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Brazilian Journal of Analytical Chemistry

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Analytical Chemistry: beyond on its own borders

Write about the importance of Analytical Chemistry for Analytical Chemists is somewhat redundant, once the community involved in this field is concious about its importance and benefits (i.e. social, educational, industrial development, and others).

Once the bases for the growing of Analytical Chemistry are well established all over the world, including, of course, Brazil, the analytical community needs to figure out a way in "increasing the sensitivity" of the perception that Analytical Chemistry is extremely important to others areas. This is not an easy task, but we need to "optimize" our partnership with industries and also with areas such as toxicological, medical, nutritional, biochemical, environmental, forensic, among others, avoiding the concept of compartimentalization of the science if we want to establish fruitfull connections with different fields of knowledge.

We have the opportunity not only to make Analytical Chemistry a bridge between researchers of different backgrounds, but also "expand" even more the knowledge. Some examples can be pointed out such as M. Tswett (botanist, considered the chromatography´s father), S. P. D. Sørensen (enzimologist that coined the term pH) or L. C. Clark Jr (chemist, considered the biosensor´s father), which contributed, since long time ago, for transdisciplinary work.

Thus, all the analytical community is invited to highlight the importance of Analytical Chemistry in different fields of applications.

In this sence, BrJAC is contributing to show the presence of Analytical Chemistry beyond on its own borders, as demonstrated through different contributions published in this Issue.

Enjoy the reading!!

Marco Aurélio Zezzi Arruda
Editor

Editorial Board

Summary

Expedient

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Letter

This section is reserved for you to send comments, suggestions or reviews about the articles or published reports by BrJAC. You may also submit comments on issues related to the Analytical Chemistry in Brazil and abroad. Join us in this project! Be part of that!

It was with great enthusiasm that I contributed to, and celebrated the release of, the first issue of the Brazilian Journal of Analytical Chemistry (BrJAC). This new journal, a pioneer in Latin America, is further evidence of the strength and dynamism of the first rate Science we are currently producing here in the sub-tropics. Working hard for decades to reverse historical trends, we have placed Brazil in a leadership position and in the center of attention in regard to Science and Technology.

With BrJAC, we show now that we are not only producing first rate Science and Technology but are also playing a major role in scientific publishing. BrJAC is notably well-structured and focused on a specific audience, representing a concrete action for integration and development of a highly important and actual scientific field in Brazil. BrJAC is also unequivocal demonstration of the health and effervescence of analytical chemistry in Brazil, and the increasing ability of Brazilian analytical chemists to produce and apply state of the art innovative analytical techniques and methods. These innovations are also attracting increasing interest both in the academic and industrial areas, for their practical utilities in various branches of the Brazilian industry and laboratories such as those of chemical, biochemical, clinical, forensic, food, and pharmaceutical analysis.

BrJAC arrives, then, at the right time, a time of important developments in Science, in chemistry, and, especially, in analytical chemistry in Brazil. BrJAC has become a means for us to display to the world our high quality science, and how we are working to encourage industry-university integration in Brazil. BrJAC will certainly boost this integration by bringing to the desks of professors and professionals the newest scientific and technical discoveries made in Brazil and throughout the world. This new scientific journal therefore presents an invitation for the Brazilian analytical chemistry community to participate in a very challenging task - establishing a Brazilian journal as a key reference in Analytical Chemistry. When completed, this mission will have a significant side effect: bringing further developments of Brazilian Chemical Science and Industry to the attention of the World.

BrJAC, you are very welcome!

Prof. Dr. Marcos Nogueira Eberlin
President IMSF, Vice-President BrMASS
Coordinator ThoMSon Mass Spectrometry Laboratory
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One of the last interviews given by Professor Paschoal Senise is scheduled to be published exclusively in BRJAC 2011; 1(5). Professor Senise received the Journal at the Chemistry Institute in March, 30th. Below is the text as it was prepared and approved for publication by him em May.

Professor Paschoal Senise

One of the most important teachers, researchers and analytical chemists of his generation, Professor Paschoal Senise is a kind of living memory of the Chemistry Institute of University of São Paulo (IQ–USP). He has built a long and solid academic career in the Chemistry Institute of USP that started back in 1935, when he and a small group of students formed the first class of Chemistry of the University. Today, he is often consulted on academic matters, because of his deep knowledge of the system, its administrative structure and also because of his relevant role in Analytical Chemistry studies in Brazil.

Although he has been retired for more than 20 years – he will turn 94 years old next August –, almost every morning he still drives his own car to the University. There, at the Chemistry Institute building, he occupies a room full of books and Chemistry magazines, and welcomes fellow teachers and former students. This routine is even more important on Fridays,

when the Professor participates on regular seminars of Analytical Chemistry Graduate Program with his colleagues, most of which are his former students.

As an extremely dedicated teacher and researcher, Professor Senise has brought innovations and teaching techniques to USP and, in two different periods in the 1970s, he was director of the Institute as well. However, even when he was in this function, Professor Senise never stopped teaching. "If there is such a thing as vocation, mine is teaching", he states, proudly and modestly. Professor Paschoal Senise embraced Analytical Chemistry and academic life with passion and dedication. This is visible in each and every word he says. To resume this passion and his incredible capacity to remember episodes with details, in 2006 he published Origins of the USP Chemistry Institute – reminiscences and comments, an interesting and important book of memoirs, sponsored by the Chemistry Institute-USP.

Why did you choose the scientific career?

I embraced the career of Chemistry even before I decided to become a scientist. My teenage dream was to be a doctor, but when I finished high school, there were no pre-medical schools to prepare students for the vestibular. We have to go back to 1935 to tell this story.

In 1934, the Philosophy, Sciences and Languages School (FFCL – USP) had been founded, and the Chemistry Department began to be structured. USP supporters, such as Júlio de Mesquita Filho, were very much concerned with the level of the teachers and decided to hire great international names, such as worldwide famous Professors Rheinboldt and Hauptmann. They made a lot of publicity of it, and it called my attention. I was only seventeen years old, and by that time little was known about the career of chemist, which was considered merely a low level technical profession. Because of my straight relationship with a fraternal friend then, Luciano Barzaghi, whose father was a chemist in the chemical industry and whose brother was already studying Chemistry at Escola Politécnica, I was aware that this career could be interesting. Both Barzaghi and I were approved in the vestibular test.

Soon after the classes at USP began, Professor Rheinboldt started to organize the courses and teach. His classes were inspiring and exciting. He had extraordinary charisma and a fantastic didactic ability, with a singular style, that fascinated us all. Also, the atmosphere was excellent, and we felt so comfortable among the colleagues, that both Luciano Barzaghi and I promptly made an option to continue studying Chemistry.

You were part of the first group of chemists graduated in USP. How did you guide your career in Chemistry?

When the Chemistry course started, many people, including professionals of other areas, joined it, because they thought that the prestigious teachers who had been hired would give great conferences and lectures. However, soon they realized it was a common course, which involved a lot of laboratory studies and demanded dedication and hard work. So, from the twelve students that really initiated, only four of us remained and graduated. We were a small group and this helped us to stay together and share scientific experiences along our careers. The group was: Luciano Barzaghi, Simão Mathias, Jandyra França and I. Soon after graduation, Barzaghi was invited to join the IPT (Instituto de Pesquisas Tecnológicas – Technological Research Institute). He then proceeded successfully in the Pottery Industry. The other three were hired as assistant teachers of Chemistry at USP.

The course had a completely different structure at that time. It lasted only three years, but, when this period finished, we were not satisfied and requested and granted another year. Soon after graduation, we initiated a PhD program, Simão

Mathias and I with Professor Rheinboldt, and Jandyra França with Professor Hauptmann. In 1942, we had all concluded our theses and were the first PhDs in Chemistry at USP. This was the actual beginning of my scientific career, so to say.

During the PhD program, I became very much interested in research, and Professor Rheinboldt strongly encouraged me. I have always had great pleasure in teaching and joyfully dedicated myself to it as well, over the years. I also directed the Institute twice, in different periods in the 1970s, and only left academic life officially, in a compulsory retirement, when I got 70 years old. However, I still come to USP almost every morning, where my ex-students and their students insist generously in maintaining a study room for me and include me in seminaries and discussions. I am glad to be close to my fellow researchers and teachers. But I also know that it is very important to make room for the new generations.

"Izaak Kolthoff, whose studies gave the scientific bases to Analytical Chemistry, used to say that some scientists thought that the analytical chemists were servants"

How, when and why did Analytical Chemistry begin to interest you as a researcher and chemist?

Since the very beginning. In fact, I built my entire academic career based on Analytical Chemistry. This happened naturally. During graduation, we had intense laboratory work, widely based upon Analytical Qualitative Chemistry. Nevertheless, neither Professor Rheinboldt nor Professor Hauptmann did real research in the field of Analytical Chemistry itself. Professor Rheinboldt had interest is Inorganic Chemistry, and had already concluded very relevant researches on sulfur. As for Professor Hauptmann, he was a real organic chemist himself.

When I was still a student, I became acquainted to the eminent Professor Fritz Feigl, an Austrian Jew, Nazi fugitive, who had travelled all around Europe and arrived in Rio de Janeiro around 1940. In Rio, he soon joined the Mineral Production Laboratory (Laboratório da Produção Mineral). Professor Feigl was the creator of the "spot test". He was a very accessible person, entirely dedicated to science, and worked in a modest tiny laboratory. His wife and son were his assistants, in different periods. Although his name has been cogitated to a nomination for the Nobel Prize, his real value as a scientist was never acknowledged as it should have been. After some time, Professor Feigl recommended me to Professor Philip W. West, of the University of Baton Rouge (1951-52), Louisiana, with whom I spent a year and a half. Professor West was a famous micro-analytical chemist. In Baton Rouge, I also worked with Professor Paul Delahay, a Belgian-American electrochemist.

After I came back from this rich experience abroad, I began to introduce some novelties in teaching and laboratory work, especially some instrumental methods that were not usual yet.

Have you noticed progresses in the Analytical Chemistry studies over the years, concerning its scientific status and the use of new the technologies?

It is possible to notice some progress. The question is that, in the past, due to the fact that it was necessary to make much analysis, there has been the false impression, over many years – even among scientists and chemists themselves – that Analytical Chemistry was a kind of subsidiary field. One of the fathers of modern Analytical Chemistry, Izaak Kolthoff, whose studies gave the scientific bases to Analytical Chemistry, used to say that some scientists thought that the analytical chemists were servants. Maybe this was a current thought – and, at some extent, still is – because

Analytical Chemistry has its origin in Alchemy and has been empirically applied for a long period of time. In the 1930s and 1940s, Kolthoff, an extraordinary scientist with a huge production, proceeded his works on physical chemistry and electrochemistry, which largely contributed to develop much more rational analytical methods and gave Analytical Chemistry a different status. Even so, there are still many people today who don't realize that Analytical Chemistry plays a fundamental role in the field of Chemistry and other sciences. This is a mistake. As for technology, we can see notable progress, even here in Brazil, though not to the same level we notice abroad. The equipment and instrumental techniques have improved a great deal, but the prices are still high. However, as we can see in many other areas, prices and sizes tend to get smaller as technology advances.

What has been done in terms of academic structure in Brazilian universities to improve teaching and research in general?

"I have always been against centralization, even when I myself became a full Professor"

With the University Reformation, which took place during the late 1960s and was implemented in 1970 in Brazil, many important changes were made. One of the most effective was the division of faculties and institutes into departments and the extinction of the "chairs" (with a single full Professor per discipline), for example. This system favored only one professor and his assistants. Until this person did not retire or die, other teachers remained subordinated to him or her. In the system of departments created then, it became possible to have more than one full professor per discipline.

I have always been against centralization, even when I myself became a full Professor. The decentralization brought by the reformation was somewhat mirrored in the North American system and had the clear goal to provide mobility in academic hierarchy. From then on, there has been a career progression and a professionalization of the teaching activity at universities. This resulted in a fantastic expansion. Today, there are many PhDs, working hard on research with their own teams, which is fundamental for the improvement of research. Also, the possibility of getting to the top of the academic career attracts and stimulates more and more people to join it. Today, there are many more PhDs than years ago, and I think this number has to grow. We have to go further, but with quality. There must be more investment in qualification and research. And we cannot do it in a hurry: in science, maturation takes time and hard work. Also, academic life is not about prestige.

Is Analytical Chemistry a strategic field of knowledge and research for everyday living and science activity nowadays?

Yes, absolutely. Take the environmental issue, for example. Any serious study on the effects of pollution has to take the content of the polluting substances into consideration. With the technology and expertise we have in Analytical Chemistry today, to identify and determine extremely low contents, it is possible to quantify substances more precisely. This makes it possible to distinguish poisoned from potable water, for instance. This affects everyone in the planet. The same happens with the food and pharmaceutical sectors. Analytical Chemistry procedures and methods are necessary and valuable, as much as the methods of other sciences, mainly to establish safe rates of substances.

At what extent do you think it is important to divulge scientific research to lay audiences?

