological tissue in different media. In addition, ultrasound-assisted extraction was compared to magnetic stirring to prove the effect of ultrasound.

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INTRODUCTION

One of the main trends in sample preparation is to seek for procedures that involve mild conditions and short times of extraction.[1] In this regard, many efficient techniques have been developed, particularly ultrasound-assisted extraction (UAE), that permit fast, reproducible, and quantitative analysis of trace elements from complex samples. UAE methods are very efficient alternatives for biological samples analysis that allow significant shortening of treatments with subsequent saving in energy, reduce solvents consumption, simplify manipulation, and involve safer procedures, thus being ecofriendly and low-cost strategies aligned with Green Chemistry principles.[2-5]

Ultrasonication can induce a physical phenomenon called cavitation, which accelerates extraction procedures, while inducing cell rupture and subsequently releasing encapsulated analytes.[4] Moreover, ultrasonication promotes several reactions that include the generation of oxidizing species and reactive radicals such as $OH^{\bullet}$ and H_2O_2 that favor the oxidation of organic matter.[6]

When cavitation bubbles implode on the surface of a given sample, micro-jets are generated, giving rise to different effects such as erosion, surface peeling, and particle breakdown. This fragmentation of the matrix results in a better contact with the extractant and an enhanced extraction yield of the analyte,[3] which is also favored by the vigorous mixing generated by the propagation of the ultrasound waves.[7]

Copper (Cu) is essential for the activity of several enzymes, cofactors, and reactive proteins in cattle. It is involved in reproduction, pigmentation, connective tissue development, cellular respiration, protection from oxidants, and iron transport.[8] Numerous naturally occurring diseases reported in grazing animals can be attributed to Cu deficiency. A broad range of Cu-responsive disorders are associated with low Cu status known as 'hypocuprosis', showing the importance of performing Cu assessments. When a given organ is responsible for the accumulation of a certain element, its weight is predictable, and it can be then biopsied. This occurs with the liver of ruminants, where Cu retention can be estimated from the rates of increase of the element concentration in this organ. Thus, enhanced Cu levels in the liver can give an estimation of the true absorption of this element in both cattle and sheep.[9] On the other hand, chronic Cu accumulation may occur, depending on its absorption rate from a supplement or feed, which also depends on the interactions with other constituents of the ration, especially sulfur (S), molybdenum (Mo) and iron (Fe).[9]

When it comes to the food science field, meat and its derivatives constitute a very important part of the human diet, providing essential nutrients that cannot be easily obtained from vegetable sources. Furthermore, organs such as liver, are even richer in minerals and vitamins when compared to other muscular tissues, being more adequate sources to meet the nutritional requirements of human beings.[10] In this context, the evaluation of an essential element such as Cu, constitutes a highly valuable input to assess the food quality of this product.

Most conventional UAE-based methods involve extractions that use dilute acids with oxidizing properties such as nitric acid (HNO_3).[11] However, an alternative approach for sample preparation of biological tissues is the use of strong alkaline reagents such as tetramethylammonium hydroxide (TMAH). This compound has been used as a 'tissue solubilizer' for various biological samples prior to analysis of Cu by flame atomic absorption spectrometry (FAAS).[12,13] Working with TMAH offers an efficient approach to the preparation of homogenized biological sample solutions, under mild conditions. Besides, only a small amount of TMAH is used, becoming an attractive alternative for the treatment of protein-rich samples. Additionally, its efficiency can be increased by using ultrasound energy.[14,15]

In this context, the aim of this work was the optimization and validation of simple and fast analytical methods for Cu extraction from bovine and ovine liver by means of UAE, using a bath or a probe and employing either dilute HNO_3 or dilute TMAH as extraction solvents, with subsequent determination by FAAS. Also, to compare the efficiency of the selected UAE methods against a microwave-assisted extraction (MAE) method in terms of greenness, reassuring the advantage of ultrasound over standard methods. Furthermore, a discussion related to the particle size distribution was also included, to give some

deeper insight into ultrasound effect over the sample in the different media assayed. The novelty of the work is related to the development of green, straightforward, and accurate strategies that can be easily implemented for both food surveillance and animal status monitoring. To the best of our knowledge, scarce information is available on the use of ultrasound technology for Cu extraction from bovine and ovine tissue samples, specially employing TMAH solubilization.

MATERIALS AND METHODS

Reagents

For alkaline extractions, dilute TMAH was prepared from 25% w w^{-1} TMAH in methanol (Sigma Aldrich, St. Louis, MO, USA). For acid extractions, dilute HNO_3 was prepared from 67% w w^{-1} HNO_3 (Merck, Darmstadt, Germany). Ultrapure water (18.2 MΩ cm resistivity) was obtained from a Millipore™ DirectQ3 UV water purification system (Bedford, MA, USA). Standard solutions for calibration curves were prepared by serial dilution of a 1000 mg L^{-1} commercial atomic absorption solution of Cu (Fluka, Hauppauge, NY, USA), using the same final HNO_3 or TMAH concentration as in the samples.

A bovine liver reference material (RM) RM-Agro E3001a (Embrapa, São Carlos, SP, Brazil) was used for optimization experiments while a bovine liver certified reference material (CRM) NIST 1577c (Gaithersburg, MD, USA) was employed for trueness and precision evaluation of the selected analytical method.

All other reagents were of analytical grade. All glassware was decontaminated with 1.5 mol L^{-1} HNO_3 before its use.

Samples

Bovine and ovine liver samples were provided by a local slaughterhouse, all coming from young animals (12 – 24 months). Samples were washed with deionized water, triturated, and homogenized using a food blender with stainless steel blades, and finally stored in polypropylene tubes at -18 °C until analysis.

Sample preparation procedures

Microwave-assisted extraction

For the total determinations of Cu, a microwave-assisted acid digestion (MAE) was performed, using a CEM Mars 6 microwave digestor (Matthews, NC, USA) equipped with 12 Easy Prep Plus™ vessels. Each vessel was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3. The power of the digestor varied between 400 – 1800 W. After reaching 200 °C in 15 min, the program was maintained at this temperature for 10 min. The maximum pressure was set at 500 psi. Obtained solutions were used for analytical determinations without further dilution. Treatments were run in triplicate. Reagent blanks were carried out.

UAE with probe (UAE-probe)

The treatment was performed using a Sonics® Vibra-Cell™ VC505 (Newtown, CT, USA) ultrasonic homogenizer equipped with a 13-mm titanium alloy probe (750 W, 20 kHz, 230 V_{AC}). A 50-mL flask was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3 or 0.36 mol L^{-1} TMAH, and the probe was immersed 2 cm inside the mixture for 10 min at a sonication amplitude of 35%. The obtained suspension was filtered through a 0.45 µm pore size nitrocellulose filter (Millipore™, Bedford, MA, USA) and transferred to a 50-mL polypropylene tube. The filtrate solution was used for analytical determinations without further dilution. The procedure was run in triplicate. Reagent blanks were carried out.

UAE with bath (UAE-bath)

Samples were prepared in a Cole-Parmer® 8893 (Vernon Hills, IL, USA) ultrasonic bath (47 kHz, 230 V_{AC}). A 50-mL flask was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3 or 0.36 mol L^{-1} TMAH, and then the vessel was immersed in the bath for 20 min. The obtained suspension was filtered through a 0.45 µm pore size nitrocellulose filter (Millipore™, Bedford, MA, USA) and transferred to a 50-mL

polypropylene tube. The filtrate solution was used for analytical determinations without further dilution. The procedure was run in triplicate. Reagent blanks were carried out. Samples were located in the middle part of the bath (12 cm from the front and 15 cm from the sides) since for baths fitted with a single transducer, the maximum ultrasonic intensity is found above the transducer.

Magnetic stirring extraction

A silent condition using magnetic stirring was performed to establish whether the observed effects were in fact due to ultrasound energy and not due to other parameters. Samples were prepared using a Thermolyne Cimarec® 2 (Dubuque, IA, USA) magnetic stirrer. A 50-mL flask was charged with 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3 or 0.36 mol L^{-1} TMAH, and the vessel was put over the stirrer for 10 min (HNO_3) or 20 min (TMAH) and stirred at 500 rpm. The obtained suspension was filtered through a 0.45 µm pore size nitrocellulose filter (Millipore™, Bedford, MA, USA) and used for analytical determinations without further dilution. The procedure was carried out in triplicate.

Analytical determinations

Copper was determined by FAAS using a Perkin Elmer AAnalyst 200 spectrometer (Norwalk, CT, USA) with a 10-cm burner and operated at 324.75 nm. A Lumina™ lamp from Perkin Elmer (Norwalk, CT, USA) was used. The flame was composed by acetylene (2.8 L min^{-1}) and air (10.2 L min^{-1}). Acetylene 99.5% was purchased from Praxair (Montevideo, Uruguay). Calibration curves were prepared at the same TMAH or HNO_3 concentration used in the samples.

Optimization of extraction conditions

Multivariate experiments were used for the optimization of UAE-based methods. A two-factor three-level (3^2) factorial design was employed to determine the best extraction conditions.[16] The two variables studied were HNO_3 or TMAH concentration and sonication time. The evaluated response was the average recovery R (%), by comparison with the informed value of the bovine liver RM (Table I). Additionally, it was verified by using analysis of variance (ANOVA).

Table I. Experimental conditions of the multivariate experimental design applied to UAE-based methods, using the bovine liver RM

Experiment	HNO_3 Concentration (mol L^{-1})	TMAH Concentration (mol L^{-1})	Time (min) Probe & Bath
1	2.8	0.36	15
2	1.4	0.24	10
3	4.2	0.48	10
4	1.4	0.24	15
5	2.8	0.36	10
6	4.2	0.48	15
7	2.8	0.36	20
8	1.4	0.24	20
9	4.2	0.48	20

Method validation

After the critical variables were optimized, the validation was performed according to the recommendations of the Eurachem Guide.[17] The figures of merit evaluated were precision, trueness, linear range, limit of detection (LOD), and limit of quantification (LOQ).

Particle size analysis

The particle size distribution of residues obtained after different sonication procedures was evaluated with a Coulter™ LS230 (Brea, CA, USA) laser diffraction analyzer, in the range 0.04 – 2000 µm, using a laser light with a wavelength of 750 nm. Samples were dispersed in distilled water prior to its introduction into the fluid module. The Fraunhofer mathematical model was selected to calculate the particle size based on the concentric ring pattern of light-scatter from the laser beam. The amount of sample needed was determined by the obscuration percentage of the laser beam passing through the sample cell, between 8 and 12%.

RESULTS AND DISCUSSION

Optimization experiments

The optimal conditions for UAE-based methods were selected after the evaluation of the per cent recovery estimated as R (%) = [obtained concentration (mg kg^{-1})/informed concentration (mg kg^{-1})] × 100, for each experiment. Simplest and fastest conditions with an R (%) statistically equal to 100% were chosen for subsequent validation.

Table II shows the R (%) values obtained for each experiment. The best results for UAE-probe, either for the acid or the alkaline medium, were those obtained under conditions of Experiment 5, allowing a faster, with less amount of concentrated acid and quantitative extraction procedure. However, for the UAE-bath, longer sonication times were needed using the same concentration of HNO_3 or TMAH employed in Experiment 5, for a quantitative extraction, as shown in recovery values obtained for Experiment 7.

Additionally, an ANOVA analysis was performed. Since the calculated F values were much greater (>20) than the critical F value of 4.066 (P = 0.05), the null hypothesis was rejected, so, the sample means differed significantly. Means were arranged in increasing order and the difference between adjacent values compared to the least significant difference. Results showed that the means obtained in experiments (5 and 7) did not differ significantly from the informed value of the RM, also demonstrated by a Student's *t*-test, being the experimental *t*-value = 1.67, below the theoretical *t* (0.05, 5) = 2.57.

Table II. Recovery results of the multivariate experimental design applied to UAE-based methods, using the bovine liver RM

Experiment	R (%) – HNO_3		R (%) – TMAH	
	Probe	Bath	Probe	Bath
1	99 ± 3	90 ± 2	99 ± 2	91 ± 4
2	93 ± 4	84 ± 4	92 ± 5	80 ± 3
3	98 ± 3	89 ± 5	93 ± 2	85 ± 3
4	95 ± 2	88 ± 3	93 ± 4	81 ± 4
5	100 ± 3	89 ± 4	101 ± 2	86 ± 5
6	99 ± 2	93 ± 3	100 ± 2	90 ± 4
7	101 ± 1	99 ± 2	100 ± 1	99 ± 3
8	95 ± 2	89 ± 3	94 ± 1	89 ± 2
9	100 ± 2	99 ± 1	100 ± 3	99 ± 2

Expressed as: Mean ± standard deviation (n = 3).

Finally, the optimum conditions for the proposed UAE methods turned out to be: 0.25 g of sample and 10.0 mL of 2.8 mol L^{-1} HNO_3 or 0.36 mol L^{-1} TMAH placed in a flask, and then sonicated for 10 min (probe) or 20 min (bath). Afterwards, the suspensions were filtered using a 0.45 µm pore membrane, and the obtained solutions used without further dilution for Cu determinations.

The addition of water when performing the alkaline extraction method was necessary to moisturize the biological tissue and promote the efficient penetration of TMAH. The hydration allowed this reagent to easily penetrate the protein matrix, dissolve it, and release Cu to the soluble phase.[13,18]

Considering that the purpose of the work was to develop green alternatives for the rapid diagnosis of animal status, the most straightforward methods able to give answers in a short period of time were highly desired. Thus, UAE-probe methods were selected for subsequent validation, according to the much shorter sonication time required for Cu quantitative extraction.

Effect of ultrasound on particle size

The impact of fragmentation was studied using the probe (20 kHz) and the bath (47 kHz). A fast fragmentation of liver samples was noticed after a few minutes of probe-based sonication, while this effect was less pronounced when the ultrasonic bath was used. As a matter of fact, a quantitative extraction was achieved in 10 min when using the probe, whereas the double of time was needed when using the bath.

Residues were collected after filtration and the particle size distribution was measured. When the probe was used, a remarkable decrease in particle size with increasing sonication time was observed, changing from 250 μm in the control sample to 130 μm (2.8 mol L^{-1} HNO_3)/90 μm (0.36 mol L^{-1} TMAH) after 10 min, leading to nearly complete solubilization of the sample after 20 min. This phenomenon is associated to inter-particle collisions and shockwaves created by collapsing cavitation bubbles in the liquid, as mentioned before. A direct consequence of this reduction in particle size is the increase of the surface area of the solid, which leads to a more pronounced mass transfer and a higher extraction yield.[1] When the bath was used, a slower decrease in particle size with the increment of sonication time was observed, changing from 250 μm in the control sample to 190 μm (2.8 mol L^{-1} HNO_3)/135 μm (0.36 mol L^{-1} TMAH) after 20 min. The differences found between UAE-probe and UAE-bath are probably due to a faster reduction of the particle size caused by a more intimate contact when using the probe. Additionally, the differences found between the acid and the alkaline medium agrees with the 'tissue solubilizer' capacity assigned to TMAH.

UAE of trace elements from biological matrices involves several effects such as fragmentation, erosion, and detexturation, most likely acting in combination. Furthermore, the transfer of elements to the liquid phase is favored by the intense mixing caused by the propagation of ultrasound waves. These physical effects lead to the higher efficiency achieved with UAE.

All UAE-based methods described here in this work were suitable for the quantitative extraction of Cu from bovine and ovine liver samples. The difference in optimum sonication times between the probe and the bath is logical, considering the higher output power of the probe, that provides more energy in the sinus of the suspension when compared to the bath. The larger remaining amount of residue and the bigger particle size obtained when working with the ultrasonic bath, leaded to a more difficult filtration process. This fact was also considered when selecting UAE-probe methods for subsequent validation.