I think information is always important. But it has to be done with criteria and in a way that takes

the position of the lay audience into consideration. Sometimes, we read news about one or other scientific achievement, especially in the medical field, but generally the information is incipient and incomplete. This can be dangerous, because it may create expectations as to the cure or a revolutionary treatment of this or that disease, for example. Scientific information has to be dealt with care and with responsibility.

Do you believe Analytical Chemistry brings a differential to scientific research? How does it contribute to improve everyday living?

"Any serious study on the effects of pollution has to take the content of the polluting substances into consideration. With the technology and expertise we have in Analytical Chemistry today, to identify and determine extremely low contents, it is possible to quantify substances more precisely."

Analytical Chemistry has this intrinsic capacity to intermingle with other sciences and to penetrate in many areas of knowledge. It has a kind of interdisciplinary essence. And this interdisciplinary path is inevitable and desirable to reach sustainable progress. In the industrial sector, for instance, Analytical Chemistry is of great importance, especially in creating new products, for example. In this particular matter, I believe there has a lot of work still to be done, here in Brazil. The contribution of Analytical Chemistry to the chemical industry can be much greater than it has been. We have to pursue both the culture of expertise and the exchange of experiences and knowledge, to stimulate a new mentality about the relationship between academic research and society. In the University, there should be more investment in research and human resources. There are many practices and procedures related mainly to the Applied Sciences, but it doesn't mean they have not been studied and performed with the care and the theoretical bases of Analytical Chemistry methodology provides them. The analytical chemist should study and practice to become more and more specialized and to achieve precision in his analyses. Besides, one cannot do serious research and work without deep study, and scientific work inexorably leads to reflection. There is a subjacent philosophy in it. This is very important to science and society.

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Analysis of street Ecstasy tablets by thin layer chromatography coupled to easy ambient sonic-spray ionization mass spectrometry

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Abstract

Ecstasy is a famous illicit drug with varying drug composition, but it usually contains 3,4-methylenedioxymethamphetamine (MDMA) as the main active ingredient. The common procedure to identify ecstasy tablets uses testing kits, but its low specificity may lead to false positives. Thin layer chromatography (TLC) is used worldwide in forensic investigations due to its simplicity, low-cost and versatility but may also lead to false positives. In this study, TLC separation of seven common ecstasy drugs: MDMA, metamphetamine, 3,4-methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxyamphetamine (MDA), amphetamine, caffeine and lidocaine was attained, and twenty five apprehended street ecstasy tablets analyzed by TLC. Easy ambient sonic-spray ionization mass spectrometry (EASI-MS) was then performed directly on the surface of each TLC spot for MS characterization. The combination of TLC with EASI-MS is shown to provide a relatively simple and powerful screening tool for forensic analysis of street drugs with fast and indisputable results.

Keywords: ecstasy tablets; MDMA; illicit drug; TLC; EASI-MS;

Introduction

Ecstasy, also known as "candy", "XTC" and "Adam", is a popular illicit drug sold worldwide in the form of colored tablets with varying logos and shapes. Ecstasy most often contain 3,4-methylenedioxymethamphetamine (MDMA, Figure 1), but 3,4-methylenedioxyamphetamine (MDA) or 3,4-methylenedioxyethylamphetamine (MDEA) are also found particularly in samples known as "Eve" tablets. These amphetamines display close chemical compositions and biological effects.

Renfroe and co-workers [1] were the first to report the chemical composition of ecstasy tablets. They analyzed, from 1972 to 1985, hundreds of tablets of ecstasy sent anonymously to their laboratory. All samples sent before 1975 were found to contain only MDA. The first tablet with MDMA was found in 1975, the second in 1976 and, during the next years the number of tablets with MDMA increased progressively. In the beginning of the 80's, MDMA was the main drug found in ecstasy tablets. Other amphetamine analogues, such as methamphetamine (Figure 1) and other psychoactive substances including ketamine have also been found in ecstasy tablets. Other drugs such as caffeine, amphetamine, lidocaine, and adulterants have been found in ecstasy tablets.

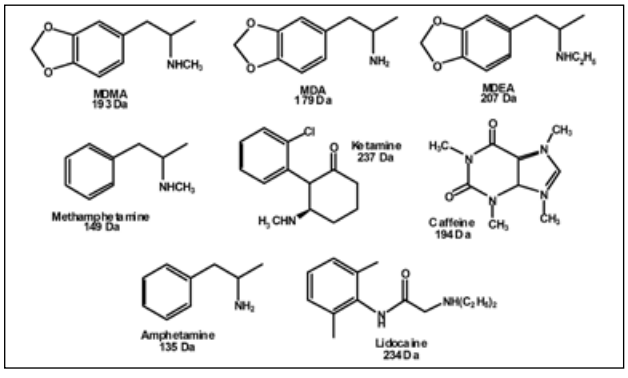

Figure 1. Structures and MW of drugs normally found in ecstasy tablets.

Forensic laboratories analyze ecstasy tablets mainly using ecstasy testing kits, which are often based on the Marquis or Simon tests and develop specific colors such as dark blue or black. These tests display, however, low selectivity leading sometimes to false-positives [2]. Additional techniques have therefore been employed to confirm the kit results such as gas chromatography (GC), GC coupled to mass spectrometry (GC-MS) [3], high performed liquid chromatography (HPLC) [4] and HPLC coupled to mass spectrometry (HPLC-MS) [5]. These instrumental techniques naturally require more skilled operators and are much more effort and time consuming.

Thin layer chromatography (TLC) is a classical, simple, low-cost, fast, and versatile separation technique [6] and has been widely used in forensic investigations. A variety of developing reagents are also available, such as ninhydrin and the Marquis reagent for anphetamines [7]. The main drawbacks of

TLC are limited resolving power and lack of a unquestionable method for structural characterization. Recently, a new class of ionization techniques for ambient mass spectrometry [8-11] has been developed. These techniques allow desorption, ionization, and MS characterization of analytes directly from their natural surfaces and matrixes [12], becoming therefore an attractive solution for direct characterization of TLC spots. Among these techniques, easy ambient sonic spray ionization (EASI) is likely the simplest, gentlest, and most easily implemented [13]. An EASI source can be constructed and installed in a few minutes from simple MS laboratory parts (Figure 2) requiring no voltages, no UV lights, no laser beams, no corona or glow discharges, and no heating, and as shown recently, even with no pumping systems [14]. EASI relies on the forces of a high velocity stream of N_2 (or even air) to accomplish analyte desorption and supersonic spray ionization (SSI) [15]. EASI has already been successfully tested with different analytes in different matrices and in various applications such as aging of ink writings on paper surfaces [16], perfumes [17], surfactants [18], biodiesel [19], propolis [20], cloth softeners [21]. EASI has been coupled to membrane introduction mass spectrometry [22], TLC [23], HPTLC [24] and has applied molecularly imprinted polymers as selective surfaces [25].

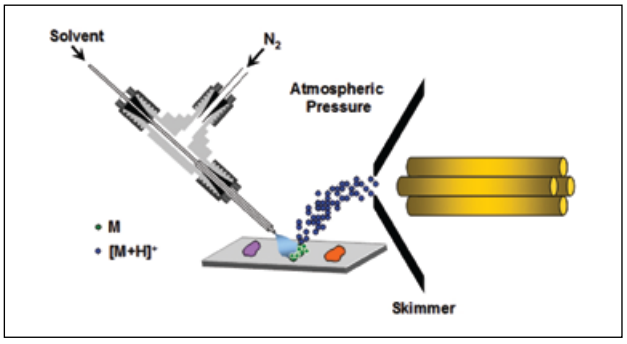

Figure 2. Schematic of TLC/EASI-MS. Supersonic spray produces a bypolar stream of very minute charged droplets (blue spray) that bombard the TLC silica surface causing desorption and ionization of the analyte molecules that rest on the target spot (green dots). Analytes are ionized often as $[M = H]^+$ or $[M - H)^-$, or both. EASI is assisted only by compressed nitrogen or air, and causes no oxidation, electrical, discharge, or heating interferences.

In this work, the coupling of TLC and EASI-MS has been tested in a "real world" forensic application. First, TLC separation has been optimized for seven standards of drugs normally found in street ecstasy tablets. A total of 25 street ecstasy tablets apprehended by the Rio de Janeiro Police Department were then analyzed by TLC/EASI-MS.

Experimental

Reagents and Samples

HPLC and P.A. grade methanol (CH_3OH), chloroform ($CHCl_3$), isopropanol ($CH_3CH(CH_3)OH$), acetic acid (CH_3COOH), and ammonium hydroxide (NH_4OH) were obtained from Merck. Twenty five street ecstasy tablets were provided by the Rio de Janeiro Civil Police. MDMA, MDEA, MDA, ketamine, caffeine, methamphetamine, and amphetamine standards solutions (1 mg mL^{-1}) were purchased from Radian (Austin, TX, USA).

Ecstasy Tablets

The ecstasy tablets were provided by the Carlos Éboli Institute of Criminalistic. The Rio de Janeiro police apprehended these tablets during the years of 2008 and 2009. The tablets displayed diameter, thickness, and weight of ca. 0.79 ± 0.11 cm, 0.44 ± 0.15 cm, and 260 ± 56 g, respectively, with a variety of shapes, logos, and colors. Tablets were pulverized and a 10 mg of the sample was partially dissolved in 10 ml of methanol. After centrifugation, the upper layer was transferred to a glass vial and analyzed by TLC.

TLC

Precoated plates (silica gel 60 GF 254, Merck, 6100 Darmstadt, Germany) were used. These plates were dried for 30 min at 80 ºC and then stored in a desiccator. A volume of ca 3 µl of a sample or standard solution were carefully applied to the TLC plate, which were developed in an horizontal chamber (Camag, Switzerland). The total developing distance was 8 cm. Four different solvent systems were tested as eluents: $CHCl_3/CH_3OH$ (50/50 v/v); $CHCl_3/CH_3OH/CH_3COOH$ (20/75/5 v/v); CH_3OH/NH_4OH (98/2 v/v); and $CH_3CH(CH_3)OH/NH_4OH$ (95/5 v/v). After experimental development, the plates were dried at 100 °C for 15 min. Spots were detected under ultraviolet (UV) radiation at 254 nm.

Limit of detection (LOD)

The LOD of MDMA in the TLC plates used was set as the minimum compound concentration that could be visualized by UV with an acceptable level of precision of ≤ 15% and accuracy of ± 15% in 10 replicates.

EASI-MS

Experiments were performed on a single quadrupole mass spectrometer (LCMS- 2010EV -Shimadzu Corp., Japan) equipped with a home-made EASI source, which is described in detail elsewhere [15]. Acidified methanol (0.1% in volume of formic acid) at a flow rate of 20 µL min^{-1} and compressed N_2 at a pressure of 100 *psi* were used to form the supersonic spray. The capillary-surface entrance angle was of ca 45°. Each TLC spot was directly analyzed by EASI-MS, without any sample preparation. Spectra were collected on each spot for about 10 s.

Gas Chromatography coupled to Mass Spectrometry (GC/MS).

GC/MS was conducted using a Thermo Scientific (Austin, Texas) Focus gas chromatograph coupled to an ITQ 700 Thermo mass selective detector. The mass spectra scan rate was 3 scans s^{-1}. The GC was operated in the splitless mode with a carrier gas (helium grade 5) flow rate of 1.5 mL min^{-1}. The mass spectrometer was operated using 70 eV electron ionization (EI) and a source temperature of 250 ºC. Both the GC injector and the transfer line were maintained at 250 ºC. The mass spectra

reported were obtained after background subtraction and by averaging ca five scans. Samples (caffeine standard solution and tablets) were diluted in HPLC grade methanol to give a final concentration of 1 mg mL^{-1}, and 1 µL was introduced via manual injection. The GC temperature program used consisted of an initial temperature of 130 °C for 1 min then increased to 280 °C at 17 °C min^{-1} and held for 11 min.

Results and Discussion

TLC separation of the seven common ecstasy tablet components was evaluated using four different solvent systems as eluents (Table I). $CHCl_3/CH_3OH$ (50/50 v/v) was inefficient since it caused spot tailing for most standards and ecstasy samples tested. $CHCl_3/CH_3OH/CH_3COOH$ (20/75/5 v/v) provided well defined spots for both the samples and standards, but MDMA, metamphetamine, amphetamine, and ketamine presented too close Rf values (0.62-0.71). The best results were obtained for $CH_3CH(CH_3)OH/CH_3OH$ (95/5 v/v) and, most particularly, for CH_3OH/NH_4OH (98/2 v/v) (Figure 3). Although close Rf values for MDMA (main drug expected in ecstasy tablets) and metamphetamine were observed, good separation and resolution was observed for MDA, MDEA, amphetamine, ketamine, and caffeine (Figure 3 and Table I).