In addition, to prove the real effect of the ultrasound over the extraction, it was necessary to perform another round of experiments using magnetic stirring. These experiments were important to assure that ultrasound energy truly influenced the proposed process. Recovery results obtained for Cu after performing the sample preparation procedure described as 'silent condition' were 27 ± 1% (HNO_3) and 24 ± 2% (TMAH), expressed as: mean ± standard deviation (n = 3). This shows that, although magnetic stirring can extract part of the analyte from the matrix, this extraction is far from being quantitative. So, the effect of ultrasound over the extraction was assured, after comparing with the quantitative recoveries observed for experiment 5 and experiment 7 in Table II.

Methods validation: MAE vs. UAE-probe

MAE was employed for total sample digestion. This method was validated for Cu determinations following the suggestions of the Eurachem Guide.[17] Results are shown in Table III. A Student's *t*-test was performed for trueness evaluation, to prove if there were statistical differences between the experimental values and the certified value of the CRM, which showed no differences at the 95 % confidence level.[19]

Repeatability expressed as RSD (%) after the analysis of the CRM (n = 6), was less than 5%. Thus, the method was considered accurate. The LOQ expressed as 10σ (n = 10, fresh weight) was 1.1 mg kg^{-1}.

Table III. Figures of merit obtained for the MAE and the developed UAE methods

Parameter	MAE	UAE-Probe	
		HNO_3	TMAH
Linear range (mg kg^{-1})	0.03 – 6.0	0.03 – 6.0	0.04 – 6.0
LOD (mg kg^{-1})	0.01[a]/0.3[b]	0.01[a]/0.9[b]	0.01[a]/1.3[b]
LOQ (mg kg^{-1})	0.03[a]/1.1[b]	0,03[a]/1.1[b]	0.04[a]/1.6[b]
Precision, RSD (%) [c]	3.8	4.5	4.0
Trueness, R (%) [c]	100	102	101
$t_{experimental}$ [c]	0.31	0.96	0.46
t (0.05, 5)	2.57	2.57	2.57

(a) Instrumental LOD and LOQ; (b) LOD and LOQ expressed in sample, fresh weight; (c) CRM NIST 1577c.

UAE-probe methods were also validated following Eurachem Guide recommendations.[17] Obtained figures of merit are shown in Table III. Six-point calibration curves were constructed to evaluate linearity. Good linearity was observed with determination coefficients (R^2) better than 0.9999. Linear range was up to 6.0 mg kg^{-1}. Individual residuals were studied, and their random distribution verified. The obtained LOQ expressed as 10σ (n = 10, fresh weight) were 1.1 and 1.6 mg kg^{-1}, respectively, being one to two orders below the expected Cu levels in liver samples. Repeatability expressed as RSD (%) after the analysis of the CRM (n = 6) was less than 5%, which was considered suitable for this application, according to the Horwitz theory related to variability at trace levels (RSD < 30%).[20] A Student's *t* test was performed for trueness evaluation, to compare the obtained values with the certified one. Results are shown in Table III. Experimental *t* values were below the theoretical *t* (0.05, 5) = 2.57, indicating that concentrations did not differ significantly from the certified value at the 95% confidence level. These figures of merit demonstrate that the developed methods were accurate for Cu determination in liver samples.

Since MAE is widely employed for elemental determinations in complex matrices, and prior validation confirmed its reliability, the values obtained using this method were considered as reference values for the analyzed real samples. Thus, the performance of the newly developed UAE methods was statistically evaluated by comparing the results obtained after the analysis of real bovine and ovine liver samples with the results obtained by MAE. A Student's *t* test was employed for this task as shown in Table IV. Experimental *t* values were below the respective theoretical *t* (0.05, 5) = 2.57 demonstrating that, at the 95% significance level, the results obtained using either UAE-probe or MAE were statistically equivalent. Thus, the proposed methods were considered adequate for the analysis of Cu in bovine and ovine liver samples.

Table IV. Comparison between the developed UAE and MAE methods by Student's *t* test

	MAE		UAE-Probe (HNO_3)		UAE-Probe (TMAH)	
Sample	**Bovine**	**Ovine**	**Bovine**	**Ovine**	**Bovine**	**Ovine**
Average	62.5 ± 8.1	105 ± 12	60.9 ± 7.9	108 ± 14	65.8 ± 7.5	104 ± 11
$t_{experimental}$	---	---	-0.37	0.20	0.77	-0.12
t (0.05, 5)	---	---	2.57	2.57	2.57	2.57

Expressed as: Mean ± standard deviation (n = 6) (mg kg^{-1}).

It is important to highlight once again at this point what has been previously described in the specialized literature on UAE methods that clearly proved its efficiency, allowing a significant decrease in extraction solvent concentration, operation time and harsh reaction conditions.[1-5] UAE makes possible the use of less acid concentration, when compared to MAE, while allowing extractions at room temperature under atmospheric pressure, instead of working at high pressure in closed reaction vessels, thus involving safer procedures.

Application to bovine and ovine liver samples

A total of 6 bovine and 6 ovine liver samples from different animals were analyzed. Copper concentrations found in bovine liver samples ranged from 58.8 ± 3.3 to 82.7 ± 3.9 mg kg^{-1} (mean ± standard deviation, fresh weight), while those found in ovine liver samples ranged from 66.2 ± 3.1 to 159.8 ± 4.3 mg kg^{-1} (mean ± standard deviation, fresh weight). The obtained Cu levels agree with those previously reported in Australian[21] and Norwegian[22] sheep, and Spanish cows[23].

The presented information is relevant, since Cu is key to the enzymatic systems, cofactors, and reactive proteins, while its deficiency is related to the development of several disorders as previously stated. Copper levels in the liver can be used to estimate deficiencies and/or poisoning to sheep and cattle. None of the animals studied in this work presented Cu concentrations in the liver below 2 mg kg^{-1} (fresh weight), a level considered indicative of Cu responsive disorder in ruminants.[9] Also, none of the samples exceeded 200 mg kg^{-1} (fresh weight), a level indicative of toxicity. Based on liver Cu concentrations found, the results suggest that sheep and cattle in Uruguay are well-managed with respect to Cu supplementation. However, it is important to perform this sort of studies, since some isolated cases of chronic Cu poisoning have been reported in sheep in our country.[24]

Taking into consideration the contribution of Cu to human diet, an intake of 50 g of liver would provide between 2.9 – 4.1 mg (bovine) and 3.3 – 8.0 mg (ovine). These values would broadly cover the recommended dietary allowance (RDA) of this essential element, that has been established by the National Institutes of Health (NIH) in 0.9 mg/day for adults over 19 years, without reaching the tolerable upper intake level (UL) of 10 mg/day, a value based on protection from human liver damage as the most critical adverse effect.[25]

Analytical Eco-Scale

To evaluate the greenness of the developed UAE methods, the analytical Eco-Scale comprehensive approach was performed, which is a good semi-quantitative tool that can be considered as an alternative to traditional Green Chemistry metrics.[26] It is based on assigning penalty points to certain parameters of the analytical process, namely, amount of reagents, hazards, energy and waste, that do not agree with an ideal green analysis. The sum of these points for the whole procedure must be included in the Eco-Scale calculation, according to the following formula: Analytical Eco-Scale score = 100 – total penalty points. The result of the calculation is then ranked on a scale. If the score is >75 it represents an excellent green analysis, if the score is >50 it represents an acceptable green analysis, and if the score is <50 it represents an inadequate green analysis.[26]

Results of the analytical Eco-Scale applied to the methods developed here in this work are shown in Table V. As it can be observed, the three methods are equivalent in terms of greenness and all of them pose a score over 75, representing excellent green strategies according to this approach. However, the UAE-probe method using dilute HNO_3 can be considered a slightly greener alternative.

Table V. Penalty points for Cu determination by developed UAE-probe and MAE methods and Eco-Scale total scores

	Method		
	UAE-Probe (TMAH)	**UAE-Probe (HNO_3)**	**MAE (HNO_3)**
	Penalty points		
Reagents			
TMAH or HNO_3 (Amount × Hazard)	6	4	4
Cu standard solutions	3	3	3
Acetylene	3	3	3
Instruments			
Probe or MW (energy)	1	1	2
Spectrometer (energy)	2	2	2
Occupational hazard (vapors)	3	3	3
Waste (Amount + Treatment)	6	6	6
Total penalty points	24	22	23
Analytical Eco-Scale score	76	78	77

CONCLUSIONS

Four efficient methods for the extraction and subsequent determination of Cu in bovine and ovine liver samples were optimized. Ultrasound-assisted methods were performed using dilute HNO_3 and dilute TMAH and without the need of external heating, thus being aligned with the postulates of Green Chemistry and can accordingly be proposed as simple and economical methods for animal monitoring, as well as for food control purposes. However, the probe-based procedures were selected for validation since they were more efficient alternatives. The greenness of the newly developed methods was assured by performing the analytical Eco-Scale approach, that showed the excellency of green analysis.

Also, it was once again highlighted the efficiency of UAE, as well as its main advantages, such as decrease in acid concentration, operation time and harsh reaction conditions, when compared to MAE. The research group will continue working on the decrease of solvent concentration, working under milder and greener conditions.

Regarding Cu levels in Uruguayan samples, results agreed with previous values informed in sheep and cattle abroad. Besides, Cu concentrations lied between the minimum level indicative of Cu responsive disorder and the maximum level indicative of Cu toxicity, suggesting that sheep and cattle in Uruguay are well-managed with respect to Cu supplementation. Furthermore, the intake of 50 g of liver would completely cover the RDA of Cu for adults over 19 years, according to data provided by the NIH. This constitutes novel information since there is scarce data available on this matter in academic search engines.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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ARTICLE

Eco-friendly UV Spectrophotometric Method for Simultaneous Estimation of Evogliptin and Metformin Hydrochloride in Bulk and Combined Tablet Dosage Form

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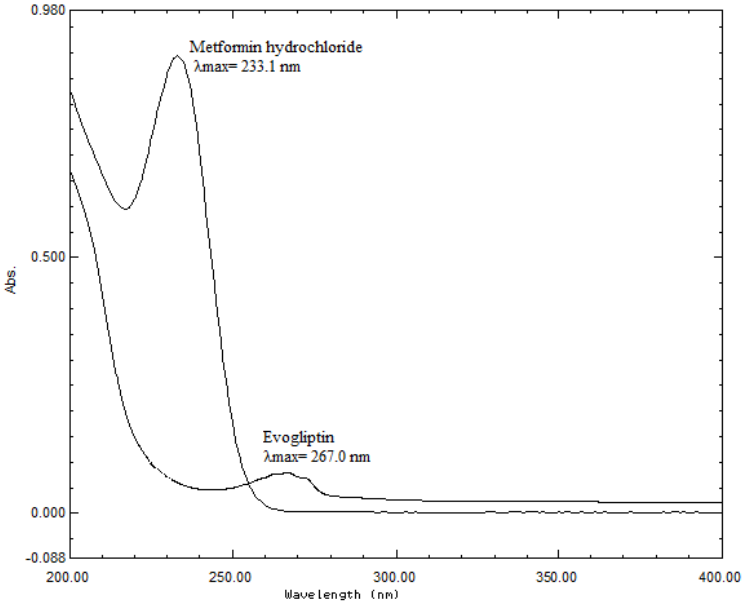

Evogliptin (EGT) is used in fixed-dose combination with metformin hydrochloride (MFH) for a better glycemic control in Type 2 diabetes mellitus. To date, no method is available for simultaneous estimation of these drugs. In the present study, an UV spectrophotometric method was developed in distilled water, an environment-friendly solvent using the simultaneous equation technique to simultaneously determine EGT and MFH in bulk and tablet dosage form. The developed method was validated and applied to commercial tablet dosage forms containing EGT and MFH in combination. With a great correlation value (R^2>0.998), the analytes displayed good linearity in the range of 10-100 µg mL^{-1}. The low percent relative standard deviation proved the methods' precision. The methods' accuracy was demonstrated by excellent recovery. Thus, the developed method was found to be simple, environment-friendly, fast, specific, precise, and accurate, and it may be effectively used for routine analysis of EGT and MFH in bulk and their combined tablet dosage form.

Keywords: evogliptin, metformin hydrochloride, simultaneous equation, UV method, analytical method

Abbreviations used in this paper: EGT: Evogliptin; HPLC: High Performance Liquid Chromatography; HPTLC: High Performance Thin Layer Chromatography; MFH: Metformin hydrochloride; RP-HPLC: Reverse Phase-High Performance Liquid Chromatography; RSD: Relative Standard Deviation; UV: Ultraviolet.

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INTRODUCTION

Metformin hydrochloride (MFH) is chemically N,N-dimethyl imidodicarbonimidic diamide hydrochloride (Figure 1A), a white crystalline hygroscopic powder freely soluble in water.[1] It belongs to the biguanide class and is used in the treatment of Type 2 diabetes mellitus (T2DM). By improving insulin sensitivity, lowering glucose absorption, and blocking hepatic gluconeogenesis, biguanides help lower blood sugar levels. Metformin has the advantage of attaining glycemic control without worsening weight gain or hyperinsulinemia and having a positive effect on blood cholesterol concentrations.[2]

Patients who do not respond to lifestyle changes and metformin have to add a second oral medication like dipeptidyl peptidase 4 (DPP-4) inhibitors to their treatment plan.[3,4] DPP-4 inhibitors boost insulin production while suppressing glucagon release by the pancreas' alpha cells by blocking glucagon-like peptide 1 (GLP-1) inactivation.[5] This helps to bring blood glucose levels back to normal. Because of their easy administration, modest effects on glycosylated hemoglobin (HbA_{1c}), and absence of severe side effects, this class of antidiabetic agents is extensively used either alone or in combination with metformin.[6] One of the potent and selective DPP-4 inhibitors is Evogliptin which is available as tartrate salt with MFH in fixed dose combination, which significantly improves glycemic control compared to monotherapies.[7] Evogliptin (EGT) is chemically (3R)-4-[(3R)-3-amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-[(2-methylpropan-2-yl)oxymethyl]piperazin-2-one (Figure 1B) which is freely soluble in distilled water.

The literature survey reveals that many UV, HPLC, and HPTLC methods have been reported for MFH alone[8–11] and in combination with other drugs.[12–16] For EGT, only one UV and one RP-HPLC method is reported for its estimation.[17,18] However, no method has been reported for the simultaneous determination of MFH and EGT in combination. UV spectrophotometer methods are favored over other analytical techniques due to their wide range of applications, ease of use, robustness, and simplicity. Furthermore, analysis can be carried out in areas outside of the main laboratory when needed using portable UV spectrophotometers.[19] As a result, the development of such a method might be utilized to estimate the combined dosage form of both medications.

The proposed method is fast, simple, environment-friendly, precise, and reproductive and may be used in tablet dosage for routine analysis of these two drugs simultaneously. The method was developed in distilled water, making the method cheap and environment-friendly. Following International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines, the proposed approach is optimized and validated. There has been a successful attempt in simultaneously estimating both of these drugs utilizing the UV spectrophotometer simultaneous equation approach in this work.

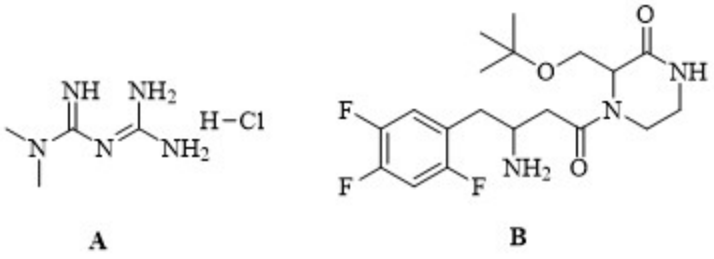

Figure 1. Chemical structure of Metformin hydrochloride (A) and Evogliptin (B).

MATERIALS AND METHODS

Instruments

The experimental study was carried out by a UV/Vis double beam spectrophotometer (UV-1800, Shimadzu) with a 1 cm matched quartz cell and loaded with UV probe software. The study employed a citizen CX 220 weighing balance with 0.1 mg sensitivity for all weighing and a sonicator (Hicon, model 1.5 L (H)).