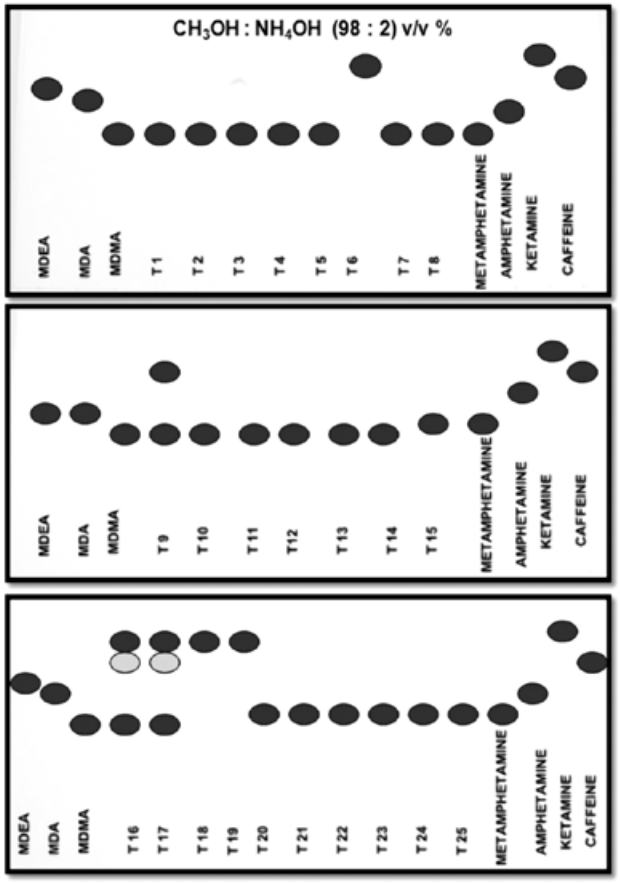

Figure 3. TLC data for the seven common ecstasy components tested as well as for the 25 samples of apprehended street ecstasy tablets using $CH_3OH:NH_4OH$ (98:2) v/v as the eluent. Spots developed by UV are represented by dark black or gray (less intense) ovals.

TLC/EASI-MS

For TLC, we selected therefore CH_3OH/NH_4OH (98:2) v/v as the best eluent and the components of each spot (Figure 3) were then subjected to desorption, ionization, and *m/z* measurements by EASI-MS in the positive ion mode using acidified methanol as the spray solvent.

Table I. Rf values for the seven drug standards as a function of different TLC eluents

Compound	$CHCl_3$: CH_3OH (50:50) v/v	$CHCl_3$: CH_3OH: CH_3COOH (75:20:5) v/v	CH_3OH: NH_4OH (98:2) v/v	$CH_3CH(CH_3)$ $OH:NH_4OH$ (95:5) v/v
MDEA	0.62	0.74	0.71	0.87
MDA	0.48	0.60	0.67	0.81
MDMA	0.37	0.64	0.56	0.62
Metamphetamine	0.35	0.62	0.57	0.62
Amphetamine	0.71	0.66	0.66	0.70
Ketamine	0.86	0.71	0.84	0.80
Caffeine	0.84	0.94	0.77	0.70

Figure 4 shows the "on-spot" EASI-MS acquired directly from the surface of the TLC spots of each of the seven standards used. Note the unambiguous characterization of each drug, mostly as a single ion (which facilitates spectra interpretation and analyte characterization) corresponding to their protonated molecules, that is, $[M + H]^+$. MDEA was the only drug that was also detected as $[MDEA + H_20 + H]^+$ and $[MDEA + Na]^+$. Signal-to-noise ratio was quite high for all standards except caffeine (Figure 4). LOD was evaluated for TLC of MDMA and found to be of 3 ± 0.3 µg. For the caffeine spot, the low sensitivity of EASI-MS seems to result from its polarity and high affinity to silica that hampered desorption from the TLC plate by the EASI droplets containing acidified methanol. We are currently searching for an EASI-spray solvent or mixture of solvents that could provide proper desorption and ionization of caffeine from the silica in TLC spots.

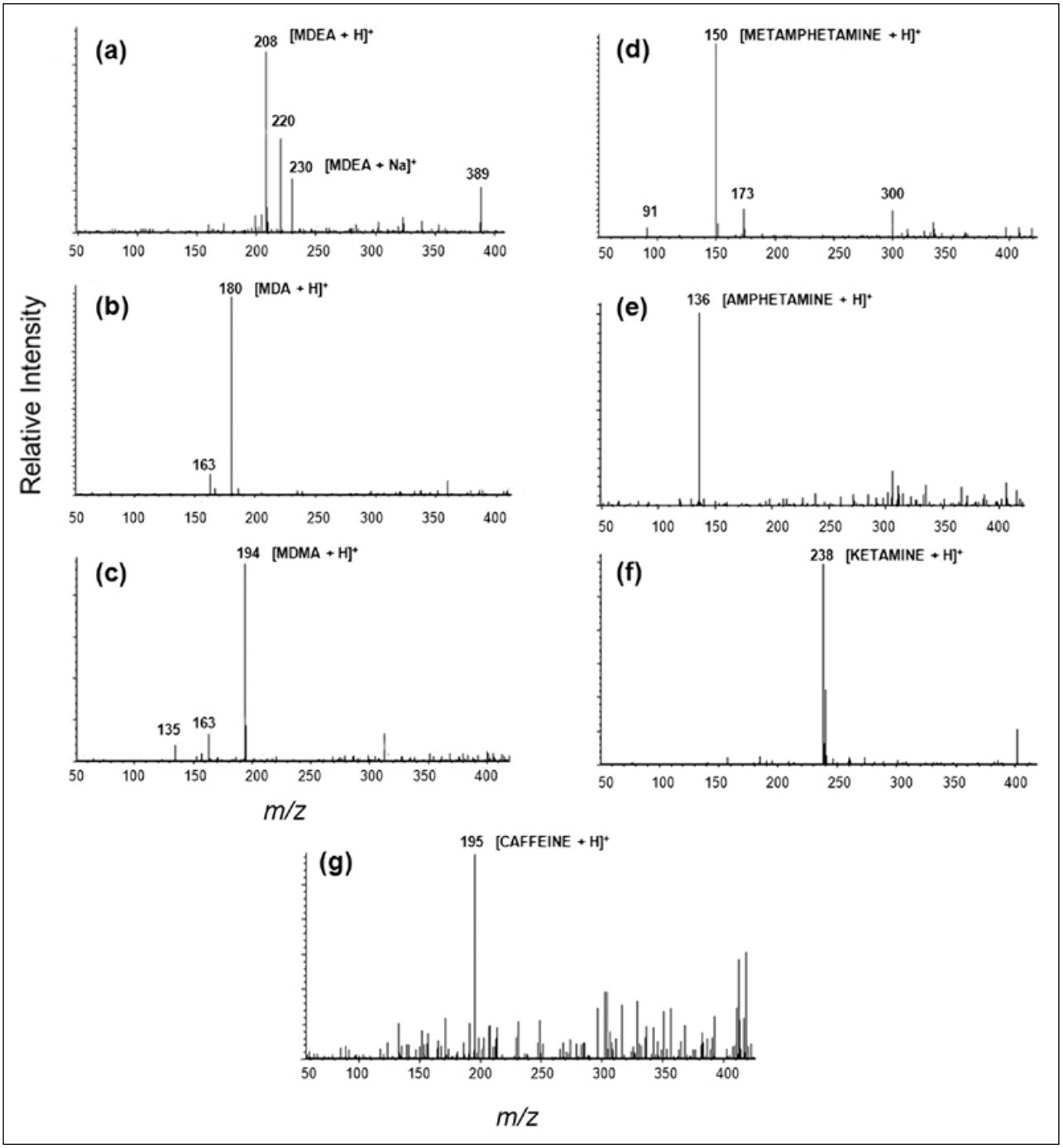

Figure 4. EASI-MS collected directly on the surface of the TLC spots corresponding to the seven common ecstasy components tested: (a) MDEA, (b) MDA, (c) MDMA, (d) metamphetamine, (e) amphetamine, (f) ketamine, and (g) caffeine.

Figure 5 shows the EASI-MS for the single TLC spot of sample T1 (Figure 3), a representative street sample of ecstasy. Note there could be doubt about the composition of this spot based on TLC alone, since both MDMA and metamphetamine displayed quite close Rf values (Figure 3). But the presence of MDMA (*m/z* 194) is unmistakably confirmed by EASI-MS. This result illustrates the importance of the TLC/EASI-MS coupling for rapid and unambiguous analysis of ecstasy tablets. Sample T6 also provided a dubious case since its single TLC spot, judging by the Rf value, could be interpreted as corresponding to either ketamine or caffeine. EASI-MS of this T6 spot (not shown) displayed very low overall abundance (mostly noise) and failed to detect therefore the intense protonated molecule of *m/z* 238 expected for ketamine (Figure 4). Since EASI-MS sensitivity to caffeine using acidified methanol as the spray solvent was found to be

very poor (Figure 4), the spot was assigned to caffeine. GC/MS data (not shown) confirmed that the main constituent of T6 was indeed caffeine.

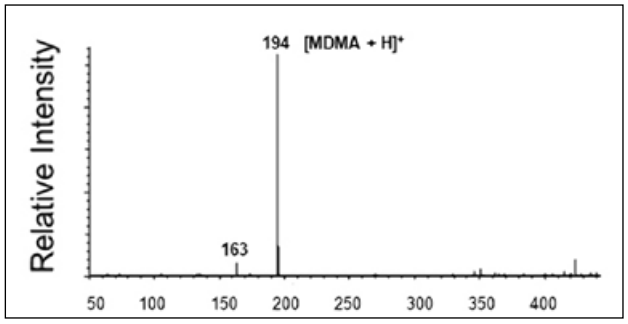

Figure 5. EASI-MS collected directly on the surface of the single TLC spot of sample T1.

Figure 3 shows that most ecstasy tablets displayed a single TLC spot with Rf values (and EASI-MS data) corresponding to MDMA. Tablets T18 and T19 displayed, however, a single spot corresponding, as far as only TLC and Rf values are concerned, to ketamine. But EASI-MS analysis for T18 (Figure 6) and T19 clearly points to an erroneous TLC attribution since the $[M + H]^+$ ion of *m/z* 235 indicates lidocaine as the main spot constituent. Both T18 and T19 samples displayed similar shape, logo, dimension, and mass indicating common origin.

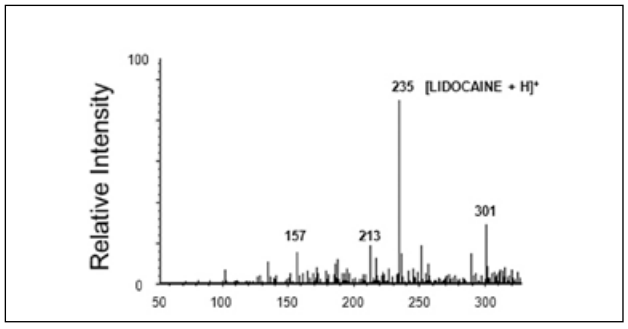

Figure 6. EASI-MS collected directly on the surface of the single TLC spot for tablet T18. A similar spectrum was collected for T19.

In contrast to most ecstasy tablets showing a single TLC spot, samples T9, T16 and T17 displayed two or three spots. Some of these spots (Figure 3) have Rf values corresponding to caffeine, and this attribution was confirmed by GC/MS (data not shown). A third spot observed for T16 and T17 with the highest Rf value could be attributed to ketamine. But again EASI-MS discarded ketamine, showing very low ion abundance and mostly noise. These spots were therefore labeled as "unknown". Tablets T16 and T17 also displayed similar shapes, logos and colors, indicating common origin.

Conclusions

Validation of methods used to detect drugs using TLC analysis is crucial to generate undisputable results, particularly in forensic investigations. TLC is a simple, low-cost, versatile, and popular technique used widely in forensic screening of illicit drugs, but may lead to false positives or erroneous attributions due to limited resolution and lack of an undisputable and selective method for structural characterization particularly for unexpected components. EASI-MS performed directly on the surface of TLC spots provides rapid and secure MS characterization. The coupling of TLC with EASI-MS constitutes therefore a valuable tool in forensic investigations, as demonstrated herein for a "real world" case involving the analysis of apprehended street ecstasy tablets. Although a few cases have required more elaborated GC/MS analysis, or a few spots remained identified, rapid screening of samples by TLC/EASI-MS provided secure identification for most samples, greatly speeding the overall analysis time and increasing its accuracy. EASI is the simplest ambient ionization technique currently available for ambient mass spectrometry [9], being easily implemented in all API mass spectrometers. Miniature mass spectrometers able to operate with ambient ionization techniques are also being made more compact and robust, and with diminishing costs [26, 27]. Therefore, the use of such compact and affordable instruments would allow widespread use of the EASI-MS technique in most forensic laboratories. TLC is also the simplest and the most popular separation technique in forensic investigations. The coupling of TLC to EASI-MS provides therefore a suitable technique for simple, rapid and secure forensic investigations. The favorable characteristics of TLC/EASI-MS indicate many advantageous applications in forensic analysis.

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Determination of Hg species in edible mushrooms using reversed phase-liquid chromatography-chemical vapor generation-inductively coupled plasma mass spectrometry

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Abstract

A method for Hg speciation in edible mushroom is described. Mercury species were determined using reversed phase liquid chromatography (LC) combined with chemical vapor generation (CVG) and inductively coupled plasma mass spectrometry (ICP-MS). Conventional extraction, ultrasound (US) and microwave (MW) radiation were evaluated for Hg species extraction using water, hydrochloric acid or L-cysteine solutions as extraction media. Extraction time, temperature (for MW extraction), concentration of HCl and L-cysteine solutions and US amplitude were investigated. Hg species interconversion was observed in HCl media, whereas the extraction was not quantitative if only water was used. Better results were obtained by using 1.0% (m/v) L-cysteine and US amplitude set at 10%. Accuracy was evaluated by the analysis of certified reference material (CRM) and analyte recovery from spiked samples. The agreement of the obtained results for CRM sample was in the range of 92 to 96% for methylmercury (CH_3Hg^+) and 95 to 102% for total Hg. Analyte recoveries from spiked samples were in the range of 93 to 109% for Hg^{2+} and 96 to 113% for CH_3Hg^+. Ultrasound assisted extraction was considered more efficient, simple and faster than conventional and MW assisted extraction. The proposed method was applied for Hg speciation in edible mushrooms and the main species was Hg^{2+}.

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Introduction

Edible mushroom may contain relatively high levels of Hg, which can be a result of natural uptake of the element due to its growing in polluted areas or use of Hg-based fungicides. Mushroom uptakes Hg and it has been used as bioindicator for environmental pollution for Hg and to assess the impact on public health.[1,2] Because of the health risks to Hg exposure, the United States Environmental Protection Agency (USEPA) established the reference value for methylmercury (CH_3Hg^+) as 0.1 μg kg^{-1} body weight/day. Additionally, the World Health Organization (WHO) set the tolerable value to 1.6 μg kg^{-1} body weight/week (0.23 μg kg^{-1} body weight/day). However, the maximum acceptable content of Hg in mushroom has not been established up to now.[3] All Hg species are cosidered toxic, while CH_3Hg^+ is one the most dangerous and usually present in the enviroment.[4] Therefore, the identification and quantification of Hg species in edible mushroom is of great interest.

Although improvements in instrumentation have been made in recent years, accurate Hg species determination may be difficult and non-quantitative analyte recovery and Hg species interconversion during sample preparation can occur.[5,6] Sample preparation is considered of primary concern in Hg speciation analysis and it needs to be carefuly evaluated for each kind of sample in order to avoid analyte losses, contamination and species interconversion.[7] Studies dealing with procedures of Hg extraction as well as extractant solutions are described in the literature.[5] Acid and alkaline solutions, thiol compounds, cysteine, thiosulfate and mercaptoethanol have been used.[7-11] Compounds containing sulfur are usually very effective owing to the high affinity of Hg to sulfur, which promotes Hg species releasing from sample matrix.[12,13]

Microwave (MW)[7,14-16] and ultrasound (US)[8,17] have been evaluated to promote Hg species extraction from

different biological matrices. Both systems promote and accelerate analyte extraction. The extraction efficiency varies with the irradiation time, temperature, characteristics of sample and extraction media. Usually, extraction using MW and US are advantageous in comparison to conventional extractions (mechanical shaking and heating) in terms of time, efficiency and reagent consumption.[6,8,18]

For Hg species determination, hyphenated techniques such as gas chromatography (GC)[14] and liquid chromatography (LC) coupled to a selective detector[19-21] have been frequently used. Most of LC methods are based on the use of silica-C_{18} as stationary phase, whereas the mobile phase contains methanol, or chelating or ion-pair reagent (especially 2-mercapthethanol or L-cysteine).[5,22] Liquid chromatography or GC coupled to inductively coupled plasma mass spectrometry (ICP-MS)[7,12,13,16,23] has been used for Hg speciation analysis and good limits of detection (LOD) and specificity are obtained. In addition, cold vapor generation (CVG) can be combined to LC-ICP-MS, wich improves the LOD.[15,24,25]

Although different methods have been proposed for Hg speciation in biological tissues, particularly in fish and seafood, little information is available about Hg speciation in edible mushroom. Thus, the main purpose of this study was to develop a method for Hg speciation in edible mushroom focused on sample preparation. Conventional extraction (mixture of sample plus extracting solution and let to stand) and also the use of US and MW radiation combined with different extractant solutions were studied. LC-CVG-ICP-MS was used for Hg species separation and quantification. Accuracy was evaluated using certified reference material and recovery tests.

Experimental

Instrumentation

The LC system used for mercury species separation consisted of a quaternary pump (Model Series 200, PerkinElmer, USA) equipped with a Rheodyne six-port injector valve, a 200 µL-sample loop and a silica-C_{18} column (Discovery C_{18} HPLC column, 250 mm x 4 mm, 5 µm, Supelco, USA). The mobile phase flow rate was 1.0 mL min^{-1} using isocratic conditions. The separation column was connected to a continuous cold vapor generation system. The Hg vapour produced was introduced directly into the ICP.

Mercury determination was carried out by means of an inductively coupled plasma mass spectrometer (PerkinElmer SCIEX, Model ELAN DRC II, Canada), equipped with a quartz torch (injector tube 2 mm i.d.) and platinum cones. Parameters of ICP-MS were adjusted in order to obtain the highest signal to background ratio for Hg (using ^{202}Hg). Operational conditions of the LC-CVG-ICP-MS system are summarized in Table I and a scheme of the proposed system is shown in Fig. 1.

Table I. LC-CVG-ICP-MS operational conditions

ICP-MS	
RF power, W	1250
Plasma gas flow-rate, L min^{-1}	15
Auxiliary gas flow-rate, L min^{-1}	1.2
Nebulizer gas flow-rate, L min^{-1}	1.10
Dwell time, ms	250
LC	
Column	C_{18} (250 mm x 4 mm, 5 µm)
Mobile phase (L-cysteine), % (m/v)	0.10 (pH 4.0)
Mobile phase flow rate, mL min^{-1}	1.0
Injected volume, µL	200
CVG-ICP-MS	
Carrier solution (HCl), mol L^{-1}	1.0 (8.0 ml min^{-1})
Reductant solution ($NaBH_4$), % (m/v)	0.2 (3.7 ml min^{-1})

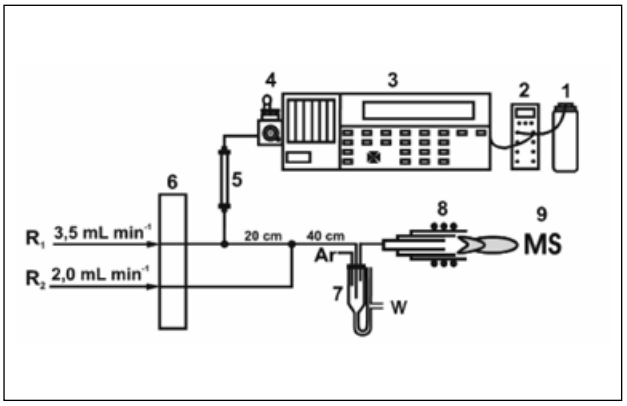

Figure 1. Squematic diagram of the LC-CVG-ICP-MS system. 1: mobile phase (L-cysteine); 2: vacuum degasser; 3: LC pump; 4: injector; 5: column (Si-C_{18}); 6: peristaltic pump; 7: gas-liquid separator (L = 10 cm; i.d. = 15 mm); 8: ICP; 9: mass spectrometer; R_1: HCl (1.0 mol L^{-1}); R_2: $NaBH_4$ (0.25% m/v).

A Multiwave 3000 microwave oven (Anton Paar, Austria) was used for sample digestion and mercury species extraction. A Fisher Sonic Dismembrator (Fisher Scientific, Model 100, 20 kHz, 100 W, USA) with an ultrasonic probe (1/8", full wave titanium probe solid, 127 mm long) was used for Hg species extraction. A pH meter (Metrohm, Switzerland) and a centrifuge (Nova Técnica, Brazil) were also used.

Reagents, standards and mobile phase

Distilled and deionized water was obtained by using a Milli-Q system (Millipore Corp., USA). Concentrated HNO_3 and HCl (Merck, Germany) were purified in a sub-boiling system (Milestone, Model Duopur, Italy). L-cysteine with purity higher than 98.5% (Vetec, Brazil) was used and solutions were prepared in water and used as extracting solution and as mobile phase. Sodium tetrahydroborate (Vetec,

Brazil) solutions (prepared in 0.2% NaOH - Merck) and ammonium hydroxide (Merck) were used for mercury reduction and pH adjustment, respectively.

A solution containing 1000 mg L^{-1} Hg (as Hg^{2+}) in 2% (v/v) HNO_3 was purchased from Merck. This solution was sequentially diluted in 1.0 mol L^{-1} HCl in order to prepare the reference solutions of Hg^{2+}. A solution containing 1000 mg L^{-1} of Hg (as CH_3HgCl) was prepared by dissolving CH_3HgCl salt (Aldrich, USA) in methanol. Intermediate solutions of Hg^{2+} and CH_3HgCl (1 mg L^{-1}) were prepared by dilution in 1.0 mol L^{-1} HCl. Working solutions used for calibration curve were prepared fresh daily by diluting the Hg stock solutions in the mobile phase solution. All the solutions were stored in polypropylene vessels, which were previously cleaned by immersion in 20% (v/v) HNO_3 solution by 24 h and rinsed with water and were kept at 4 °C in the dark before use.

The mobile phase used for mercury species separation by LC was adapted from reference [13] which contains 0.10% (m/v) L-cysteine at pH 4.0, adjusted with NH_4OH.

Sample preparation

Five species of edible mushroom were purchased at the local market and freeze-dried. Samples were ground in a cryogenic mill (SPEX, SamplePrep, USA), transferred to polypropylene flasks and stored 4 °C in the dark until analysis. Particle size of the powdered samples was lower than 100 µm.

Total mercury determination

About 500 mg of dried sample were transferred to quartz vessels of microwave oven and 6 mL of HNO_3 were added. The mixture was irradiated for 20 min at 1000 W (ramp of 10 min) and 0 W for 20 min (cooling step). The maximum temperature and pressure were 280 °C and 80 bar, respectively. After cooling, digests were transferred to graduated polypropylene vessels and the volume completed to 30 mL. Certified reference material (Dogfish Liver, DOLT-3 from National Research Council Canada - NRCC) and blanks were analyzed in parallel using the same conditions selected for samples. Total mercury concentration in digested samples was determined by CVG-ICP-MS.

Procedures for Hg species extraction

Ultrasound, microwave and conventional procedures were evaluated for Hg species extraction from CRM and mushroom samples. Standard Hg^{2+} and CH_3Hg^+ solutions addition were also tested in order to evaluate possible interconversion or losses of the Hg species.

About 500 mg of dried and powdered mushroom were accurately weighed and transferred to polypropylene vessels (15 mL) or quartz flasks of the microwave oven. Water (6 mL), HCl (6.0 mL of 1.0, 3.0 and 6.0 mol L^{-1}) and L-cysteine (6 mL from 0.1 to 3% m/v) were added to each aliquot of the sample. Subsequently, the mixture was submitted to the following treatments: a) shaken during 2 min and standing for 12 h, or b) US treatment up to 3 min using US amplitude of 10, 20 and 30%, or c) irradiation with microwave at 500 W for 5 min (ramp of 5 min) at 60, 80, 100 and 120 °C. In the following step, the mixture was transferred to polypropylene vessels and the pH adjusted to 4.0 using 10% (v/v) NH_4OH. Final volume of the mixture was completed to 20 mL with water. Then, the mixture was centrifuged by 10 min at 3000 rpm and filtered through a 0.45 µm-glass fiber filter. Aliquots of obtained solutions were immediately injected in the chromatograph. To evaluate the accuracy, analyte recovery tests and certified reference material were used. The certified sample and solutions spiked with the analytes were submitted to the same procedure used for the mushroom samples.

Results and discussion

Total Hg determination

Total Hg concentration in the certified sample and mushroom was determined by CVG-ICP-MS after digestion in microwave oven. The obtained results are summarized in Table II. No statistical difference was obtained between the results after microwave digestion and value of certified reference material. Total mercury found in mushroom samples was used as reference to the value found when the speciation of Hg was performed.