Reagents and chemicals

The drugs MFH and EGT were procured as gift samples from Anwita Drugs and Chemicals Pvt. Ltd., Telangana and Reine Life Science, Gujarat, respectively. To assess the purity of the drugs, melting point was determined which was found as per the literature. Thus, the drugs were used without further purification. Throughout the study, calibrated glasswares were used. Purified water was acquired using reverse osmosis and filtration via a milli-Q® system (Millipore, Milford, MA, USA) using 0.45 µm membrane and utilized to make all solutions.

Marketed formulation

The marketed formulation studied was Valera M tablets manufactured by Alkem Laboratories Ltd. purchased from the local market. Each tablet contains 500 mg Metformin hydrochloride and Evogliptin Tartrate equivalent to 5 mg Evogliptin.

Preparation of standard stock and working solutions

MFH (10 mg) and EGT (10 mg) accurately weighed were placed into two separate 100 mL volumetric flasks, dissolved in little distilled water, sonicated, and diluted up to 100 mL with distilled water (stock solutions: 100 µg mL^{-1} MFH and EGT). Then, a working standard solution (10 µg mL^{-1}) of MFH and EGT was prepared by diluting 1 mL of stock solutions to 10 mL with distilled water.

Development of simultaneous equation

To determine the λmax, the working standard solutions of both the drugs were scanned individually across the entire UV range (400–200 nm). Both the drugs displayed considerable absorption at the λmax of another drug; thus, the wavelengths 233.1 nm and 267.0 nm were selected for developing simultaneous equations. Both drugs' stock solutions were diluted separately with distilled water to yield a series of standard solutions ranging from 10 to 100 µg mL^{-1} for EGT and 1 to 10 µg mL^{-1} for MFH. The absorbances at the selected wavelengths were measured, and the absorptivities (A 1%, 1 cm) for both drugs at both wavelengths were calculated. The absorbances and absorptivities at these wavelengths can be substituted in equations (1) and (2) to find the concentration of drugs.

$$Cx = A_2\, ay1 - A_1\, ay2/ax2ay1\text{-}ax1ay2 \qquad (1)$$

$$Cy = A_1\, ax2 - A_2\, ax1/\, ax2ay1\text{-}ax1ay2 \qquad (2)$$

Where, A_1 and A_2 are the absorbances of sample solutions at 267.0 nm and 233.1 nm, respectively. ax1 and ax2 are the absorptivities for MFH at 267.0 nm and 233.1 nm, respectively, and ay1 and ay2 are the absorptivities for EGT at 267.0 nm and 233.1 nm, respectively. Cx and Cy are concentrations of MFH and EGT, respectively in µg mL^{-1} in the sample solution.

Validation of method

The validation of the analytical procedure was done according to ICH guidelines in terms of linearity, precision, accuracy, ruggedness, and tablet analysis.[20]

Linearity

From stock solutions of both drugs, ten dilutions were prepared in the range of 10-100 µg mL^{-1}. The absorbances of all the resulting solutions were recorded at the λmax of that particular drug, and a calibration curve was drawn to get the linearity and regression equations.

Precision

The precision of the established method was tested by calculating the relative standard deviation (%RSD) for working standard solutions of EGT and MFH repeated thrice on a similar day (intra-day) and in three days (inter-day). The repeatability test was also done for both the drugs (system Precision), in which a homogenous sample was analyzed six times in the same day.

Accuracy

Three dilutions of the stock mixture solution were prepared to test the accuracy of the developed method. These known concentration solutions were then analyzed as unknown samples using the developed method. To test the developed method's accuracy further, the pre-analyzed sample was separately spiked with an extra 80%, 100%, and 120% of the drug concentrations. The mixtures were analyzed again using the developed method, and %RSD was calculated.

Ruggedness

In this parameter of validation, two analysts performed the same analysis method for the same sample to find out the ruggedness of the method. Then %RSD was calculated.

Analysis of combined tablet dosage form

The developed method was performed on the marketed formulation of EGT and MFH, each tablet containing 5 mg of EGT and 500 mg of MFH. Ten tablets were crushed, extracted with water, and filtered; finally, volume was made up to 100 mL. Further dilutions were made with water to form a working standard solution equivalent to 5 μg mL^{-1} of EGT and 500 μg mL^{-1} of MFH. Aliquots of definite concentration were withdrawn from this working standard solution in six replicates (in Beer-Lambert's Law limit), and absorbance was measured at 267 and 233.1 nm.

RESULTS AND DISCUSSION

Method development

In the current case, the UV spectrophotometric method was established according to the simultaneous equation method. The scanning showed that MFH and EGT exhibit λmax at 233.1 nm and 267.0 nm, respectively (Figure 2). In this study, a partial simultaneous equation method was applied. Absorptivities of both drugs at both wavelengths were calculated by dividing absorbance by concentration (μg mL^{-1}) and put in equations 1 and 2. The absorptivity values determined for MFH are 0.004597 (ax1), 0.072336 (ax2) and for EGT, 0.074493 (ay1), 0.004802 (ay2) at 267.0 nm and 233.1 nm, respectively. The equations developed were:

$$Cx = A_1(0.0074493) - A_2(0.004802)/0.005366 \quad (3)$$

$$Cy = A_1(0.072336) - A_2(0.004597)/0.005366 \quad (4)$$

Using the above equations 3 and 4, the concentrations of EGT and MFH can be directly calculated by putting the values A_1 and A_2 (absorbance of the test sample at 233.1 nm and 267.0 nm, respectively) and solving them for Cx (concentration of MFH) and Cy (concentration of EGT).

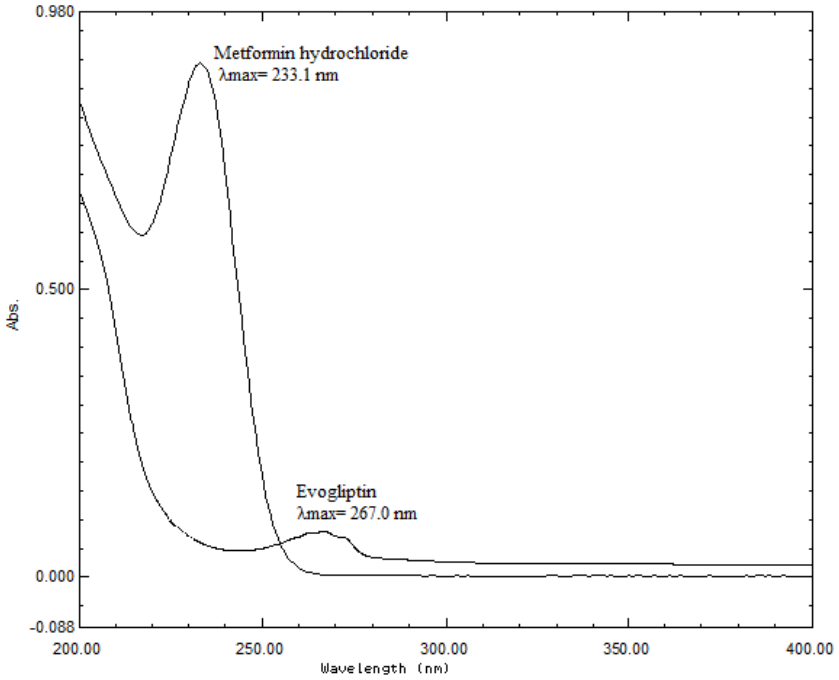

Figure 2. UV Scan of Metformin hydrochloride and Evogliptin.

Method validation

Linearity

The linearity was found to be 10-100 µg mL^{-1} for both EGT and MFH (Table I). The linearity of the established technique was resolute, and the linear regression data for the calibration curve of EGT and MFH showed good linearity (R^2 = 0.9986) and (R^2 = 0.9983), respectively over the concentration range of 10-100 µg mL^{-1} (Figures 3 and 4).

Table I. Linearity study

Concentration (µg mL^{-1})	Absorbance (Mean ± SD)	
	Evogliptin	Metformin
10	0.087±0.000	0.65±0.03
20	0.151±0.002	1.34±0.04
30	0.219±0.008	2.28±0.02
40	0.277±0.002	2.86±0.03
50	0.367±0.020	3.85±0.02
60	0.443±0.003	4.37±0.01
70	0.515±0.003	5.17±0.01
80	0.579±0.015	5.94±0.02
90	0.657±0.004	6.59±0.01
100	0.743±0.008	7.27±0.04

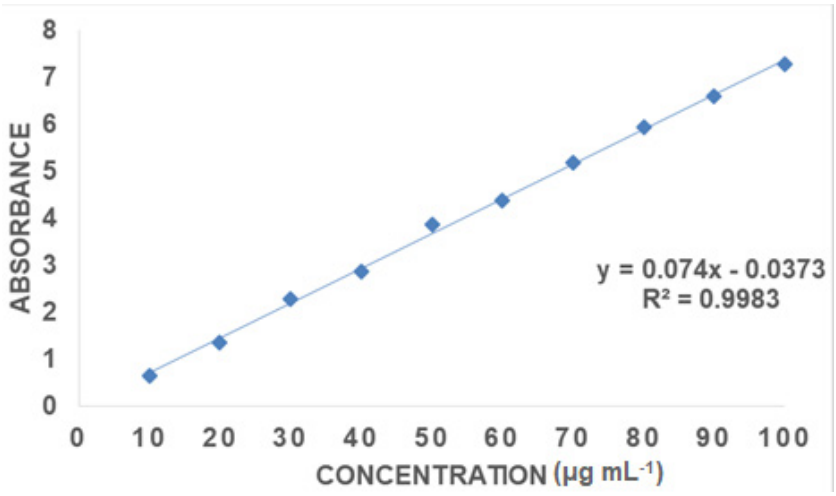

Figure 3. Calibration curve for Metformin HCl.

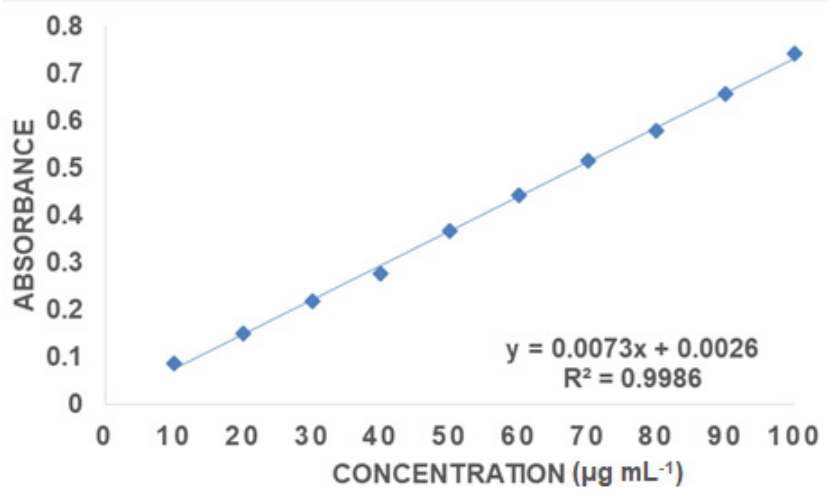

Figure 4. Calibration curve for Evogliptin.

Precision

The developed method was validated for precision by measuring absorbances intra-day and inter-day (Table II) and for repeatability by measuring absorbances six times (Table III).

Table II. Precision Result (Method) for EGT and MFH

	Intra-day study			Inter-day study		
	Morning	**Afternoon**	**Evening**	**Day 1**	**Day 2**	**Day 3**
			EGT (10 µg mL^{-1})			
Avg. Abs	0.088	0.086	0.086	0.084	0.082	0.082
SD	0.001	0.001	0.002	0.002	0.002	0.002
%RSD	1.1	1.1	0.9	0.5	0.9	0.6

(continues on the next page)

Table II. Precision Result (Method) for EGT and MFH (continuation)

	Intra-day study			Inter-day study		
	Morning	**Afternoon**	**Evening**	**Day 1**	**Day 2**	**Day 3**
			MFH (10 µg mL^{-1})			
Avg. Abs	0.722	0.725	0.0729	0.730	0.723	0.726
SD	0.002	0.003	0.001	0.001	0.002	0.001
%RSD	0.5	0.8	0.5	0.1	0.6	1.2

Table III. Precision Result (System) for EGT and MFH

Concentration (µg mL^{-1})	**Absorbance**	
	EGT	**MFH**
10	0.087	0.727
10	0.085	0.725
10	0.087	0.723
10	0.083	0.722
10	0.085	0.722
10	0.087	0.725
Mean	0.085	0.724
SD	0.001	0.002
%RSD	1.1	0.2

Accuracy

To test the accuracy and reproducibility, recovery experiments were performed using the standard addition technique by adding 80, 100, and 120% of the drug's concentration in pre-analyzed samples. Data is summarized in Table IV.

Table IV. Accuracy result for EGT and MFH

Level (%)	**EGT (10 µg mL^{-1})**			**MFH (10 µg mL^{-1})**		
	Absorbance	**%Recovery**	**Mean % recovery**	**Absorbance**	**%Recovery**	**Mean % recovery**
80	0.065	99.84	99.59	0.592	99.93	99.95
80	0.062	99.90		0.595	99.94	
80	0.061	99.04		0.593	99.98	
100	0.085	100.11	100.31	0.727	100.02	99.87
100	0.087	100.80		0.728	100.03	
100	0.088	100.01		0.725	99.56	

(continues on the next page)

Table IV. Accuracy result for EGT and MFH (continuation)

Level (%)	EGT (10 µg mL^{-1})			MFH (10 µg mL^{-1})		
	Absorbance	%Recovery	Mean % recovery	Absorbance	%Recovery	Mean % recovery
120	0.090	100.02	99.93	0.814	100.01	99.94
120	0.091	99.87		0.822	99.98	
120	0.090	99.90		0.815	99.83	

Ruggedness

The developed method was rugged as %RSD was found to be less than 2. The results are reported in Table V.

Table V. Ruggedness result for EGT and MFH

	EGT (10 µg mL^{-1})			MFH (10 µg mL^{-1})		
Analyst	Absorbance	SD	%RSD	Absorbance	SD	%RSD
Analyst 1	0.087	0.001	0.1	0.726	0.002	0.2
	0.085			0.726		
	0.085			0.722		
Analyst 2	0.084	0.0005	0.5	0.729	0.003	0.4
	0.085			0.722		
	0.085			0.725		

Tablet estimation

The developed method was used to estimate the marketed formulation of the composition of EGT 5 mg and MFH 500 mg. Using the developed simultaneous equations, the concentration of EGT and MFH were estimated in commercial formulation. These data are incorporated in Table VI.

Table VI. Tablet analysis of EGT and MFH using developed method

Formulation	Drugs	Label claim (mg)	Drug found (mg)
Tablets (Valera M 500)	Evogliptin	5	4.96±0.038
	Metformin hydrochloride	500	499.93±0.050

Optical characteristics of UV spectrophotometric method

The summary of optical characteristics and validation parameters of the UV method is shown in Table VII.

Table VII. Summary of optical characteristics of UV method

Parameters	Results	
	EGT	**MFH**
Detection wavelength (nm)	267.0 nm	233.1 nm
Beer's Law limits (μg mL^{-1})	10-100	10-100
Regression equation	y = 0.0073x + 0.0026	y = 0.074x - 0.0373
Correlation coefficient	0.9986	0.9983
Slope (m)	0.0073	0.074
Intercept (c)	0.0026	0.0373
Precision (%RSD)		
Intra-day	<2	<2
Inter-day	<2	<2
Accuracy (% mean recovery)		
80% level	99.59	99.95
100% level	100.3	99.87
120% level	99.93	99.94
Ruggedness		
2 Analysts (% RSD)	<2	<2

Discussion

As per literature review, to date, no method is available for simultaneous estimation of EGT and MFH. Thus, the present study focused on developing a simple, cheap, eco-friendly and accurate method for the estimation of these drugs in combination. The UV scanning showed that MFH and EGT exhibit λmax at 233.1 nm and 267.0 nm in water, respectively and both the drugs showed considerable absorbance at the λmax of another drug. Thus, a simultaneous equation was developed by measuring absorbances of EGT and MFH at 233.1 nm and 267.0 nm. The linear regression data for the calibration curve of EGT and MFH showed good linearity (R^2 = 0.9986) and (R^2 = 0.9983), respectively over the concentration range of 10-100 μg mL^{-1} (Figures 3 and 4). The system and method precision were found within limits according to ICH guidelines. The found values of %RSD were less than 2%, approving the great precision of the established method (Table II and III). The percentage recoveries were close to 100% for this method indicating excellent accuracy of the method (Table IV). For the tablet dosage form, the analysis obtained was in good uniformity in the claimed amount in the marketed sample. Thus, the method developed is simple, accurate, sensitive, and precise and can be used effectively in the simultaneous determination of EGT and MFH in a tablet dosage form.

CONCLUSIONS

In Type 2 Diabetes mellitus, combination therapy gives improved glycemic control when compared to monotherapies. Evogliptin (EGT), a DPP-4 inhibitor, is used in fixed-dose combination with metformin hydrochloride (MFH). However, no method is available for their simultaneous determination. Thus, in the present study, a method was developed using UV spectrophotometry for the simultaneous estimation of

evogliptin and metformin hydrochloride in bulk as well as in tablet dosage form. The method was found to be simple, sensitive, and reproducible. Throughout the estimation, distilled water was used as a solvent, making this method cost-effective and environmentally friendly. The results of the statistical analysis confirmed that the method is accurate and precise. The RSD for all parameters was found to be less than two, establishing the method's validity. Thus, the developed method can be used for routine estimation of evogliptin and metformin hydrochloride simultaneously.

Conflicts of interest

Authors declare no conflicts of interest.

Acknowledgement

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FEATURE

The 16th Edition of Analitica Latin America Expo Received Around Seven Thousand Attendees

Analitica Latin America Expo is one of the main meeting points worldwide for professionals involved with Analytical Chemistry. The event brings together different market segments, such as pharmaceuticals, cosmetics, food, petrochemicals, agribusiness, and others.

Attendees at the entrance of the Analitica Latin America Expo. Photo: Analitica Latin America

Visitors to the 16th edition of Expo Analitica Latin America, held from June 21 to 23, 2022, were introduced to the novelties and trends presented by 400 exhibitors in an area of 14 thousand square meters.

The previous edition of Analitica Latin America Expo was held in 2019. In 2020, the Analitica Latin America Expo was not held because of the COVID-19 pandemic and in 2021 it was held only as a pavilion within the International Technology Exhibition for the Pharmaceutical Industry (FCE Pharma) and the International Technology Exhibition for the Cosmetic Industry (FCE Cosmetique), the main meeting point for business in the pharmaceutical and cosmetic sectors, respectively.

Attendees circulating in the exhibitor area. Photo: Analitica Latin America

On the first day of the event, the 16th Analitica Latin America Expo brought together 15 exhibitors with 50 potential buyers in a reserved space favorable to the consolidation of partnerships. Over two hours, about 190 meetings were held in order to facilitate the negotiation between the two parties. The meeting moved around US$ 200,000.

João Paulo Picolo, the president of NürnbergMesse Brasil and promoter of the event stated that “Analitica Latin America Expo is a consolidated event in the Brazilian and international calendar. In addition to Brazilian visitors, the Expo receives visitors from around 20 countries, more than 60% of them from Latin America. Visitors and exhibitors were eager for this edition and it was very good to see everyone’s satisfaction as they circulated through the corridors, during the three days of the event”.

The 8th Analitica Latin America Congress, held in parallel with the 16th Analitica Latin America Expo, had a partnership with the Brazilian Meeting on Analytical Chemistry (ENQA), which promoted a preview of the 20th ENQA to be held over September 25-28, 2022, in Bento Gonçalves, RS, Brazil.

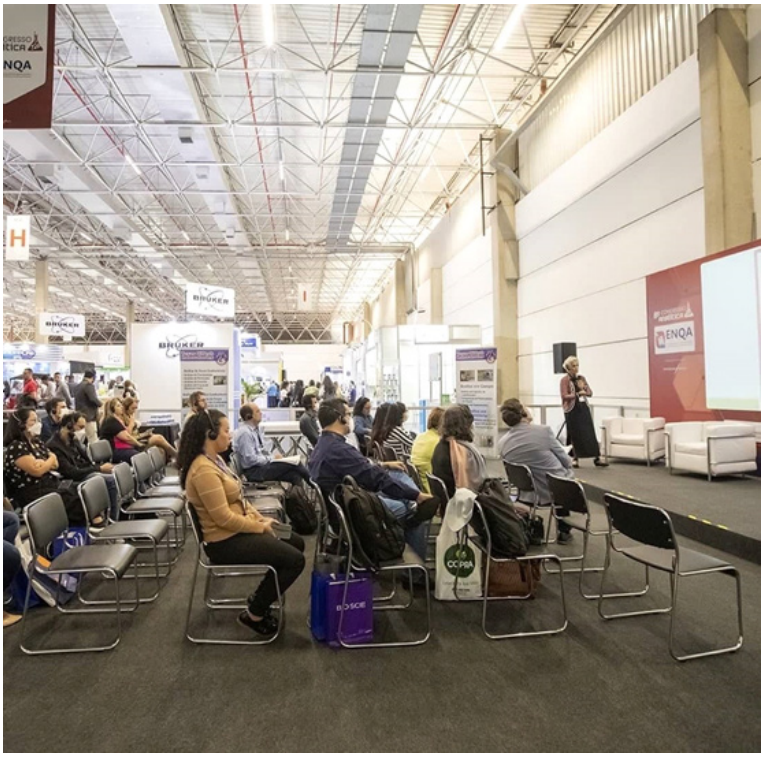
Lecture by Prof. Dr. Lauro Tatsuo Kubota, from the Institute of Chemistry, University of Campinas (IQ-Unicamp, SP, Brazil) Photo: Analitica Latin America

The 8th Analitica Congress hosted around 100 participants and involved ten hours of content presented by speakers recognized for their important scientific contributions to the field of analytical chemistry. The topics of interest in the areas of food, pharmaceuticals, alternative energies, green chemistry, and fuel quality control were addressed.

According to the organization, the choice of speakers was strategic so that attendees could participate in discussions on the most relevant topics in analytical chemistry at the time. Professionals from the private sector and professors from universities with recognized academic quality were selected to share their knowledge.

The speakers included Caroline Ravazzi, MSc, from the Customer and Quality Compliance, Nestlé Quality Assurance Center, and Prof. Dr. Lauro Tatsuo Kubota, from the Institute of Chemistry, University of Campinas (IQ-Unicamp), SP, Brazil.

The program for the first day of the 8th Analitica Congress included discussions on the quality control of vaccines, nanotechnology applied to pharmaceuticals and cosmetics, the analysis and quality control of food, and nanotechnology applied to the food and agribusiness industries.



Participants attending another lecture at the Analitica Congress.
Photo: Analitica Latin America

On the second day, the lectures of the 8th Analitica Congress addressed the challenges of the analytical chemistry segment with debates on bioanalytical and sustainable development goals, challenges and achievements of the elemental spectrometric analysis of fuels and oily residues, microanalysis, bioanalytical strategies applied to agriculture, the treatment of bacterial infections and in forensic chemistry, recent advances in electrochemical biosensors, speciomics, challenges and perspectives in the direct analysis of solids for elemental determination, and digital microfluidics platforms as a new generation of devices for bioanalytical applications.

Companies present at Analitica Latin America Expo

The attendees had the opportunity to visit the booths of around 400 exhibitors that presented the main innovations in technology for laboratories, analysis, biotechnology, and quality control. These included Thermo Fisher Scientific, Nova Analitica, Allcrom/Bion, LAS do Brasil, Agilent, Waters, Merck, and many other companies.

Next edition of the Analitica Latin America Expo & Congress

At the end of the event, NürnbergMesse Brasil announced that the 17th edition of Analitica Latin America Expo will be held from September 26 to 28, 2023.

Source: With information from Analitica Latin America.

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SPONSOR REPORT

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

John Schmelzel[1], Daniel Kutscher[2], Carl Fisher[3]
Thermo Fisher Scientific [1]San Jose, CA, USA [2]Bremen, Germany [3]Sunnyvale, CA, USA

This report was extracted from the Thermo Scientific Application Note 44407

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.

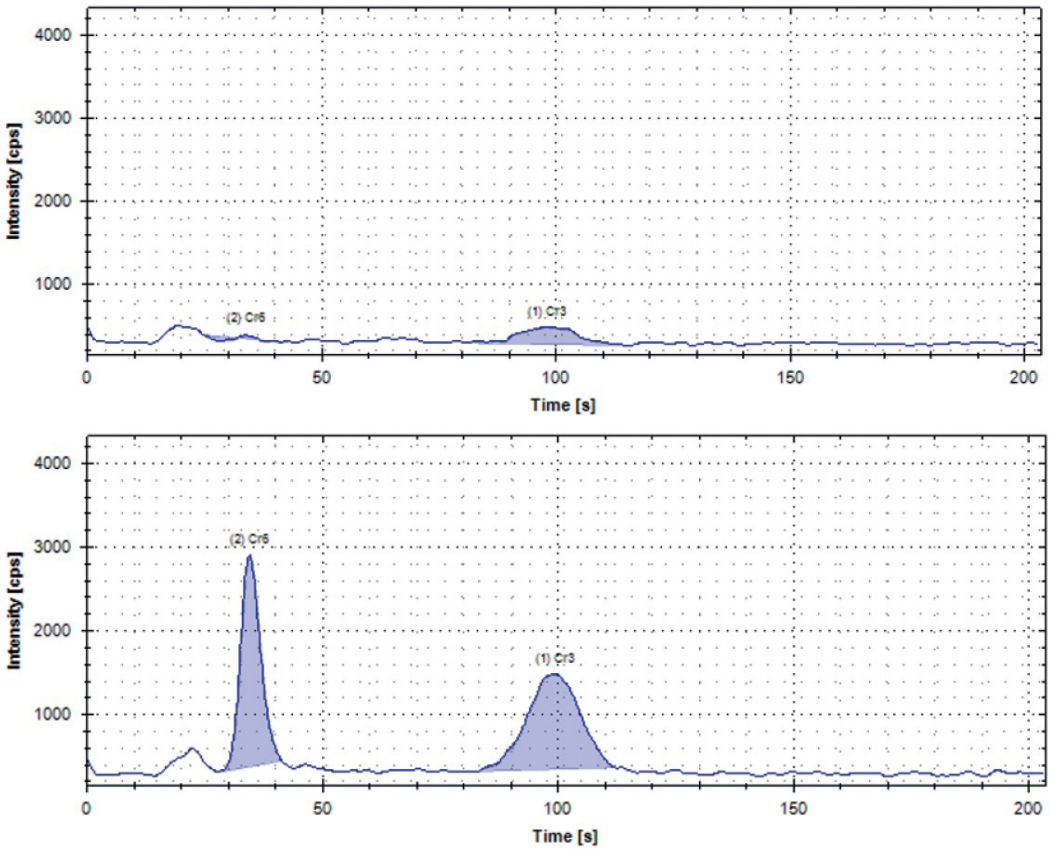

Figure 1. Chromatograms showing the injection of (from top to bottom) a blank, a standard solution containing 0.1 µg L^{-1} of both species (top), tap water, and spiked tap water (0.1 µg L^{-1}). For better comparability, all are scaled identically.

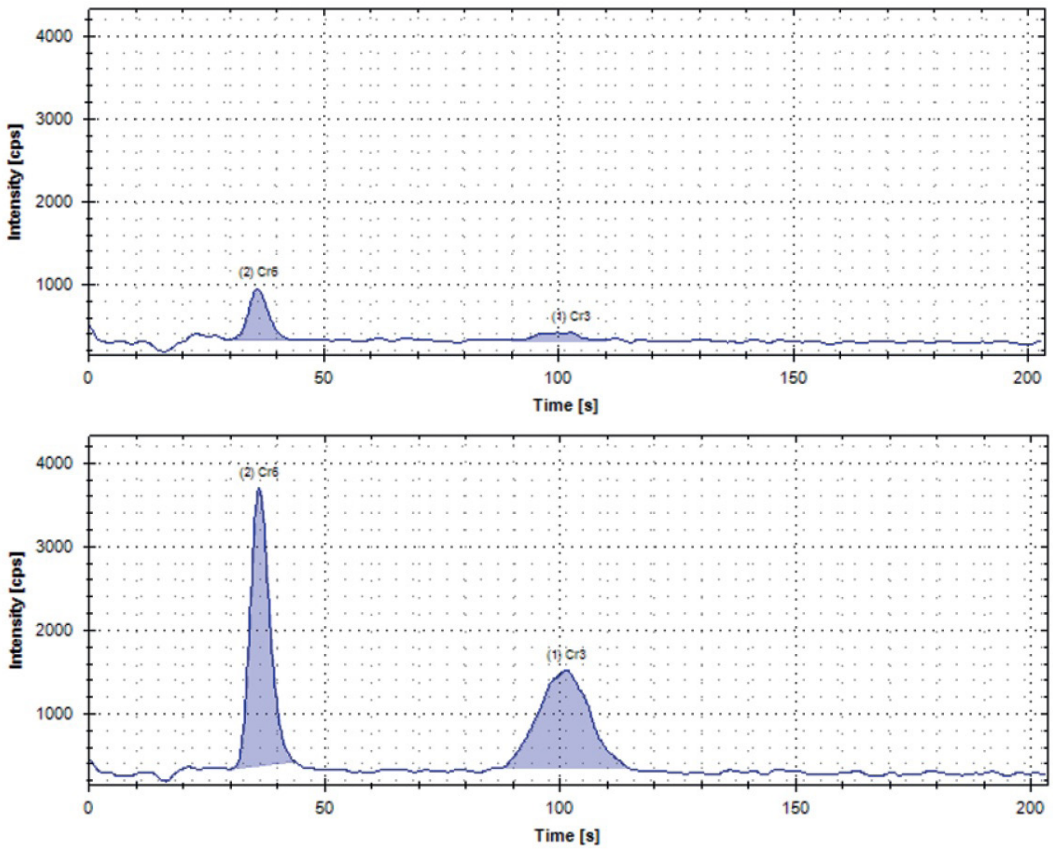

Figure 1. Chromatograms showing the injection of (from top to bottom) a blank, a standard solution containing 0.1 µg L^{-1} of both species (top), tap water, and spiked tap water (0.1 µg L^{-1}). For better comparability, all are scaled identically. (Continuation)

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 application note, 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.

REFERENCES

1. Agency for Toxic Substances & Disease Registry, Toxic Substances Portal – Chromium, https://www.atsdr.cdc.gov/phs/phs.asp?id=60&tid=17 (Accessed 2018).
2. Séby, F.; Charles, S.; Gagean, M.; Garraud, H.; Donard, O. F. X. *J. Anal. At. Spectrom.*, **2003**, *18*, pp 1386-1390.
3. Xing, L.; Beauchemin, D. *J. Anal. At. Spectrom.*, **2010**, *25*, pp 1046-1055.
4. Thermo Fisher Scientific – Application Note 43098.

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SPONSOR REPORT

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Unstoppable analysis of pesticides residues in black tea using triple quadrupole GC-MS

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This report was extracted from the Thermo Scientific Application Note 000560

Keywords: Pesticides, tea, gas chromatography-mass spectrometry, GC-MS, triple quadrupole, TSQ 9610 mass spectrometer, NeverVent Advanced Ionization ion source (AEI), TRACE 1610 GC, AI/AS 1610

Goal: The aim of this application note is to demonstrate the performance of the Thermo Scientific™ TSQ™ 9610 triple quadrupole mass spectrometer coupled to the Thermo Scientific™ TRACE™ 1610 GC for trace level determination of pesticide residues in black tea.