Mercury species extraction assisted by US

Effects of the extracting media composition (water, HCl and L-cystein), sonication at different US amplitudes, time of sonication, as well as the mass of sample were investigated. Solutions containing Hg^{2+}or CH_3Hg^+ or a mixture of both were evaluated in parallel to check possible interconversion of the Hg species. It was observed that Hg species interconversion occurred in HCl media, mainly when the mixture was sonicated for a period longer than 1 min (the period of sonication was extended up to 6 min). With higher HCl concentration (3.0 and 6.0 mol L^{-1}) CH_3Hg^+ was almost completely converted to Hg^{2+}, even using low US amplitude (10%) and time of sonication (1 min). With 1.0 mol L^{-1} HCl the interconversion of CH_3Hg^+ to Hg^{2+} was complete at 20% of US amplitude. These results are not in agreement with previously published results where US bath was used instead of US probe.[9] The main reason for the different results found in the present work could be due to the energy delivered to the solution that is higher when a US probe is used when compared to US baths.

No mercury species conversion was observed using water and L-cysteine medium. However, only L-cysteine was able to extract mercury species quantitatively from mushroom samples. It can be explained by the high affinity of Hg to the sulphydryl group of L-cysteine that improves Hg species extraction.[5] It was observed that Hg species were well extracted with 1.0% (m/v) L-cysteine under sonication at 10% US amplitude. In order to evaluate the effect of the sonication time, the concentration of L-cysteine solution (1% m/v) and the US amplitude (10%)

were kept constant, while the period of time of sonication was varied from 0.5 to 3 min. The effect of the time of sonication on Hg^{2+} and CH_3Hg^+ in standard solution was also evaluated where no analyte losses or interconversion of Hg species have occured. The same was observed for Hg in the certified sample and mushroom samples. Therefore, the time of sonication for Hg species extraction was kept in 1 min.

Due to the low concentration of Hg in mushroom samples (Table II), the effect of the sample mass on Hg species extraction was evaluated in order to improve the LOD of the method. Volumes of 1% (m/v) L-cysteine solution (1.0, 3.0 and 6.0 mL) were used for analyte extraction keeping the sample mass up to 500 mg. No mercury species interconversion was observed, but the analysis became difficult for volumes lower than 3.0 mL mainly due to the high viscosity of the mixture. Quantitative Hg species recovery was observed up to sample masses of 500 mg, using 6 mL of extractant solution. This condition was used for further experiments.

Mercury species extraction assisted by MW radiation

Temperature can affect Hg species stability and also analyte extraction. Therefore, the microwave oven conditions were adjusted for a maximum temperature of 120 °C using water, HCl and L-cysteine as extractants. Mercury species interconversion has occured in 6.0 mol L^{-1} HCl media, even at 60 °C. They were stable in 1.0 mol L^{-1} HCl in the investigated temperature range, while CH_3Hg^+ was converted in 3.0 mol L^{-1} HCl at temperatures higher than 100 °C. As cited, CH_3Hg^+ was stable in 1.0 mol L^{-1} HCl, but it was observed that the extraction of Hg species in mushroom and certified sample was not quantitative.

Concerning the other extractant solutions, Hg species interconversion in water was observed only at 120 °C, while no interconversion was observed in any condition for L-cysteine. Besides, L-cysteine has demonstrated to be the best media for Hg species extraction from mushroom. As previously mentioned, the good performance of L-cysteine is due to the strong afinity between sulfur and Hg.[26,27] According to the results obtained in this step of the study, 1% (m/v) L-cysteine, heating at 60 °C for 5 min (ramp of 5 min) were stablished for Hg species extration assisted by MW radiation.

Mercury species extraction by using conventional extraction

Conventional extraction of Hg species was evaluated by using the same sample mass, volume of extractants and respective concentrations used for US and MW procedures. The extracting solution was spiked with Hg^{2+} and CH_3Hg^+ and the mixture was let to stand up to 12 h at room temperature. It was observed that Hg species losses or interconversion have occurred only in 6.0 mol L^{-1} HCl. For Hg species extraction from the certified sample or mushroom, the mixture was manually shaken for 2 min and then allowed to stand for 12 h at room temperature.[13] However, as previously found extraction with 1% (m/v) L-cysteine solution was more efficient.

Determination of inorganic and methylmercury in mushrooms

In order to evaluate the accuracy of the extraction procedures, a certified reference sample was analyzed and recovery tests of Hg species performed. Results found in the speciation analysis step were also compared with the total Hg concentration found in the digested samples (Table II). For this purpose the sum of Hg species concentration was calculated and compared with the total Hg concentration in order to check the mass balance. According to Table II, the concentrations of Hg species found in the reference sample submitted to conventional extraction, or US or MW assisted were in good agreement with the certified values. However, when MW radiation was used for Hg species extraction from mushroom, the sum of the concentrations of Hg species was generally lower than the total Hg concentration determined by CVG-ICP-MS. In the case of conventional extraction and US application, the sum of Hg species was always higher than 92% of the total Hg found by CVG-ICP-MS.

Chromatograms obtained for samples submitted to the three different extraction procedures using 1% (m/v) L-cysteine are presented in Fig. 2. As can be seen, only Hg^{2+} and CH_3Hg^+ were detected in mushroom. The retention time for both Hg species was the same in mushroom and certified sample. It was also observed that complete separation of the Hg species was achieved in 5 min, for samples and standard solutions.

The concentration of mercury species was determined by external calibration, using peak area signal intensity for each Hg species. Calibration curves for both Hg species were linear up to 5.0 μg L^{-1} of Hg. Detection limits (based on 3σ of the baseline noise, in peak area) of Hg^{2+} and CH_3Hg^+ were 0.41 and 0.35 ng g^{-1} (as Hg), respectively. Detection limit was estimated considering 500 mg of sample in 20 mL extraction solution and a volume of 200 μL of solution injected in the chromatograph.

Conclusions

It was demonstrated that Hg species in HCl medium are easily interconverted or not quantitatively extracted by using conventional, US or MW assisted extraction. Hg species are more stable in water and L-cysteine. In water, CH_3Hg^+ is only converted to inorganic mercury when submitted to MW irradiation at temperature higher than 120 °C. No interconversion of Hg species occurs when US and 1.0% (m/v) L-cysteine were selected. In this case, Hg recovery from mushroom and certified sample (dogfish liver) was higher than 92%. However, recovery of Hg was only 62% when MW assisted extraction and L-cysteine 1% (v/v) were used. Therefore, it was concluded that US assisted extraction using L-cysteine can be recommended

for Hg speciation analysis in mushroom. Extraction of Hg species was quantitative, no interconversion of Hg species was observed and the time of extraction (1 min of sonication) was considered suitable for routine analysis.

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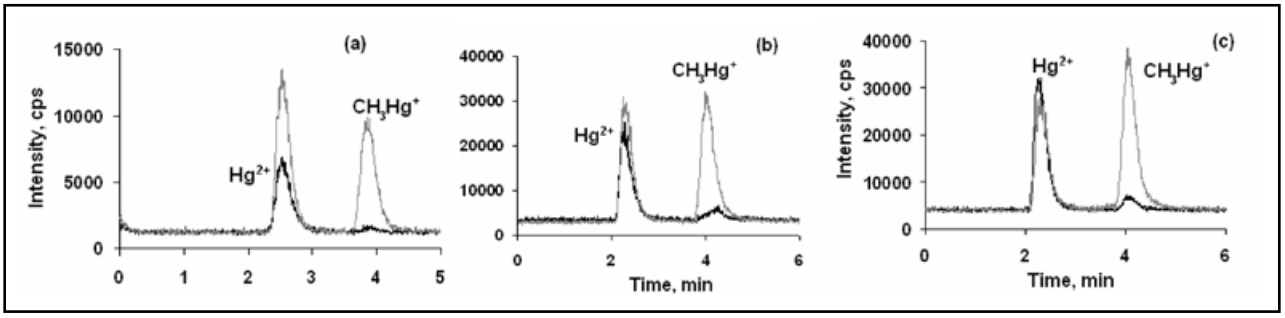

Figure 2. Chromatograms of a standard solution (1.0 µg L^{-1} Hg) and mushroom sample. (a) US assisted extraction; (b) MW assisted extraction; (c) conventional extraction. Gray line: standard solutions; black line: mushroom sample.

Table II. Concentration (in ng g-1) of mercury species and total mercury in mushroom and certified sample (DOLT-3) using conventional extraction, US and MW radiation with 1% (m/v) L-cysteine. Analyte measurements by LC-CVG-ICP-MS. Results are the average and standard deviation from 3 consecutive determinations.

	US (20%, 1 min)			MW (100 °C, 10 min)			Conventional Extraction			Digestion
Sample	CH_3Hg^{+**}	Hg^{2+}	Hg_{Total}^{***}	CH_3Hg^{+**}	Hg^{2+}	Hg_{Total}^{***}	CH_3Hg^{+**}	Hg^{2+}	Hg_{Total}^{***}	Hg_{Total}
Agaricus bisporus	4.85 ± 0.59	35.2 ± 0.1	40.0 ± 0.6 (100%)	7.73 ± 0.30	22.5 ± 0.1	30.2 ± 0.3 (76%)	4.21 ± 0.07	34.7 ± 1.3	38.9 ± 1.3 (97%)	39.9 ± 2.0
Pleurotus citrinopileatus	7.20 ± 0.96	19.1 ± 1.0	26.3 ± 1.4 (92%)	8.52 ± 1.30	17.0 ± 0.8	25.5 ± 1.5 (88%)	5.36 ± 0.47	20.2 ± 1.4	25.6 ± 1.5 (88%)	28.4 ± 1.2
Pleurotus eryingii	7.31 ± 0.33	10.6 ± 0.6	17.9 ± 0.5 (93%)	6.67 ± 0.25	8.39 ± 0.15	15.1 ± 0.3 (73%)	4.65 ± 0.51	18.6 ± 0.7	23.2 ± 0.9 (111%)	19.2 ± 2.3
Pleurotus ostreatus	5.18 ± 0.41	16.9 ± 1.43	22.1 ± 1.5 (92%)	3.20 ± 0.34	11.3 ± 0.4	14.5 ± 0.5 (59%)	1.95 ± 0.15	21.1 ± 1.6	23.0 ± 1.6 (94%)	24.4 ± 0.5
Pleurotus djamor	5.38 ± 0.32	17.9 ± 1.1	23.3 ± 1.2 (92%)	3.55 ± 0.53	7.15 ± 0.66	10.7 ± 0.8 (42%)	2.50 ± 0.33	22.5 ± 0.8	25.0 ± 0.9 (98%)	25.6 ± 0.8
DOLT-3*	1465 ± 47 (92%)	1646 ± 58	3111 ± 75 (95%)	1482 ± 90 (93%)	1646 ± 93	3128 ± 129 (96%)	1403 ± 46 (88%)	1917 ± 97	3320 ± 107 (102%)	3258 ± 85

* Certified value: Hg_{Total} 3370 ± 140 ng g^{-1}; CH_3Hg^+ 1590 ± 120 ng g^{-1}
** As Hg
*** Sum of CH_3Hg^+ e Hg^{2+} concentration

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Liquid chromatography for bioseparations: Fundamentals, developments and applications

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Abstract

This review describes recent developments in liquid chromatography separation techniques that focus on biomolecules (DNA, peptides, enzymes and proteins). Different modalities and materials are discussed in terms of reserved phase, ion exchange, size exclusion, affinity, and hydrophilic and hydrophobic interaction chromatographic modes. On-line or off-line bi-dimensional chromatographic separation modes as well as the latest developments are also discussed.

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1. Introduction

The identification, quantification and characterization of biomolecules are extremely challenging, with gel electrophoresis (GE), capillary electrophoresis and liquid chromatography (LC) commonly being employed for such tasks [1-3].

Although the complexity of the analytes, their dynamic ranges in the different samples, as well as their fragilities, LC is a powerful and popular technique because of its robustness, reproducibility, large number of chromatographic separation modalities and the possibility of hyphenation to other analytical techniques, offering advantages as the biomolecules (*i.e.,* proteins) can be separated under certain conditions in which biological activity is maintained [4]. Additionally, on-line or off-line LC separations as well as two-dimensional approaches can be carried out with different separation modalities [5], renders chromatography a versatile technique for biomolecule separation.

Thus, the dissemination and maturation of this tool for biomolecule analysis is crucial to the progress of proteomics, metabolomics and metalloproteomics, among others areas.

This review focuses on some modalities of LC separation for bioseparation purposes, including those applied for two-dimensional separations as well as the latest developments in separation modalities and chromatographic materials, including a diversity of examples in terms of DNA, peptides, enzymes and protein analyses.

2. Reversed phase chromatography

The reversed phase (RP) retention mechanism is the most widely used chromatographic approach, based on the relative hydrophobicity of proteins [6, 7]. The separation depends on the binding of a solute molecule present in the mobile phase to the hydrophobic ligands in the stationary phase. It is considered a powerful technique for the analysis of proteins and peptides due to a number of factors, including speed, sensitivity and good resolution, being able to resolve closely related molecules. Additionally, the flexibility that is given by the multiple mobile phases that are suitable for this chromatographic mode makes it possible to manipulate selectivity through these changes [8-10].