INTRODUCTION

Products of botanical origin, including black tea, have become an increasingly prevalent part of the worldwide health culture with their global market forecast to reach more than $230 billion by 2027. Manufacturers must ensure that these botanicals are safe for consumption, which requires routine/robust trace analysis of pesticide residues. Pesticides are chemicals used for crop protection against a variety of pests such as weeds, fungi, rodents, and insects. Because of their extensive use, pesticides can be found in the air, soil, water, and ultimately in the food chain. Despite their use being highly regulated, misuse of pesticides can lead to unwanted contamination of food and have possible impacts on both human and environmental health.

Laboratories performing the analysis of pesticides in botanical Table 1. GC-MS/MS and autosampler experimental conditions for matrices face numerous challenges. A prominent obstacle to accurate pesticide residue determination is matrix interference. As most botanical ingredients exist in a form of concentrated extracts, smaller sample sizes are needed to overcome heavy matrix interference, in turn requiring highly sensitive instrumentation to detect trace amounts of pesticide residues. Sensitivity is key in this analysis as the regulatory maximum residue levels (MRLs) must be met. The typical global MRL level for pesticides in food is 0.01 mg/kg. High-throughput laboratories must consistently hit the MRL levels in matrix with minimal user intervention on the GC-MS/MS system. Unplanned and prolonged instrument downtime can delay the return of results to the manufacturer of the botanicals. It is therefore essential that the GC-MS/MS produces confident and reliable results over time to ensure consumer safety.

In this study, the suitability of the TSQ 9610 triple quadrupole GC-MS/MS system was assessed for the analysis of more than 200 pesticides in black tea at trace concentrations supplemented with SPE cleanup. The linearity, accuracy, precision, limit of quantitation, and injection reproducibility of 20 selected analytes representative of the different pesticide classes in black tea matrix were demonstrated.

EXPERIMENTAL

Data was acquired with a TSQ 9610 triple quadrupole mass spectrometer equipped with a Thermo Scientific™ NeverVent™ Advanced Electron Ionization (AEI) ion source was coupled to a TRACE 1610 gas chromatograph equipped with a Thermo Scientific™ iConnect™ Split/Splitless (iConnect-SSL) injector and a Thermo Scientific™ AI/AS 1610 liquid autosampler. The NeverVent technology allows for ion source cleaning, filament replacement, and column exchange without breaking instrument vacuum, therefore ensuring minimum downtime to the laboratory workflow. Chromatographic separation was achieved on a Thermo Scientific™ TraceGOLD™ TG-5SILMS analytical column, 30 m × 0.25 mm i.d. × 0.25 µm fused silica capillary, with 10 m SafeGuard, (P/N 26096-1421). The MS/MS method monitored a minimum of three transitions for each compound. Additional method details are in Table 1.

Table 1. GC-MS/MS and autosampler experimental conditions for the analysis of pesticides

Parameter Value	Parameter Value
Injection liner	Thermo Scientific ™ LinerGOLD™ Splitless Liner, Single Taper with Quartz Wool, 4 mm (P/N 453A1925-UI)
GC column	TraceGOLD TG-5SILMS analytical column, 30 m × 0.25 mm i.d. × 0.25 µm fused silica capillary column with 10 m SafeGuard, P/N 26096-1421
Injection volume	1 µL
Injection temperature	260 °C
Splitless time	1 min
Column flow	1.4 mL/min, He
Run time	26.5 min
MS source temperature	320 °C
Emission current	10 µA
Aquistion mode	timed-SRM

Compounds were evaluated using SANTE/12682/2019 (Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed[1]) guidelines:

- Compound recovery should be within a mean recovery of 80–120%. If recovery within this range cannot be achieved, correction for recovery is necessary, or an explanation given for why a correction factor was not applied.
- Ion ratios for detected compounds must be within ±30% of the average of matrix-matched calibration standards.
- Detected compounds must have a signal-to-noise ratio (S/N) of at least 3:1.

In this application, difficult-to-analyze compounds, in addition to compounds from different pesticide classes (organophosphates, organochlorides, synthetic pyrethroids, and herbicide methyl esters), are highlighted in the results.

Data acquisition, processing, and reporting

Data were acquired, processed, and reported using the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.3. Integrated instrument control ensures full

automation of the analytical workflow combined with an intuitive user interface for data analysis, processing, customizable reporting, and storage in compliance with the Federal Drug Administration Title 21 Code of Federal Regulations Part 11 (Title 21 CFR Part 11). The advanced reprocessing capability of Chromeleon CDS ensures immediate and easy data analysis, offering the possibility to easily flag and check the compliance of the results with the SANTE/12682/2019 criteria.[1]

Sample preparation

Sample preparation involved the addition of water and acetonitrile to a black tea matrix, followed by QuEChERS extraction. Final clean-up was performed with solid-phase extraction using a cartridge consisting of graphitized carbon black (GCB) and primary−secondary amine sorbent (PSA). The resulting extract solutions were concentrated and re-diluted with toluene for a GC-MS/MS analysis.

RESULTS AND DISCUSSION

Chromatography

Black tea is an extremely complex sample, and it is important that the matrix interferences are removed during sample preparation to produce good chromatography and subsequent quantitation of pesticides at the MRLs defined by the regulations. A timed-selected reaction monitoring (t-SRM) acquisition method allowed for simultaneous acquisition of multiple characteristic ions for each pesticide, maintaining high sensitivity and selectivity to discriminate between the target compounds and the matrix, thus ensuring a confident and selective identification of analytes. Figure 1 shows the t-SRM acquisition of pesticides at 5 µg/kg in black tea.

Linearity

The TSQ 9610 NeverVent AEI is equipped with the Thermo Scientific™ XLXR™ detector, which offers an extended dynamic range and longer lifetime compared with its predecessor. In this experiment, linearity was evaluated for select analytes using matrix-matched calibration curves ranging from 0.01 to 200 ng/mL. The lowest standard to meet SANTE/12682/2019 criteria ranged, for the selected analytes, from 0.01 to 2 ng/mL.

Figure 2 shows an example calibration curve for 4,4’-DDT (0.5 to 200 ng/mL (ppb)) and cypermethrin I (2.0 to 200 ng/mL (ppb)). Table 2 shows the calibration result for the 20 selected pesticides.

To be compliant with the current regulation, it is essential that analytical testing laboratories meet the MRL of 10 ppb (0.01 mg/kg). To demonstrate the reliability of the instrument at such low concentration, the peak area repeatability of a 10 ppb black tea extract was assessed by using n=12 repeated injections. The % RSD of the calculated concentrations for all 20 compounds was <20%, with the majority of compounds giving RSDs <10%. Figure 3 shows a summary of results at the MRL including the %RSDs for n=12 injections and the average percentage recovery for each compound. Appendix 1 shows the chromatograms of the lowest matrix-matched standard conforming to SANTE/12682/2019 guidance for each of the selected pesticides.

Recovery and precision

Analyte recovery was assessed by spiking black tea at three levels (10, 25, and 50 ppb) before extracting with QuEChERS. Six extractions were performed at each level. Calculated recoveries for the spiked compounds were between 80% and 120%, with calculated precision ≤10% with the exception of folpet, which is thermally labile and therefore known to break down in the GC injector. Utilizing the PTV injector can help reduce the breakdown of labile compounds. Table 3 summarizes the recovery and precision results.

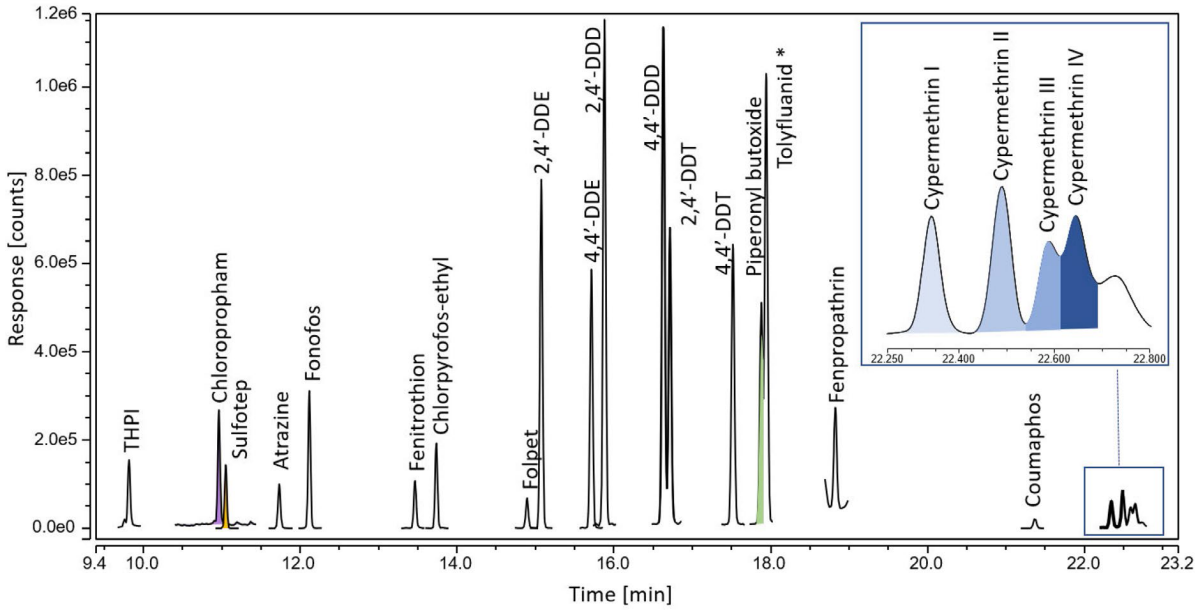

Figure 1. t-SRM acquisition for black tea sample pre-spiked at 5 µg/kg. *A matrix interferent was found to co-elute with tolyfluanid; therefore, the results for this compound were not included in the present work.

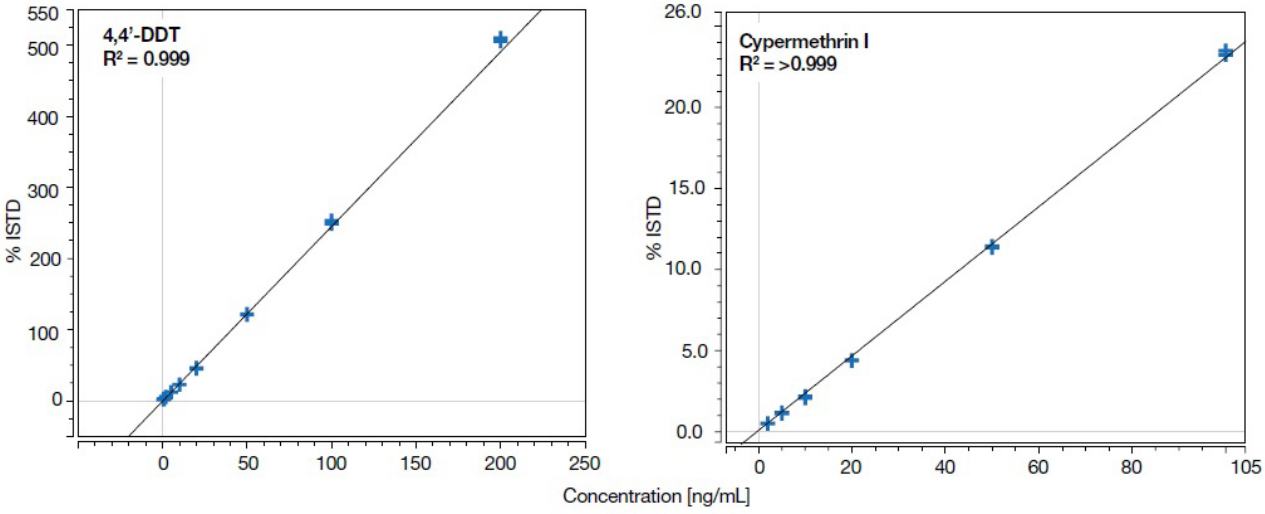

Figure 2. Calibration curve for 4,4'-DDT (range 0.5–200 ng/mL (ppb)) and cypermethrin I (2.0–200 ng/mL (ppb)).

Table 2. Calibration results for 20 selected pesticides in black tea (each point was injected in duplicate)

Peak name	Internal standard	Calibration type	R^2	Linear range (ppb)
Atrazine	Internal PCB 18 ISTD	Lin, WithOffset, 1/A	>0.999	0.5–200
Chlorpropham	Internal PCB 18 ISTD	Lin, WithOffset, 1/A	>0.999	0.5–200
Chlorpyrifos-ethyl	Internal PCB 52 ISTD	Lin, WithOffset, 1/A	0.999	0.5–200
Coumaphos	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	0.999	1.0–200

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Table 2. Calibration results for 20 selected pesticides in black tea (each point was injected in duplicate) [continuation]

Peak name	Internal standard	Calibration type	R^2	Linear range (ppb)
Cypermethrin I	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	>0.999	2.0–200
Cypermethrin II	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	>0.999	2.0–200
Cypermethrin III	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	0.998	2.0–200
Cypermethrin IV	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	0.999	2.0–200
DDD, 2,4'-	Internal Triphenylmethane ISTD	Lin, WithOffset, 1/A	0.999	0.1–200
DDD, 4,4'-	Internal Triphenylmethane ISTD	Lin, WithOffset, 1/A	>0.999	0.5–200
DDE, 2,4'-	Internal Triphenylmethane ISTD	Lin, WithOffset, 1/A	0.999	0.05–200
DDE, 4,4'-	Internal Triphenylmethane ISTD	Lin, WithOffset, 1/A	0.999	0.01–200
DDT, 2,4'-	Internal Triphenylmethane ISTD	Lin, WithOffset, 1/A	0.999	0.5–200
DDT, 4,4'-	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	0.999	0.5–200
Fenitrothion	Internal PCB 52 ISTD	Lin, WithOffset, 1/A	0.996	0.5–200
Fenpropathrin	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	>0.999	2.0–200
Folpet	Internal PCB 52 ISTD	Lin, WithOffset, 1/A	0.997	2.0–200
Fonofos	Internal PCB 18 ISTD	Lin, WithOffset, 1/A	>0.999	0.5–200
Piperonyl butoxide	Internal Triphenylphosphate (TPP) ISTD	Lin, WithOffset, 1/A	0.999	0.5–200
Sulfotep	Internal PCB 18 ISTD	Lin, WithOffset, 1/A	>0.999	0.1–200
Tetrahydrophthalimide (THPI)	Internal PCB 18 ISTD	Lin, WithOffset, 1/A	0.998	1.0–200

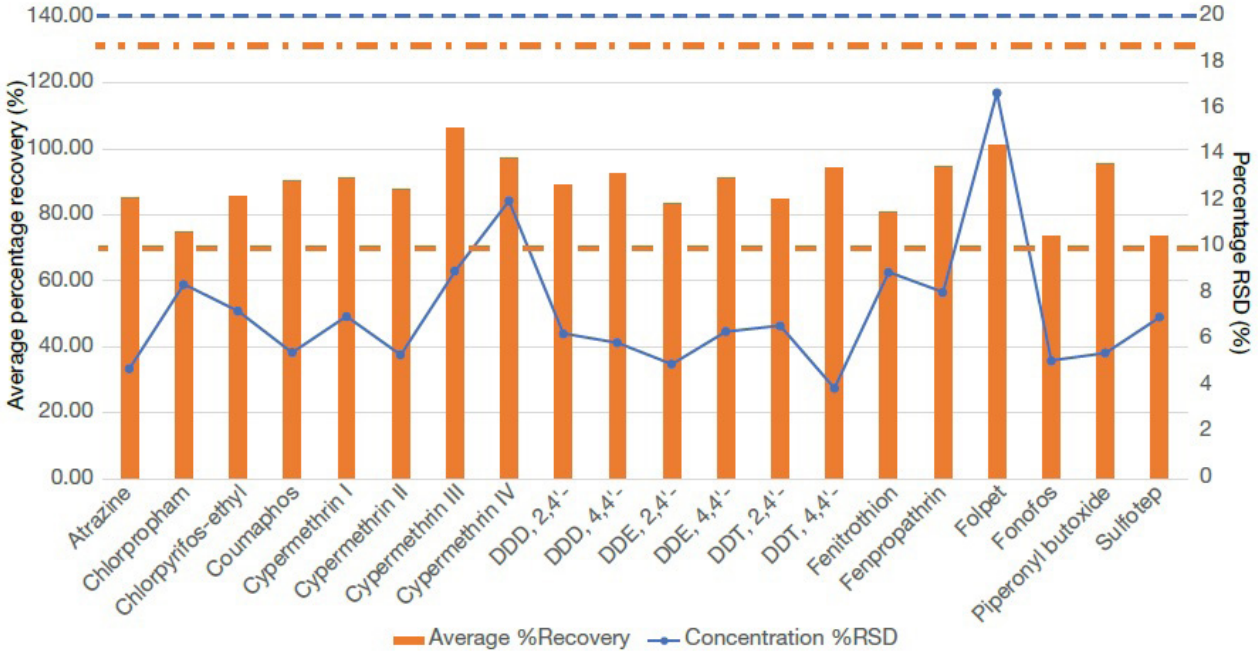

Figure 3. MRL results with %RSDs for n=12 injections and the average percentage recovery for each pesticide.

Table 3. Precision and spike recovery at 10, 25, and 50 ppb (ng/mL) (n=6 extracts at each level)

Compound	Concentration %RSD			Average %Recovery		
	10 ppb spike (n=6)	25 ppb spike (n=6)	50 ppb spike (n=6)	10 ppb spike (n=6)	25 ppb spike (n=6)	50 ppb spike (n=6)
Atrazine	3.4	4.1	5.5	83.3	84.5	85.5
Chlorpropham	7.8	7.0	2.5	77.7	78.7	74.3
Chlorpyrifos-ethyl	4.7	6.0	3.5	84.0	78.9	81.5
Coumaphos	6.1	6.5	5.5	91.3	92.0	91.1
Cypermethrin I	8.0	3.4	4.1	92.9	91.5	91.3
Cypermethrin II	5.0	3.4	3.7	88.3	89.6	89.2
Cypermethrin III	9.7	4.1	3.6	101.2	91.6	90.7
Cypermethrin IV	7.7	5.9	6.2	88.9	87.9	90.1
DDD, 2,4'-	3.4	5.0	2.2	93.3	91.8	91.3
DDD, 4,4'-	3.1	5.7	2.6	97.0	95.3	93.6
DDE, 2,4'-	3.8	4.7	2.2	86.1	83.4	81.6
DDE, 4,4'-	5.8	4.2	3.5	94.5	88.4	85.6
DDT, 2,4'-	3.8	4.4	3.8	89.6	90.2	87.6
DDT, 4,4'-	3.6	3.0	4.2	96.9	92.7	89.3
Fenitrothion	9.8	7.7	3.8	83.2	93.0	90.9

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Table 3. Precision and spike recovery at 10, 25, and 50 ppb (ng/mL) (n=6 extracts at each level) [continuation]

Compound	Concentration %RSD			Average %Recovery		
	10 ppb spike (n=6)	25 ppb spike (n=6)	50 ppb spike (n=6)	10 ppb spike (n=6)	25 ppb spike (n=6)	50 ppb spike (n=6)
Fenpropathrin	6.0	7.5	5.0	99.1	92.2	92.6
Folpet	20.6	10.3	6.6	107.6	86.2	61.1
Fonofos	4.4	3.8	6.1	76.0	73.2	72.6
Piperonyl butoxide	4.8	3.6	4.3	98.5	95.7	95.0

Robustness

It is important that the results produced over time are consistent and minimal user intervention is required to operate the system. Mass calibration and resolution tuning are two of the most important aspects ensuring system performance. The Thermo Scientific™ SmartTune™ tool allows the user to check the tune status of the system with a few mouse clicks in an easy and quick fashion. To demonstrate instrument robustness, black tea extracts at a concentration of 50 µg/kg were injected continuously over 124 injections. Neither inlet nor ion source maintenance was performed on the system during this extended sequence, which was equivalent to over 2.5 days of continuous analysis. Peak area %RSDs were <10% for all representative analytes (Figure 4 and Table 4) with the exception of folpet, which produces inconsistent results due to breakdown in the hot GC inlet.

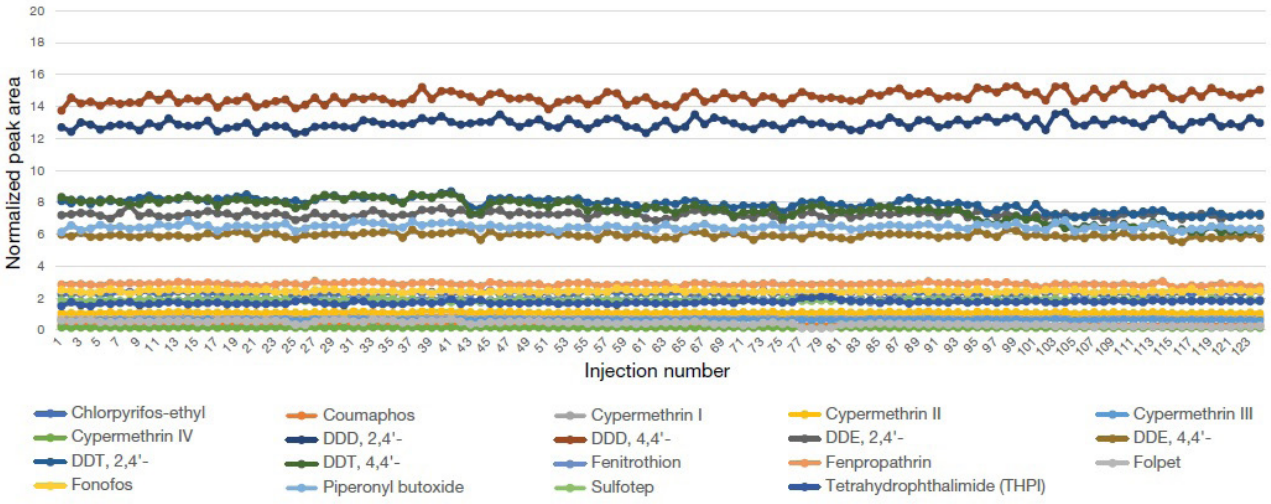


Figure 4. Normalized peak area response (analyte peak area / ISTD peak area) obtained for n=124 consecutive injections of matrix samples spiked at 50 µg/kg.

Table 4. %RSD of pesticides at 50 µg/kg over n = 124 injections

Compound	%RSD (n = 120)
Atrazine	4.3
Chlorpropham	3.6
Chlorpyrifos-ethyl	3.6

(continues on the next page)

Table 4. %RSD of pesticides at 50 µg/kg over n = 124 injections [continuation]

Compound	%RSD (n = 120)
Coumaphos	4.4
Cypermethrin I	2.8
Cypermethrin II	2.9
Cypermethrin III	3.6
Cypermethrin IV	6.0
DDD, 2,4'	2.1
DDD, 4,4'	2.4
DDE, 2,4'	2.3
DDE, 4,4'	2.6
DDT, 2,4'	5.0
DDT, 4,4'	9.0
Fenitrothion	4.1
Fenpropathrin	2.9
Folpet	34.1
Fonofos	2.8
Piperonyl butoxide	2.4
Sulfotep	2.7
Tetrahydrophthalimide (THPI)	5.9

CONCLUSIONS

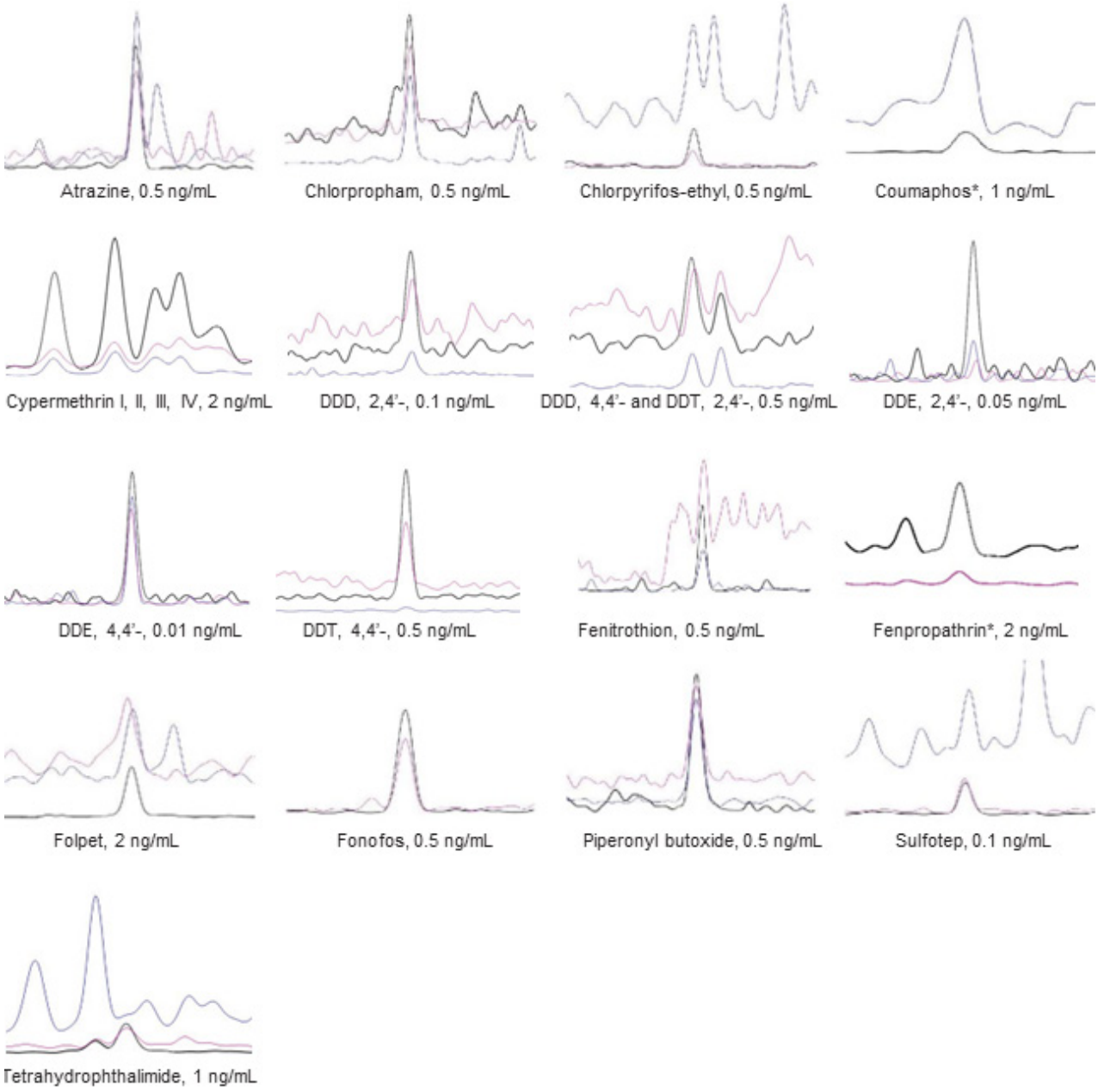
The results obtained in these experiments demonstrate that the TSQ 9610 mass spectrometer equipped with the NeverVent AEI ion source in combination with the TRACE 1610 GC and the AI/AS 1610 liquid autosampler delivers excellent analytical performance for multi-residue analysis of pesticides in black tea:

- The excellent sensitivity and good linearity between 0.01 ng/mL and 200 ng/mL allowed detection and accurate quantitation of numerous pesticides in the challenging black tea matrix.
- Peak area %RSDs of calculated concentrations at 10 ppb (0.01 mg/kg) were <10%, which are in compliance with SANTE/12682/2019 (Analytical guidelines).
- Calculated recoveries at three different spiking levels (10, 25, 50 ppb (ng/mL)) were within the 80–120% limits established in the SANTE guidelines.
- The TSQ 9610 mass spectrometer uptime is improved due to the NeverVent technology, allowing for laboratory productivity to be maximized with an uninterrupted workflow.

REFERENCE

1. SANTE/12682/2019, Analytical quality control and method validation procedures for pesticide residues analysis in food and feed. https://ec.europa.eu/food/system/files/2020-01/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf

Appendix 1. Chromatograms of lowest matrix-matched standard conforming to SANTE guidance



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Microwave assisted extraction of pesticides from environmental samples

This report was extracted from the Milestone Application Report ETHOS X - Pesticides - EPA 3546 Method

Pesticides are extensively used in modern agriculture, which could lead to serious consequences due to their biomagnification and persistent nature. Several governments require their analysis in environmental samples. Microwave assisted solvent extraction is a well-established sample preparation technique applied in several official methods. Milestone's ETHOS X equipped with fastEX-24 eT rotor was used in this study to prove its efficacy in the extraction of pesticides from environmental matrices

INTRODUCTION

Pesticides are pivotal chemical compounds for the modern agriculture production, used to control pests' diffusion. Depending on their target function, pesticides are used to treat insects, rodents, fungi and unwanted plants (weeds). For example, since 1940s, organochlorine pesticides and organophosphate pesticides are used extensively not only in agriculture but also for mosquito control.

The action mechanism of these chemicals is mostly designed to disturb the physiological activities of the target organism, leading to dysfunction and reduced vitality. Despite their fundamental use in the modern agriculture, these molecules can cause neurological damage, endocrine disorders, and have acute and chronic health effects on human[1]. Moreover, some of these chemicals belong to the class of persistent organic pollutants (POPs) with high persistence in the environment[2].

EPA 3546[3] outlines the procedure for extracting water insoluble or slightly water-soluble organic compounds from soils, clays, sediments, sludges, and solid wastes.

EPA 3546 is a specific method for Microwave Assisted Solvent Extraction (MASE), a well-established sample preparation technique that enables extractions with reduced solvent volume and time. This application note represents a guideline for the extraction of the priority pesticides from both standard reference materials and spiked materials using the official method EPA 3546.

EXPERIMENTAL

Equipment

- Milestone's ETHOS X.
- fastEX-24 eT rotor.[4]
- 100-mL disposable glass vials.
- SFS-24 (Simultaneous Filtration System).
- GC-MS/MS
- HPLC MS/MS

Figure 1. Milestone ETHOS X with fastex-24 eT (left) and SFS-24 filtration system (right).

Standard and reagents

Standards, surrogates and internal standard were purchased by Sigma Aldrich. Grade solvent pesticide were used. Sodium sulfate anhydrous, silica gel (activated for at least 16 h at 130 °C) and glass wool or paper filter were used in the clean-up procedure. According to the analytical method EPA 8270e[5], internal surrogates and standards were used.

Table 1. Pesticides Stock solution

Analyte	CAS-No	Analyte	CAS-No
α-BHC	319-84-6	Endosulfan Sulfate	1031-07-8
γ-BHC	58-89-9	Endrin aldeide	7421-93-4
β-BHC	319-85-7	Endrin ketone	01/10/7378
δ-BHC	319-86-8	Heptachlor Epoxide	1024-57-3
Aldrin	309-00-2	Methoxychlor	72-43-5
Heptachlor	76-44-8	Demeton	8065-48-3
γ-\α-chlordane	5103-74-2\57-74-9	Dimethoate	60-51-5
α-Endosulfan	1031-07-8	Malathion	121-75-5
4,4'-DDE	72-55-9	Parathion	56-38-2
Dieldrin	60-57-1	Parathion-methyl	298-00-0
Endrin	72-20-8	Mevinphos	7786-34-7
β-Endosulfan II	33213-65-9	Phorate	298-02-2
4,4'-DDD	72-54-8	Fenitrothion	122-14-5
2,4'-DDT	789-02-6	Isocarbophos	24353-61-5
4,4'-DDT	50-29-3	Methidathion	950-37-8
Mirex	2385-85-5	Toxaphene	8001-35-2
Dichlorvos	62-73-7		

Table 2. Internal Standard Solution

Analyte	CAS-No
Isoproturon-d6	217487-17-7
Biphenyl-d10	1486-01-7
Atrazina-d5	163165-75-1
Phenanthrene-d10	1517-22-2
Pirimicarb-d6	1015854-66-6
PCB 138	35065-28-2
Triphenylphosfate	115-86-6

Samples

The clay loam 1 - CRM847 certified reference material were used for the determination of pesticides. For pesticides not included in the certified materials a spiking stock solution on blank soil was used.