The HPLC analysis of intact proteins is typically carried out using silica-particulate RP columns, because porous silica packings can withstand high pressures, are compatible with most organic and aqueous mobile phase solvents, and offers high mass loadability [11, 12]. An experimental system for analysis of proteins usually consists of an *n*-alkyl-silica-based stationary phase from which the solutes are eluted with concentration gradients of water and organic solvents such as acetonitrile, both containing an ionic modifier such as trifluoracetic acid (TFA) or formic acid (FA) in the mobile phase, that can lead to improvement of the chromatographic efficiency [13, 14].

The extensive use of RP [8] for the purification of peptides and small polypeptides (molar masses < 10 kDa) has not been replicated to the same extent for larger molecules (molar masses > 10kDa) and for globular proteins. The structural features of these proteins produce undesirable adsorption effects, changing peak shapes in RP separations on silica-based sorbents, and leading to unsatisfactory resolutions or low recoveries. This happens mainly because the silanol groups are acidic and this ionic interaction results in an ion exchange retention mechanism. Problems increase with the size and/or the hydrophobicity of a protein because of the numerous interactions, therefore,

the silica-based columns used in RP separations of proteins demand high coverage on their surfaces and high inertness toward polar groups. The literature reports that the application of a C18 column for the separation of ewe milk proteins ranging from 14 to 66 kDa provides low resolution when it comes to the identification of isoforms [15]. Packings with shorter alkyl chain lengths, such as C4 phases, are favorable for hydrophobic proteins, because of the faster kinetic desorption between proteins and stationary phase [16], as demonstrated for the separation of histone isoforms with molar masses varying from 11 to 22 kDa [17].

The development of materials presenting macropores or gigapores (between 20 and 400 nm), aiming at the separation of high molar mass proteins or separation from ones presenting post-translational modifications should be highlighted. In the early 1990s [11] macroporous polymeric monolithic stationary phases were also introduced for protein separations as an alternative to classical packed-column technology. The favorable characteristics of monoliths include: simplicity of their in-situ preparation from liquid precursors and the possibility to create capillary columns; excellent robustness, since the monolith is one continuous structure that can be covalently linked to the column wall (no need for retaining frits); and the possibility to tune the morphology such that the best compromise between efficiency and permeability can be obtained [11]. The success of poly(styrene-co-divinylbenzene) monolithic columns for the RP gradient-elution separation of biomacromolecules has already been demonstrated. Good separations were also achieved using perfusion RP chromatography, with POROS R2/H commercial columns, packed with poly(styrene-divinylbenzene) particles, suitable for RP separation of biomolecules, and allowed intact soybean and maize proteins ranging from 5.1 to 45.8 kDa to be fractionated, providing data about similarities and differences between the studied samples [18].

As already pointed out, the combination of the traditionally used acidic buffering systems and hydrophobicity of the *n*-alkylsilica supports, which can result in denaturation of proteins, have often discouraged the use of RP methods for these separations. On the other hand, these same characteristics are considered by some authors a great opportunity to study the folding/unfolding processes and conformational stability of polypeptides and proteins in aquo-organic solvents and hydrophobic environments [10, 19, 20]. Using these characteristics McNay and Fernandez [10, 19] could find good correlations between protein unfolding and peak width and also showed that column pore size can cause conformational changes only on medium and high molar mass proteins.

3. Ion exchange chromatography

Ion exchange chromatography (IEX) is an efficient chromatographic mode for purifying or fractionating various biomolecules, which can be separated based on differences in their ionic charges [21]. The retention mechanism involves electrostatic attraction between charged analytes and oppositely charged functional groups on the stationary phase [6, 22, 23].

Proteins and other biomolecules are ionized to different extents, depending on the pH of a given solution. Furthermore, for any given protein, the pH at which the total net charge is equal to zero is known as isoelectric point (pI). So, under a set of defined mobile phase conditions, a mixture of proteins or peptides may be separated using ion exchange [22, 24]. Neutral molecules or molecules carrying the same charge as the ion exchange support are removed by washing, and charged biomolecules are recovered by elution with a buffer of either higher ionic strength or different pH conditions [25].

Several advantages in the use of IEX can be mentioned and include: high resolving power; fast separations; high recoveries (in general); non-denaturing buffer components. The process can also be used as a concentration step to recover proteins from a diluted solution. Besides, it can be a highly selective chromatographic technique, being able to resolve, for example, proteins that differ by only a single charged group [21].

Few disadvantages can be pointed out, and include the necessity to load the samples in the support under low ionic strength and controlled pH, which sometimes requires an extra buffer exchange step to be inserted. The instrumentation employed must be resistant to salt-induced corrosion so that, in some situations, a buffer exchange procedure is necessary to promote a reduction in salt concentration of recovered protein solutions. Also, high salt concentrations can lead to irreversible protein absorption to the resin, resulting in loss, as well as incompatibility with detection techniques such as MS [4, 25].

Ion exchangers are composed of a solid phase porous structure supporting ionizable chemical groups. Many stationary phases have been described and are classified into two categories: 1) anion exchangers, which have positively charged groups immobilized onto a chromatographic support, and will attract negatively charged analytes; and 2) cation exchangers, that have negatively immobilized groups, which attract positively charged analytes [26]. These techniques are often divided into two subtypes: strong (cation [SCX] and anion [SAX]) exchangers and weak (cation [WCX] and anion [WAX]) exchangers, depending on the type of functional group. Differentiation between weak and strong ionic groups is made on the basis of pH range over which the groups display their electric charge. Strong groups, typically containing sulphonic acid groups (cation exchange) or quaternary amino groups (anion exchange), are ionized at all working pH values, while weak groups can be either charged or uncharged, depending on the pH [6]. These functional groups are most commonly supported in functionalized silica and synthetic polymeric resins, although some other inorganic materials are sometimes

used. The major drawback to the use of a silica support is the pH limitation imposed by the instability of silica at high pH (above pH 8) and low pH (below pH 2). This question must be carefully controlled for proteins, since at pH lower than the pI the total net charge on the protein is positive and the protein would bind to a cation exchanger. At pHs higher than the pI, the total net charge is negative, and the protein would bind to an anion exchanger [25].

Innumerous applications, from analytical scale column chromatographic separations through preparative scale column separations at the industrial level can be achieved [27]. IEX has found use for both small and large biomolecules, ranging from peptides to proteins with molar mass up to 60 kDa, including immunoglobulins, plasmid DNAs and viruses [23, 28], and for all these applications the interactions with ionizable chemical moieties of these molecules are exploited, which allows to modulate the interaction with the ion exchange material according to the ionic strength and pH [22]. Also, the chosen elution conditions must allow the maintenance of the protein structure throughout the separation procedure.

Often used in combination with other separation techniques or chromatographic modes, IEX contributes to improvement in proteome coverage by facilitating the detection of low abundant proteins or specific classes of proteins through enrichment strategies [4]. Membrane proteins, which are often hard to be identified due to their uncommon characteristics such as alkaline pI, are included in this group [29, 30].

Das and co-workers [31] clearly demonstrate that the use of SCX as a first separation technique allowed the identification of considerably more proteins in HEK293T cells than when sodium dodecyl sulphate – polyacrilamide gel electrophoresis (SDS-PAGE) was used. In general, the authors identified 2.5 to 3 fold more proteins when using SCX and, considering only nuclear proteins, the difference increased to *ca.* 7 fold.

Some specific ion-exchange resins exhibit high resolution at relatively short retention times, while maintaining the biological activity of the molecule. Among them are latexed ion exchangers and tentacular materials, which are considered to be universally applicable due to their pH compatibility. The use of these materials minimizes hydrophobic interaction with proteins, a fact that results from decreasing protein retention with high salt concentrations in the mobile phase [32]. Tentacular materials can be used for the following samples: basic proteins, monoclonal antibodies [33], deamidation products, and protein isoforms, among others [32]. These stationary phases are being used for standard samples and, until now, applications for proteomic studies have not appeared in the literature, although they might be applicable in the future, due to the fact that tentacular ion exchange groups improve ionic interactions with biomolecules compared to usual stationary phases [34].

4. Size exclusion chromatography

Size exclusion chromatography (SEC) is another widely employed separation mode where macromolecules are separated according to their hydrodynamic radii (Stokes radii). It is generally used with the goal of providing a prior sample fractionation, eliminating interfering compounds and reducing sample complexity. For these purposes several matrices with different porosities/crosslinking degrees can be used as packing materials, including agarose, dextran or polyacrilamide, allowing the fractionation of peptides, proteins and protein complexes [35].

Ideally, interactions between proteins or peptides and the stationary phase should not occur [35] since they may limit the performance for some experiments, as demonstrated for the investigation of proteins in a molten-globule state using a dextran-based column [36]. In this specific case, proteins did not show a tightly packed interior resulting in non-specific interactions with the column, which resulted in changes in the expected retention times for different proteins [37]. Fortunately, this undesirable behavior can be minimized by controlling the separation conditions employed, such as ionic strength of the mobile phase [38, 39]. In other situations, interactions with the column packing material exist but do not cause limitations for the proposed experiments.

Among studies found in the literature, SEC can be employed as an interesting alternative at different steps of sample preparation procedures for samples analyzed using several analytical techniques. The manipulation of biological samples containing high salt concentrations, as well as the application of different precipitant agents containing high ionic strength do not allow the application of capillary electrophoresis or two-dimensional gel electrophoresis (2D-PAGE), especially the isoelectric focusing step, owing to the Joule effect. The presence of salt also prevents the ionization of molecules when analyzed through mass spectrometry techniques, especially when electrospray is used, resulting in the suppression of ionization of proteins and peptides as well as formation of an unstable electrospray process [40]. In these cases, SEC can be employed as a tool for electrolyte elimination and further protein purification [41] as well as in procedures to promote buffer exchange, including on-line settings [40], allowing subsequent protein solubilization using highly volatile buffers. It is noteworthy that the purification of proteins using SEC is a hard task due to the low selectivity of the separation technique resulting in separations with low resolution. The optimization of mobile phase composition using, for example, non-ionic surfactants [42], can be an alternative for minimizing this limitation.

The application of SEC in peptidomic studies, which corresponds to the analysis of endogenous peptides and their post-translational modifications, as well as the analysis of fragments of proteins and proteins of low molar mass, has also been highlighted in recent years due to the increased

interest of researchers when considering this fraction of the proteome [43]. In this case, proteins with molar masses higher than a desired value are eliminated, reducing sample complexity due to the elimination of potential interfering molecules, increasing the identification coverage after fractionation [44].

Applications of SEC related to the determination of molar mass of proteins can also be found in the literature. Generally, a calibration curve is plotted by correlating the retention volume of the proteins as a function of the logarithm of their molar masses. In this case, to determine the molar mass of a protein, the use of retention volumes, which deals with the molecules under a hydrodynamic point of view, is possible since it is directly related to the Stokes radii, which explores physical-chemical aspects. Unfortunately, these two parameters are not directly correlated with molar mass of proteins, what is currently done for constructing the calibration curve. It is observed that the Stokes radii (R) is related to the protein molar mass (M) through Equation 1, since the frictional ratio (f/f_0) and specific volume are taken into account ($\overline{v}$). Thus, when calibration curves are plotted, it is assumed that these two parameters are the same for all proteins, resulting in uncertainties that in some cases can reach 20% [45], as in the case of metallothioneins linked to different metallic ions [46]. To minimize these limitations, efforts have been made to estimate the uncertainty involved in molar mass determinations, using, for example, more than one detection system [47].

$$R = \left(\frac{f}{f_0}\right)\left(\frac{3\overline{v}\ M}{4\pi\ N}\right)^{1/3} \quad (1)$$

On the other hand, variations in predicted retention times can be used to obtain information related to the dimensions of macromolecules in a given solvent [48], to the degree of protein fragmentation [49], or even to the formation of aggregates [50-52], since the pressures applied during the separations are lower than in other chromatographic methods. For these purposes, the application of macromolecular characterization software, such as ASTRA, may be considered [53].

Still exploiting the lower pressure of SEC, this method can be employed for the analysis of metalloproteins, contributing to the evaluation of interactions involving proteins and metal ions [39]. In this situation, SEC is an important tool that promotes protein fractionation and allows determination of metal distribution in different sample fractions using mass spectrometry facilities [54].

5. Affinity chromatography

Affinity chromatography is widely applied for the separation of specific classes of proteins or peptides, to reduce the complexity of the matrix, as well as to eliminate interfering molecules, facilitating the handling of the sample during its preparation step [55].

One of the most widespread applications exploiting this principle of separation consists in the depletion of high abundant proteins [56]. When proteomics studies are developed using complex matrices (*e.g.* blood plasma samples), target proteins are usually in small concentrations. In this case, the presence of albumin and immunoglobulins makes the analysis more difficult by masking less abundant proteins [57]. Different chromatographic columns are commercially available for this purpose. Among them, the use of immobilized dyes, such as Cibracon Blue [58, 59], where low cost consists in the main advantage, can be highlighted. However, low specificity can be pointed out as the main limitation of this kind of column [60]. On the other hand, columns containing one or more immobilized polyclonal antibodies, such as the Multiple Affinity Removal System (MARS) commercialized by Agilent Technologies, can be another interesting alternative for depletion [61], and in this case the main advantage consists in its high specificity for removal of the target protein, despite its higher cost when compared with systems that employ dyes for depletion.