Sample preparation

The samples were collected and stored in accordance with the requirements of EPA 3546. Decant and discard any water layer on a sediment sample. Discard any foreign objects such as sticks, leaves, and rocks. Mix the sample thoroughly, especially composited samples. Grind or otherwise reduce the particle size of the waste so that it either passes through a 1 mm sieve or can be extruded through a 1 mm hole.

Ground samples, wet or dried, were weighed directly into the 100 mL extraction disposable glass vials of the fastEX-24 eT rotor. 30 mL of acetone-hexane (1:1) was used as extraction mixture. An aliquot of the internal standard solution was added to the samples just prior to solvent addition then the glass vials were closed (automatic capping tool available).

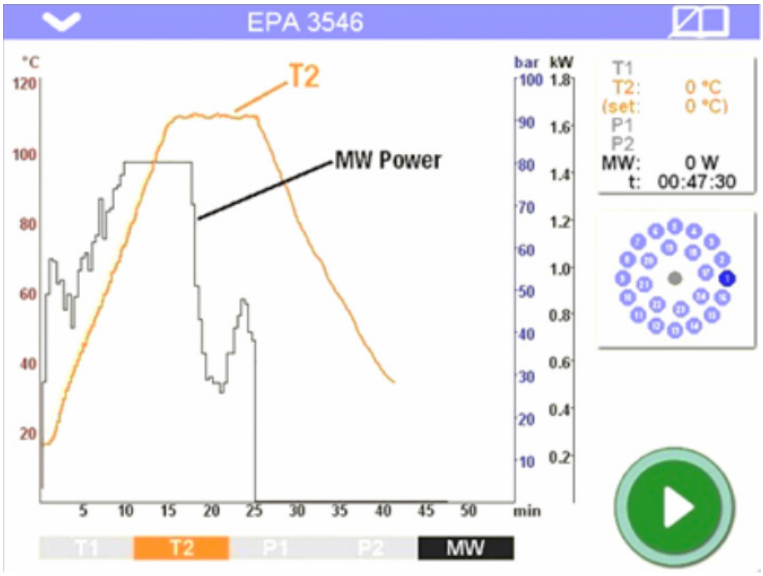

Figure 2. Microwave run profile.

After the extraction, samples were filtered with milestone SFS-24 simultaneous filtration system using sodium sulfate anhydrous. The vials were rinsed with additional solvent aliquots. SFS-24 allows to filter 24 samples simultaneously with different types of filters available. Extracts and rinse solution were collected together.

The extract was subsequently concentrated with nitrogen flow. If purification is not required, concentrate directly until 0.5 mL and add the appropriate surrogate standard solution to achieve the surrogate standard concentration. If purification is necessary, concentrate the extract directly until 2 mL. Purify the solution according to the method (EPA 3610, 3620, 3630, 3640, 3660). Finally, the extracts obtained by ETHOS X were concentrated for analysis.

Analitical conditions

Based on the pesticide compositions both GCMS/MS and HPLC MS/MS with triple quadrupole were used.

GC-MS/MS is equipped with a split-splitless injector, autosampler and triple quadrupole mass spectrometer. Sample injection volume was 1 µl. A 30 m x 0.25 x 0.25 RXI 5MS Capillary column (Restek) was used for the analyses. A five steps ramp oven program was used:

Table 4. GC-MS/MS oven program

Rate (°C/min)	Temperature (°C)	Plateaus (min)
20	50	2
30	150	0
7	260	0
20	290	11

Helium was used as the carrier gas at a linearity velocity of approximately 65 cm/s.

HPLC MS/MS was used to ensure the recovery data and to better quantify some compounds. An oven temperature of 40 °C is used with an injection volume of 0.25 µL.

Mobile phase:

- Water 0,1% formic acid 5 mM ammonium formate.
- Methanol 0,1% formic acid 5 mM ammonium formate.

Table 5. HPLC-MS/MS gradient method

Time (min)	Water (%)	Methanol (%)
0.2	95	5
11	0	100
13	0	100
13.1	95	5

RESULTS AND DISCUSSION

Results from extractions of Clay loam 1 - CRM847 are shown in Table 6. Recovery for all compounds is in the range 70-120% of the certified standard reference material.

Table 6. Pesticides recovery from Clay loam 1 - CRM847 (1g) (n=4)

Analyte	Certified value (µg/kg)	Ethos X (µg/kg)	Recovery (%)	RSD (%)
δ-BHC	138	128.89	93.4	8.7
α-BHC	221	188.95	85.5	7.0
β-BHC	295	342.2	116	2.2
α-Chlordane	309	330.63	107	3.3
γ-Chlordane	171	182.28	106.6	2.4
4.4'-DDD	120	107.28	89.4	3.1
4-4'-DDE	315	290.74	92.3	3.3
4-4'-DDT	92.1	84.27	91.5	11.5
Dieldrin	53.3	54.79	102.8	9.6
Endosulfan I	211	162.25	76.9	4.8
Endosulfan II	225	160.65	71.4	7.9
Endosulfan Sulfate	159	159.63	100.4	4.0
Endrin Ketone	170	139.57	82.1	6.3
Endrin	162	156.81	96.8	4.4
Heptachlor epoxide	127	119.25	93.9	3.7
Methoxychlor	290	260.13	89.7	6.6

Additionally, a pesticide mixture was spiked to a blank soil in order to test the performance of the fastEX-24 eT on a wider list of pesticides (Table 7).

Table 7. Pesticides recovery from Spike solution (n=4)

Analyte	Spike concentration (µg/kg)	Ethos X (µg/kg)	Recovery (%)	RSD (%)
α-BHC	50	52.00	104.0	4.6
γ-BHC	50	50.00	100.0	8.1
β-BHC	50	60.00	120.0	3.2
δ-BHC	50	56.67	113.3	4.1
Aldrin	50	53.33	106.7	5.2
Heptachlor	50	56.67	113.3	2.6
alfa clordano	50	48.40	96.8	4.8
gamma clordano	50	52.67	105.3	6.1
4.4'-DDE	50	56.67	113.3	3.2

(continues on the next page)

Table 7. Pesticides recovery from Spike solution (n=4) [continuation]

Analyte	Spike concentration (µg/kg)	Ethos X (µg/kg)	Recovery (%)	RSD (%)
Dieldrin	50	53.33	106.7	1.3
Endrin	50	52.05	104.1	1.9
β-Endosulfan II	50	50.00	100.0	4.1
4.4'-DDD	50	56.67	113.3	3.8
2.4'-DDT	50	46.65	93.3	2.4
4.4'-DDT	50	45.70	91.4	2.9
Mirex	50	53.05	106.1	5.1
Dichlorvos	50	40.00	80.0	9.3
Demeton	50	41.45	82.9	6.8
Dimethoate	50	52.50	105.0	4.2
Malathion	50	45.70	91.4	3.6
Parathion	50	43.95	87.9	2.7
Parathion-methyl	50	53.33	106.7	2.9
Mevinphos	50	54.20	108.4	3.9
Phorate	50	46.67	93.3	4.6
Fenitrothion	50	56.67	113.3	5.8
Isocarbophos	50	46.25	92.5	6.4
Methidathion	50	43.15	86.3	8.9
Endosulfan Sulfate	50	46.90	93.8	10.6
Endrin aldeide	50	39.45	78.9	3.5
Endrin Ketone	50	39.70	79.4	4.9
Heptachlor Epoxide	50	47.30	94.6	6.3
Methoxychlor	50	53.35	106.7	5.5
Toxaphene	50	41.80	83.6	4.9

CONCLUSION

The results demonstrate the efficiency of the ETHOS X with fastEX-24 eT rotor for the pesticides extraction from environmental matrices. High recovery rate for all the tested molecules showed the great extraction efficiency.

The fastEX-24 eT enables simultaneous solvent extraction of up to 24 samples in only 40 minutes (cooling step included). In turns this means that is able to extract over 200 samples in 8-hour workday. Contamination, memory effects, and cleaning are completely eliminated due to the use of disposable glass vials. The use of contactless temperature control ensures high reproducibility and full recovery of the target analytes for full compliance with Official Methods.

Thanks to the unique design, fastEX-24 eT is easily applied to even more challenging matrices such as solid wastes and plastics. ETHOS X provides extracts with the lowest solvent usage and significant time saving compared to all the other extraction techniques.

The ETHOS X with all its unique features fully addresses the need of environmental laboratories in terms of productivity, ease of use, running costs, and extraction quality.

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ABOUT MILESTONE

At Milestone we help chemists by providing the most innovative technology for metals analysis, direct mercury analysis and the application of microwave technology to extraction, ashing and synthesis. Since 1988 Milestone has helped chemists in their work to enhance food, pharmaceutical and consumer product safety, and to improve our world by controlling pollutants in the environment.

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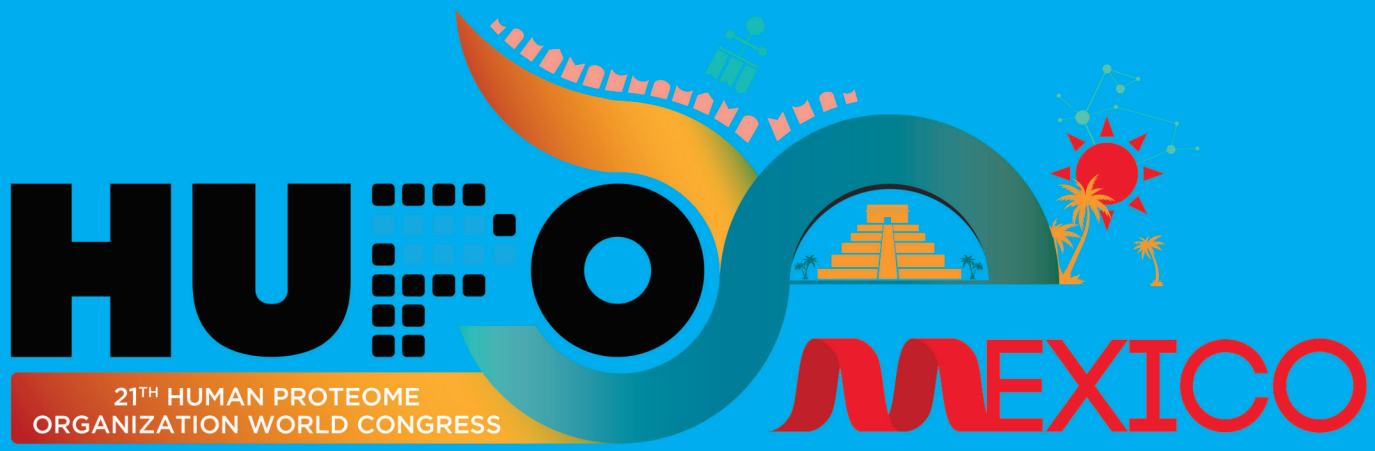

The HUPO 2022 Congress will take place December 4 – 8, 2022 in beautiful Cancun, Mexico. After two virtual editions, we are thrilled to invite you to join us again in a face-to-face meeting with virtual elements that will enhance participant's experience and allow anyone from anywhere to join one of the largest international gatherings in the field of proteomics.

The Congress program will offer both exciting education and engaging networking opportunities that we all have missed so much over the last couple of years. In addition to abstract presentations and lectures by world renowned speakers, we will be offering a variety of interactive sessions and networking options. The HUPO World Congress is an excellent opportunity for initiating or strengthening ongoing co-operations and knowledge.

Cancun is recognized throughout the world for its spectacular white sand beaches, its fascinating sea in turquoise blue tones, unique natural places, Mayan culture, water activities and typical handicraft markets. The wonderful natural attractions such as the majestic mangroves, the lush jungle and the mystic cenotes stand out and provide countless adventure activities. We hope you take some extra time before or after the Congress and take advantage of the many opportunities Cancun and its surroundings have to offer.

We can't wait to see you again in Cancun in December 2022.

Registration: https://2022.hupo.org/registration/

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IC-ICP-MS Analyzer for Speciation Analysis

While total elemental content can be analyzed by ICP-MS, speciation analysis of trace elements is ideally performed by a Thermo Scientific IC-ICP-MS system. The speciation analyzer includes a metal-free IC system with high resolution ion exchange columns and simple online connectivity, together with high sensitivity ICP-MS and integrated software.

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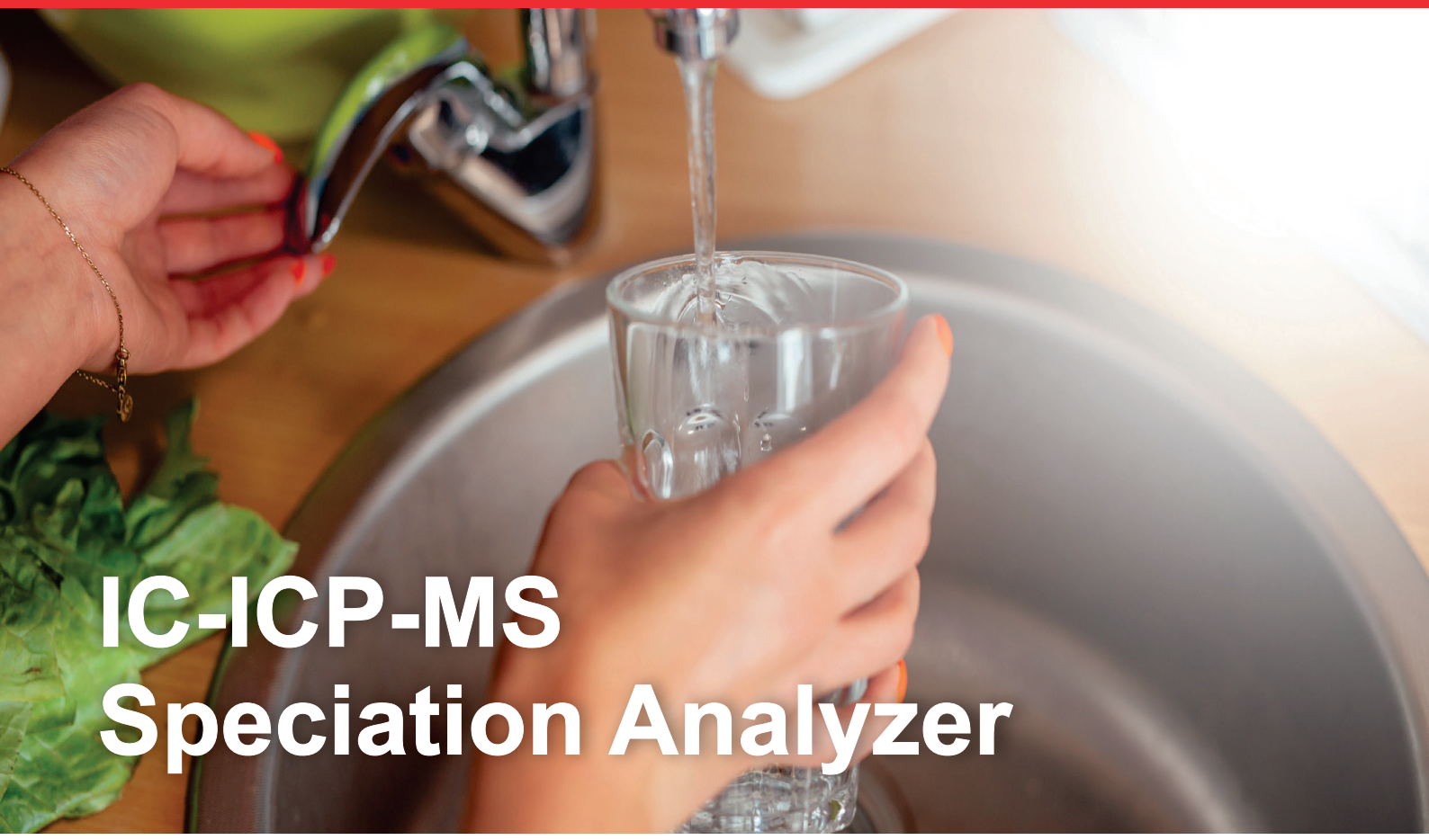

IC-ICP-MS Speciation Analyzer

Speciation Analysis

Distinguishing between chemical forms of an element is critical for multiple industries, including the food, environmental, and pharmaceutical sectors. In the past, measuring the total amount of an element was suffcient. Unfortunately, the effects of an element extend far beyond its absolute amount. Different forms of an element can exhibit very different physicochemical properties, including varying toxicities. The process of separation and quantification of different chemical forms of an element, more specifically termed speciation analysis, delivers a better understanding of the environmental or health-related impact associated with a particular sample. Speciation analysis can be split into two components: separation of individual ionic species by ion chromatography (IC), and trace elemental detection and quantification using inductively coupled plasma mass spectrometry (ICP-MS). This combined method is termed ion chromatography inductively coupled plasma mass spectrometry (IC-ICP-MS).