This chromatographic strategy can also be employed for evaluation of post-translational modifications [62]. In this case, proteins containing specific modifications (*e. g.,* phosphorylation, glycosylation) are partially purified, eliminating most of the macromolecules that are not of interest for a given study [63-65]. This procedure reduces the complexity of the sample, allowing further application of additional separation procedures, as well as mass spectrometry analysis, it is noteworthy that not all post-translational modifications can be studied using this strategy (*e.g.,* methylation of arginines) due to the smaller changes in the physical properties of the peptides [66].

Regarding the analytical development of new column packing materials, the isolation of glycosilated proteins can be mentioned as one of the most exploited topics. It consists in an efficient alternative to promote the selective enrichment of glycoproteins at low cost [67]. Here, resins containing immobilized sugar binding proteins, called lectins, can be employed for the enrichment of glycoproteins enabling their study in complex samples such as blood plasma [68]. However, limitations related to unspecific interactions involving peptides from high abundant proteins could also be observed.

Also exploiting affinity chromatography is Immobilized Metal Affinity Chromatography (IMAC). Initially proposed for the purification of proteins containing histidine tags [69], it is one of the most adopted strategies for the purification and / or enrichment of phosphoproteins and phosphopeptides [70], being used before the steps of protein precipitation or dialysis [71]. The popularity of this method can be attributed to its compatibility with other separation and characterization techniques such as LC-ESI MS [72, 73] and MALDI MS [74, 75], that are employed later.

In this chromatographic method the reversible, and ideally selective, adsorption of biomolecules that have affinity for the metallic ions immobilized on chelating agents conjugated to a solid support occurs [76]. Thus, phosphoproteins or phosphopeptides, that have spare electrons, can be coordinated with the chelate through electrostatic interactions involving unoccupied orbitals of metallic ions, as shown in Figure 1. The most widely used chelating agents consist in nitroloacetic acid (NTA) [77, 78] and iminodiacetic acid (IDA) [79, 80], immobilized on an uncharged support to circumvent unspecific interactions between proteins and the chromatographic column [81, 82]. Such compounds will chelate different metallic ions, being the most common Fe(III) and Ga(III) [81, 83-86]. However, depending on the required experimental separation conditions and sample complexity, different metallic ions can be used for this purpose, including Cu(II), Zn(II) and Al(III) [83, 87, 88]. The application of Zr (IV) as immobilized material on an uncharged metallic support [81] proved to be an interesting alternative for the enrichment of rat liver phosphopeptides, producing better results than those found with the more common materials.

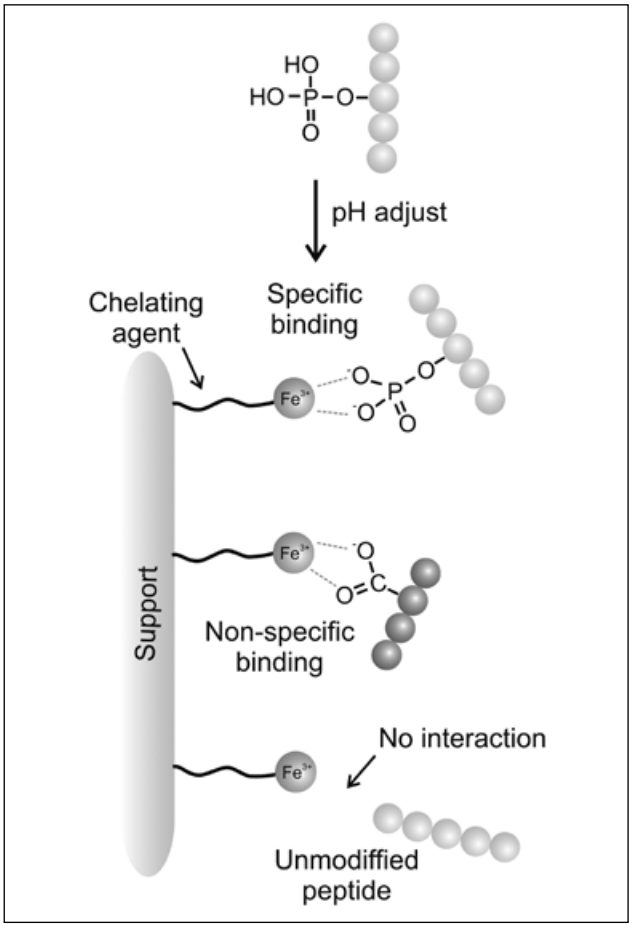

Figure 1. Illustration describing the occurrence of specific and non-specific interactions in an IMAC column. Specific biding occurs involving only the immobilized metal ion and phosphorilated peptides.

Several parameters influence the separations, including pH, ionic strength and mobile phase composition [71, 79]. These parameters modulate the electrostatic interactions involving biomolecules and the immobilized metal ions. However, even with the optimization of experimental parameters, it is common to observe non-specific interactions involving the packing material and amino acid side chains [80]. The presence of histidines [89, 90] or carboxyl groups [76] in the primary structure of a protein presents problems in studies that attempt to observe phosphorylations. On the other hand, this characteristic can be exploited as one of the main tools for the enrichment of recombinant proteins [71, 91].

The literature reports the esterification of carboxylic acids, leading to the formation of a methyl ester, as one of the most widely employed alternatives to circumvent the limitations found in phosphoproteomics studies that use IMAC as the separation alternative [92, 93]. However, the conditions for ester formation must be strictly controlled to avoid incomplete synthesis or side reactions that increase matrix complexity. The application of two-dimensional systems, using a strong cation exchanger for the separation at the first dimension [94, 95], can also be an interesting alternative for minimizing the problems of non-selective interactions with the IMAC column. In this case, separations must be carried out in acidic medium, since phosphorylated peptides will present charge +1 while the non-phosphorylated ones will be double charged [96].

An alternative to IMAC for the enrichment of phosphorylated peptides consists in the application of affinity chromatography using titanium dioxide. Initially proposed by Pinkse *et al.* [97] for the selective enrichment of phosphoproteins, the method exploits the selective interaction of phosphate groups with porous TiO_2 microspheres. The peptides are trapped in the column using an acidic solution and desorbed with an alkaline reagent. Unfortunately, non-specific interactions are also quite common when this strategy is adopted. However, the literature reports the use of additives to minimize the observed problems [98, 99], such the addition of 2,5-hydrobenzoic acid (DHB) to the mobile phase, which minimizes the adsorption of non-phosphorylated peptides owing to competition for the column sites. Ideally, the enrichment of phosphorylated peptides is not affected. Yu *et. al.* [95] demonstrated the efficiency of DHB and other additives to reduce the non-selective adsorption of peptides in the column. The authors studied aspects related to the concentrations of reagents, as well as presented interesting discussions based on conformational aspects.

Recently, the development of other metal oxide microspheres highlights the potential of these materials for the enrichment of phosphopeptides, and include the use of SnO_2 [100], Nb_2O_3 [101] or ZrO_2 [102].

6. Hydrophilic Interaction Liquid Chromatography (HILIC)

The acronym HILIC was first used in 1990 by Alpert [103], to describe a chromatographic mode that uses a hydrophilic stationary phase and hydrophobic mobile phase (mostly organic), suitable for separation of mixtures of proteins, peptides, amino acids, oligonucleotides and carbohydrates. This technique has been used since the 70's for the analysis of sugars and oligosaccharides [104].

This chromatographic mode is similar to normal phase (NP) chromatography, where the stationary phase is polar, and apolar solvents are used in the mobile phase, resulting in increased retention when the polarity of the analytes or stationary phases increases or/and the polarity of mobile phase decreases [105, 106]. However, unlike NP, where the aqueous part of the mobile phase is present at minimum amount, a water content higher than 5% is crucial for the HILIC retention mechanism [107]. Retention is believed to be caused by partitioning of the analyte between a water enriched layer of stagnant eluent on the stationary phase and a relatively hydrophobic bulk eluent, where the main component is organic, often 5-40% water in acetonitrile [104]. Other water-miscible organic solvents, such as methanol and isopropanol, have been tested for proteomics analysis, but resulted in poorer chromatography or no analyte retention [108].

The most employed stationary phases include underivatized silica, that contain functional groups such as siloxanes and silanols; hydrosilated silica (silica hydride); hybrid silica-organic phases. A large variety of silica-based or polymeric HILIC columns have already been designed [108, 109]. However, other polar chemically bounded stationary phases can also be used for these purposes and include amide-, diol-, and cyano-, polyethylene glycol, sulfoalkylbetaines, cyclodextrins and polypeptides, among others [104, 109].

An exponential growth in published papers on HILIC reflects the rising interest in this separation technique [110]. Among the reasons for this trend is the widespread use of mass spectrometry (MS) coupled to HPLC, as HILIC mobile phase compositions are well compatible with the most widespread MS ionization techniques and provides high sensitivity [111].

Recently, HILIC was introduced as one dimension for two-dimensional separations in proteomics analysis, specially coupled to RP chromatography [108]. On-line coupling of these stationary phases is considered difficult, because RP and HILIC mobile phases are not directly compatible [108], but recent work presented by Lam *et al.* [112] showed that this coupling in a micro-flow scheme is feasible. In this case, solvent incompatibility was overcome through the use of constant pressure online solvent mixing, allowing the system to perform efficient separations for both hydrophilic and hydrophobic compounds. This scheme generated near-identical coverage of peptides and glycopeptides for the characterization of proteins and glycoproteins when applying both on-line and off-line configurations. According to the authors, this simpler approach can be useful for detection of highly heterogeneous compounds.

Intact protein analyses in HILIC are not common, mostly because integral proteins are well separated by other chromatographic techniques, and also due to solubility limitations [104]. HILIC is often used for the separation of histones and their modified forms, which are quite conserved, highly basic proteins that organize and compact DNA in eukaryotes to form chromatin. In this specific case, RP chromatographic resolution depends mainly on hydrophobic interactions of the column material and hydrophobic regions of the proteins that have the most conserved domains, and not on polar regions that are less conserved between species, which can explain the insignificant change or influence in chromatographic behavior in RP even when extensive structural changes occur [104, 113]. Taking that characteristics into account, Sarg *et al.* [114] used a HILIC PolyCAT A column to detect micro sequence variations in histone subtypes isolated from various human tumor cell lines, that can affect important cellular functions *in vivo*, and demonstrated the high resolving power of this separation technique in the analysis of these proteins.

Although relatively new, this technique appears to solve separation problems that were difficult to handle in various fields, and has been considered, since the beginning, as useful as RP and IEX [103].

7. Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography exploits the reversible interaction of hydrophobic regions on the surface of proteins and large peptides, especially non-polar amino acids such as tryptophan, methionine and alanine, with a stationary phase containing hydrophobic immobilized ligands, such as phenyl, butyl and octyl groups [115]. This chromatographic mode is not as widespread as the others described earlier, due to its specific applicability, which is related to the separation of proteins with marked hydrophobic characteristics.

The main difference to distinguish between HIC and RP chromatography consists in the fact that HIC exploits the use of aqueous mobile phases containing moderate salt concentrations, especially cosmotropic ions. Thus, HIC consists in an excellent alternative for the separation of proteins that were previous precipitated using high ionic strength solutions, such as ammonium sulphate, or for samples previously fractionated using SCX, since the salt content is high in most situations [116].