WEBINAR WEBSITE

Benefts of IC-ICP-MS

- 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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GC-MS that's ready to run when you are.

VIDEO **WEBSITE**

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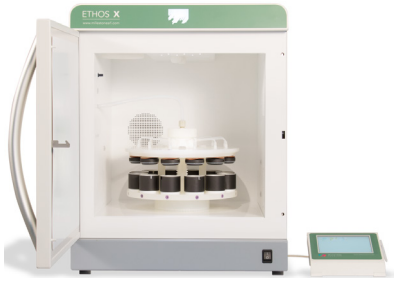
ETHOS X – Advanced Microwave Extraction System for Environmental Laboratories



Microwave-assisted solvent extraction offers superior lab efficiency in the determination of organic pollutants with microwave green extraction technique. Typical applications include chlorinated pesticides, semi-volatile organics, PAHs, PCBs, chlorinated herbicides, phenols, organophosphorus pesticides, dioxins and furans.

Determination of organic pollutants in environmental matrices is a common task for thousands of laboratories worldwide, as it leads to controlling and protecting our environment from high levels of contaminants. This analysis is often done to evaluate the effectiveness of a remediation process, to assess the contamination in waste, in waste landfills and for general environmental monitoring. Therefore, every day environmental laboratories deal with several challenges to ensure high quality data and fast turnaround time while maintaining their competitiveness.

Extraction of pollutants from solid matrices is often performed with techniques that limit the productivity and have high running costs. Many laboratories still use the Soxhlet method that was developed in 1879!

Milestone listened to the needs of environmental laboratory professionals by developing the ETHOS X with the fastEX-24 rotor, which allows for simultaneous extraction of 24 samples in 40 minutes with minimal solvent usage. By using large volume disposable glass vials, the fastEX-24 rotor simplifies handling and allows to achieve lower detection limits.

- High throughput: 24 samples in 40 minutes.
- Superior return of investment. Substantial reduction in solvent.
- Simple handling. Disposable glass vials.
- Consistency & Reproducibility. Consistent and reproducible results.
- Safety & Reliability.

Achieving lower detection limits with higher sample amount

The ETHOS X with fastEX-24 rotor extracts up to 30 grams of sample with minimal solvent volume, helping analysts to accomplish their tasks.

The Milestone fastEX-24 rotor uses disposable glass vials, eliminating the need for cleaning and the possibility of memory effect between different runs. The 100 mL vials can accommodate the extraction of a large sample amount. The easy to handle and affordable cost of the vials leads to high productivity at a very low running cost. ETHOS X system easily adapts to existing extraction chemistry through the use of a unique, patented material, called Weflon. Stir bars of Weflon are heated by microwaves and they subsequently transfer this heat to the non-polar solvent, which is not heated by microwaves.

COMPLIANCE

Several official methods describe the use of microwave closed-vessel technology to enhance the extraction efficiency of organic pollutants, such as US EPA 3546, ASTM and other national methods. The ETHOS X with fastEX-24 further enhances the performance of microwave technology for the extraction of water-insoluble or slightly water-soluble organic compounds from soils, clays, sediments, sludges, and solid wastes.

MILESTONE
HELPING CHEMISTS

ETHOS X

ENVIRO

Chlorinated pesticides Semivolatile organics PAHs PCBs Chlorinated herbicides (phenoxyacid herbicides) Phenols Organophosphorus pesticides and chlorinated herbicides Dioxins and furans Petroleum Hydrocarbons

ELEVATED SAMPLE THROUGHPUT

DISPOSABLE VIALS

MINIMUM SOLVENT USAGE

SUITABLE FOR US EPA METHOD 3546 AND ASTM METHOD D5765-05

ADVANCED MICROWAVE EXTRACTION SYSTEM FOR ENVIRONMENTAL APPLICATIONS

The overall concept of the ETHOS X for environmental applications has been developed to fully comply with the requirements of US EPA and ASTM methods.

This system has been developed by studying the working routine of several thousand contract laboratories around the world performing solvent extraction, with the aim of helping them, by offering an integrated solution able to render their activity easier, faster and safer.

A completely new rotor has been specifically developed by Milestone to fully accomplish the US EPA method 3546 requirements. This new rotor consists of a 24-position carousel, which holds large pressure vessels made of an innovative and unique inert polymer material. At the core of the vessel there is a disposable and inexpensive 100 mL glass vial. A self-regulating pressure cover assures safe operations of the system. Temperature and pressure are monitored and controlled in all vessels by non-contact sensors.

With an installed power of 1900 Watt, the ETHOS X is the most powerful microwave platform system available for extraction.

VIDEO

WEBSITE

Is your Laboratory following US EPA 3546?
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MILESTONE

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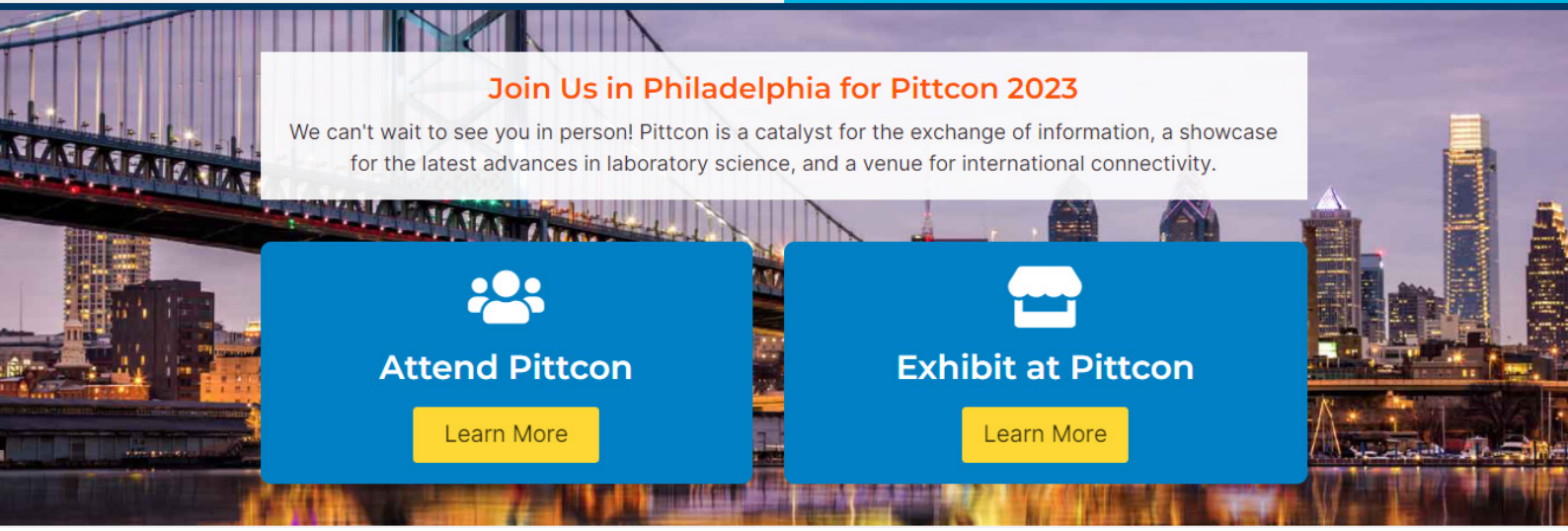

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Eliminate tedious tasks, accelerate timelines, expand testing capability and improve the overall output of your lab with automated analyzers. In this article, wet chemistry analyzers that are specifically designed to eliminate operator burden in busy analytical testing labs are discussed. By Roberto Marcos Perchin, KPM Analytics. Access **here**

VIDEO AT THE SCIENTISTS' CHANNEL

How innovative analytical technologies are helping to meet the global demand for safer food production

In this video, Prof. Fernández-Alba discusses the dynamic world of pesticide residues, and highlights the need for cutting-edge technologies and accelerated workflows to achieve more, faster, and better analyses. By Amadeo Rodríguez Fernández-Alba, professor in analytical chemistry at the University of America, and head of the European Reference Laboratory for pesticide residues. Access **here**

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This webinar will introduce a fully integrated in situ solution in a field emission scanning electron microscope (FESEM) and explore automated workflows that can be used to generate meaningful data with high levels of reproducibility and precision.

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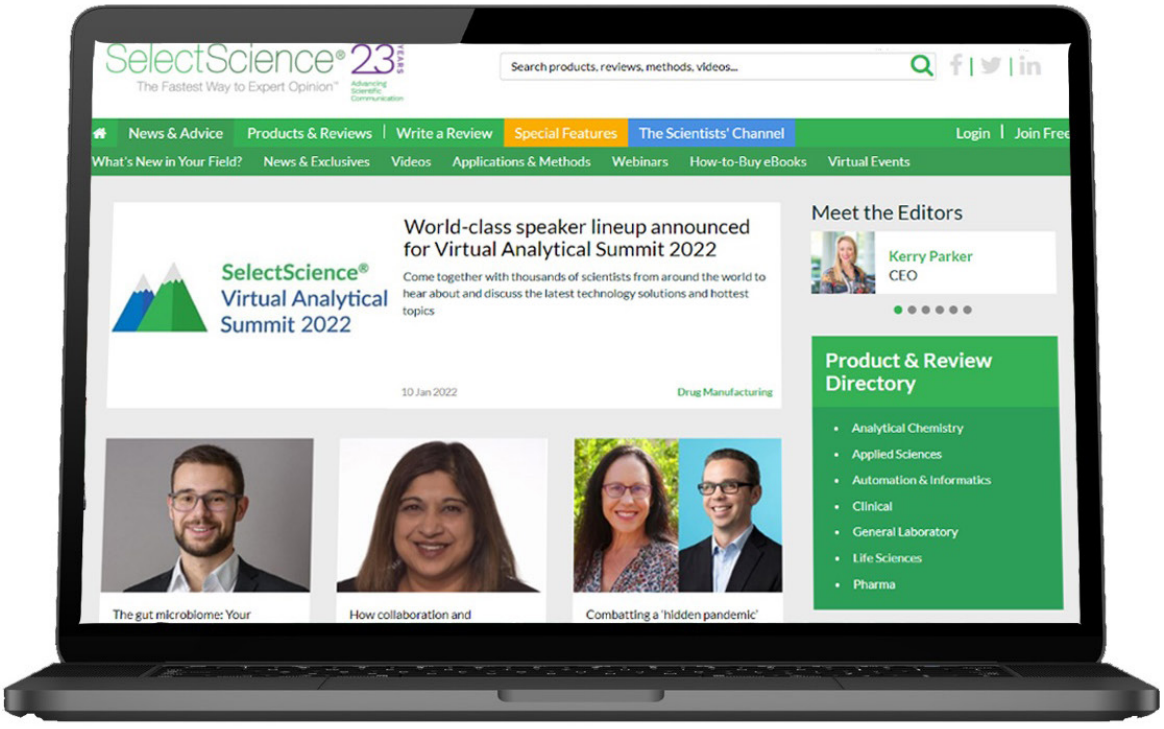

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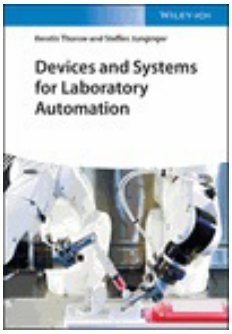

Devices and Systems for Laboratory Automation

Kerstin Thurow, Steffen Junginger, Authors

August 2022. Publisher: Wiley-VCH GmbH

Structured Overview on the Available Systems and Devices for Laboratory Automation. Choosing the right systems and devices for the automation in any given laboratory is an essential part for the process to succeed. This book provides an introduction into laboratory automation and an overview of the necessary devices and systems. Read more

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Herman Tang, Editor

November 2021. Publisher: Wiley & Sons, Inc

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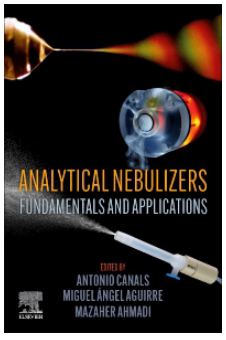
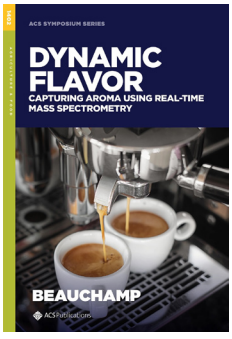


Analytical Nebulizers: Fundamentals and Applications

Antonio Canals, Miguel Angel Aguirre, Mazaher Ahmadi , Editors

February, 2023. Publisher: Elsevier Science

This book presents the fundamentals of analytical nebulizers, including types, aerosol generation, characterization, and design information of various classes of nebulizers such as nanonebulizers, multinebulizers, electrosprays, and ultrasonic nebulizers. Read more



Dynamic Flavor: Capturing Aroma Using Real-Time Mass Spectrometry

Jonathan D. Beauchamp, Editor

November, 2021. Publisher: American Chemical Society

Advanced analytical technologies offer flavor chemists the opportunity to explore and understand complex mixtures in great detail. This work covers three real-time mass spectrometric techniques applied to food-flavor assessments: APCI-MS, PTR-MS, and SIFT-MS. Read more

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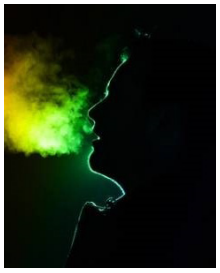

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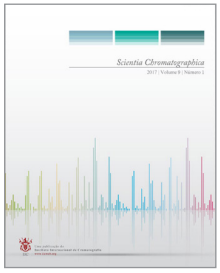

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Scientia Chromatographica is the first and to date the only Latin American scientific journal dedicated exclusively to Chromatographic and Related Techniques. With a highly qualified and internationally recognized Editorial Board, it covers all chromatography 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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7th Congreso Uruguayo de Química Analítica
Montevideo, Uruguay
https://sites.google.com/view/cuqa7/inicio

December 10 – 15
III Ibero American Conference on Mass Spectrometry (IBERO 2022)
Rio de Janeiro, RJ, Brazil
https://www.ibero2022.com/

EVENTS in 2023

March 18 – 22, 2023
PITTCON Conference & Expo
Philadelphia, PA, USA
https://pittcon.org

May 28 – 31, 2023
46th Annual Meeting of the Brazilian Chemical Society (RASBQ)
Águas de Lindóia, SP, Brazil
http://www.sbq.org.br

August 27 – 31, 2023
EuroAnalysis 2023
Geneva, Switzerland
https://www.euroanalysis2023.ch/

September 26 – 28, 2023
Analitica Latin America Expo & Conference
São Paulo, SP, Brazil
https://www.analiticanet.com.br/

GUIDELINES FOR AUTHORS

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