Separations using HIC occur based on hydrophobic interactions between immobilized hydrophobic ligands and hydrophobic solvent-exposed regions of proteins, exploiting the salting-out effect. At the beginning of the separation process, the concentration of cosmotropic ions in the mobile phase is high enough to promote an elevation of stability of water/water interactions, due to the preferential solvation of these ions (which can be roughly con-

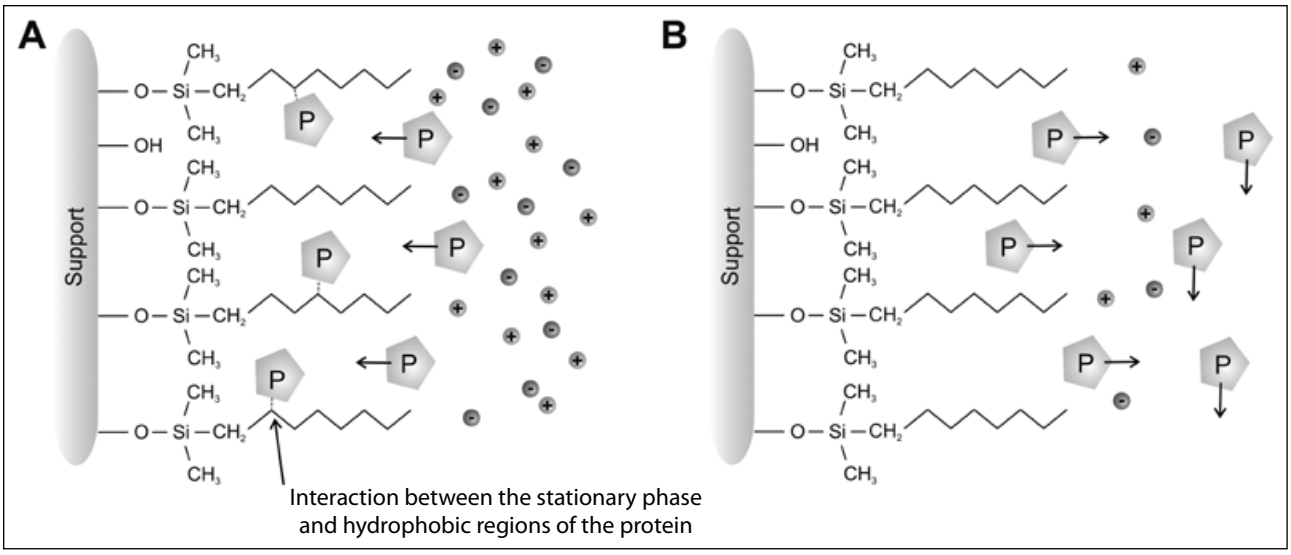

Figure 2. Schematic representation for the separation of hydrophobic proteins through HIC. (A) in the presence of high concentration of cosmotropic ions, and (B) after reducing the salt concentration, promoting the gradual elution of proteins.

sidered point charges). This process promotes the exclusion of water molecules from protein surfaces, resulting in effective interaction between macromolecules and the nonpolar stationary phase, as can be seen in Figure 2. As the salt concentration is gradually reduced, the interactions between the non-polar regions of proteins and the stationary phase decrease and the elution of proteins occurs [115]. Consequently, the type of ligand immobilized on the column support and its surface concentration are parameters that must be strictly selected to provide adequate separations. Columns containing lower substitution degrees may not provide adequate separation, while higher substitution degrees demand high salt concentrations, resulting in protein denaturation or precipitation [117].

Other parameters have significant influence for the separation of proteins using HIC and include solvent polarity (that also modulates the interaction of proteins with the chromatographic column), the type of salt (according to the lyotropic series), pH of mobile phase, temperature and the presence of additives in the mobile phase, such as chaotropic agents or surfactants [118, 119]. Regarding the use of additives, the literature shows that the addition of arginine in the mobile phase [120] can contribute to increase protein recovery and reduce its aggregation.

The recent literature reports the application of HIC in combination with other chromatographic methods especially for the purification of antibodies [121] and enzymes [122-124]. The fractionation of intact proteins in different matrices, including whey [125], fish tissue [126], vegetal material and derivatives [127, 128] and blood plasma [129] are also reported, as well as applications regarding the isolation of proteins containing post-translational modifications [130].

8. Two-dimensional separation strategies

The high complexity of biological matrices requires application of powerful tools for the separation or fractionation of proteins. In most studies, one-dimensional chromatographic systems are not selective enough to promote adequate separation of proteins and peptides. In these cases, the application of multidimensional systems consists in the alternative that promotes separations with greater selectivity. Considering that several studies require coupling with mass spectrometers, multidimensional separations play an important role to promote adequate sample fractionation, allowing the introduction of small protein fractions into the MS through time and enabling the ionization and further identification of biomolecules [56, 131]. It is important to emphasize that the selectivity of a multidimensional system increases when completely independent separation mechanisms are exploited in each chromatographic dimension (orthogonal separations). Moreover, the physical-chemical characteristics of proteins and column packaging material present significant influences in this aspect.

Multidimensional separations can be carried out through on-line and off-line approaches. Experiments that exploit off-line configurations can promote a greater fractionation of the sample, ideal for highly complex matrices. This strategy also allows reducing the interferences caused by the addition of salts into mass spectrometers. In this configuration, the eluent is usually monitored using an UV detector and several fractions are collected, lyophilized and dissolved in an appropriate buffer for further separation in a second dimension [132].

Considering on-line systems, proteins are gradually eluted from the first separation column and the eluent immediately flows to an interface, which generally contains

a trap column for protein concentration and mobile phase compatibilization. Then the proteins are eluted from the trap column and the separation is carried out in the second dimension. The main advantage provided by on-line systems consists in the reduction of protein losses along the separation stages, resulting in increases in protein identifications. Additionally, on-line configurations can also be considered less time consuming, since the separation procedure for a specific matrix has been previously optimized to maintain the orthogonality in the separation [132]. Different configurations can be adopted, as shown in Figure 3, to increase analytical frequency, concentrate protein fractions or allow a prior sample clean-up procedure.

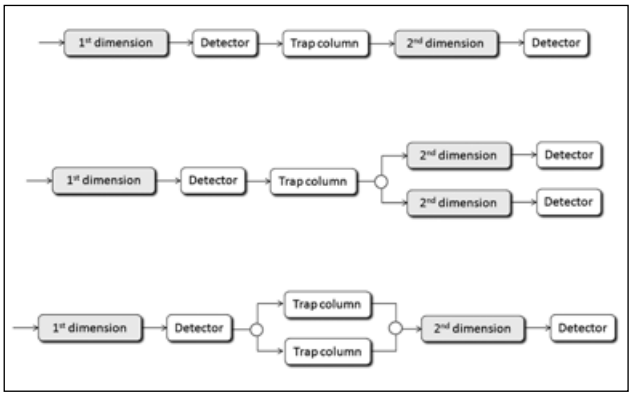

Figure 3. Different configurations employed for the separation of proteins using on-line two-dimensional chromatographic systems. The use of multiple trap columns or multiple columns for the separation in the second dimension allows increase in the analytical frequency or a higher sample fractionation during the separation in the first dimension.

Both on-line and off-line configurations can be adopted for the separation of biomolecules using two experimental approaches: top-down, in which intact proteins are fractionated using, *e.g.,* liquid chromatography, followed by their characterization; or bottom-up in which an enzymatic digestion step is initially performed followed by the fractionation and characterization of the obtained peptide mixture [56]. A widely employed bottom-up strategy is called Multidimensional Protein Identification Technology (MudPIT). This approach exploits the fractionation of protein digests using a strong cation exchange column followed by the separation of the fractions obtained using a reversed phase column [56].

The information summarized in Table I lists some recent papers that employ two-dimensional systems. Some aspects are highlighted, such as the target proteins or class of proteins, the mechanisms of separation adopted in the first and second dimensions, and the strategies adopted for the separation and detection of proteins.

9. Conclusions

Liquid chromatography has been consolidated as the most widespread alternative for the separation of protein mixtures from biological matrices. A good reason for that is the possibility to apply different chromatographic modes, where different characteristics of the samples can be taken into account for the separations, and there is also the possibility to use multidimensional configurations, allowing adequate protein fractionation for most situations. The combination of RP and IEX, especially when SCX columns are employed, has proven to be an efficient alternative for the fractionation of intact proteins and peptides, mainly when the characterization of complex mixtures is required. The application of on-line and off-line configurations can be considered for these purposes, and the adoption of each strategy depends on the number of collected fractions, the time required for the separation and the compatibility between mobile phases.

Independent of the adopted configuration, procedures focusing on the separation of peptides are preferentially used. In this case, a prior step focusing on enzymatic digestion of the proteins is performed, making manipulation of the hydrophobic fractions of the proteins easier and their further characterization through mass spectrometry techniques.

Also, efforts for the development of new materials should be pointed out, including affinity-based columns, which are often employed for the separation of specific classes of modified proteins or peptides, or monolithic columns with controlled porosity. However, in most cases, the application of these materials is still restricted to some specific research groups that work on the development of new stationary phases.

Finalizing, although liquid chromatography is known as one of the most used techniques in the identification of proteins, it is in constant development, and a great diversity of researches still being carried out in order to improve resolution, speed, among other factors, to help in proteomics studies.

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Table I. Recent applications of different two-dimensional chromatographic systems for the separation of intact proteins or protein digests. Information regarding the employed chromatographic mode for the separation in the first and second dimensions, as well as the adopted detection systems, are also shown.

Reference	Separated compounds	1st dimension	2nd dimension	Configuration; Detection
[98]	Endogenous phosphorilation in proteins from *Drosophila melanogaster*.	Affinity (TiO_2)	RP – C18	On-line; MS/MS, MS^3
[133]	Digest from a secretome of epithelial ovarian cancer cells.	SCX	RP – C18	Off-line; MS/MS
[134]	Digested cellular palmitoylated proteins previously purified with a streptavidin column.	SCX	RP – C18	On-line; MS/MS
[135]	Digest from rat hippocampal proteins.	SCX	RP – C18	Off-line; MS
[136]	Global protein expression in *Xanthomonas axonopodis* pv. *Citri*.	SCX	RP – C18	Off-line; MS/MS
[137]	Post-translational modified core histones.	WCX-HILIC	RP – C18	On-line; UV, MS/MS
[138]	Whole protein secreted by methicillin resistant *Staphylococcus aureus*.	SCX	RP – C18	On-line; MS
	Proteins from methicillin resistant *Staphylococcus aureus*		RP – C18	Off-line; MS/MS
[139]	Pegylated peptides in human plasma.	SCX	RP –Perfluorinated phenyl	On-line; MS/MS
[140]	Commercially available *Escherichia coli* protein sample.	Monolith – PS-DVB; pH 8.0	Monolith – PS-DVB; pH 2.0	Off-line; UV
[141]	Pooled human plasma proteins.	Affinity	RP – C18	On-line; UV,MS/MS
[142]	Digested *Nasonia vitripennis* venom extracts.	SCX	RP – C18	Off-line; MS/MS
[143]	Peptides obtained from secreted proteins of human thyroid cancer cells.	SCX	RP – C18	On-line; MS/MS
[144]	Manganese bound to low molar mass proteins and other molecules extracted from *Pinus pinea*.	SEC	SAX	Off-line; UV, MS/MS
[145]	Proteins extracted from *Saccharomyces cerevisiae*.	SEC	RP – C18	Off-line; UV, MS/MS
		SAX	SEC	Off-line; UV, MS/MS
		HIC	SEC	Off-line; UV, MS/MS
[146]	Human serum plasma.	WCX	HIC	On-line; UV
[147]	Separation of proteins extracted from human liver tissue.	SCX	RP-C8	Off-line; UV, MS/MS
[148]	Study of phosphorylation in histones H1.	RP – C18	HILIC	Off-line; UV
[149]	Present different configurations for the fractionation of wood protein digest.	SCX	RP – C18	On-line; MS/MS
				Off-line; MS/MS
[150]	Digested proteins from breast cancer cells.	SCX	RP – C18	Off-line; UV, MS/MS
[151]	Digested from bone tissue.	SCX	RP – C 18	On-line; MS/MS
[152]	Separation of phosphopeptides from *Hordeum vulgare* and *Arabidopsis thaliana* using different separation strategies for the evaluation of tonoplast proteins regulation.	SCX	IMAC	Off-line; MS/MS
			TiO_2	
[153]	Isolation of antifungal proteins from *P. edulis* (passion fruits) seeds.	SCX	HIC	Off-line

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Analysis of biodiesel microemulsions using ICP OES with axial configuration and argon-oxygen plasma

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Abstract

In this work an analytical procedure was developed for analysis of biodiesel microemulsions using inductively coupled plasma optical emission spectrometry (ICP OES) with axial configuration. Microemulsions were prepared using 0.5 mL of 20 % v v^{-1} HNO_3, 0.5 mL of Triton X-100, and 1.0 mL of biodiesel sample and diluted with n-propanol to a final volume of 10 mL. Oxygen addition to the composition of the auxiliary gas to reduce Swan band emissions and possible matrix effects was studied and the best conditions were reached at a flow rate of 165 mL min^{-1}. Nebulization gas flow rate for ICP OES was kept at 0.7 L min^{-1} based on both signal-to-background ratio (SBR) and signal intensities for Ca, K, Mg, Na, P and S determinations. Analytical performance was evaluated and good linear correlations ($r > 0.99$) were observed for several monitored emission lines. The accuracy of the procedure was checked by addition and recovery experiments for different types of biodiesel samples and recoveries ranged from 103.3 to 117.5, 93.5 to 113.5, 76.3 to 92.5 and 88.3 to 116.1% for Ca, Mg, P and S determinations, respectively. However, accuracy for Na and K determinations was not adequate, probably due to the complex matrix composition with high carbon content and the strong ionization of both analytes in the adopted plasma conditions.

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1. Introduction

Population growth, development of new technologies and the uncertainty about their availability have increased demands for fuels [1]. Moreover, environmental and health concerns have encouraged research to identify new alternative sources of energy, since continuous burning of fossil fuel can cause environmental contamination due to the high emission of the gases NO_x, SO_x, and CO_x and metals, such as Cd, Co, Cu, Pb, V, and Ni [2,3].

Biodiesel is an alternative for fossil sources of energy. It is basically a blend of alkyl esters produced from natural and renewable sources, such as different oleaginous seeds or grains and animal fats, mainly by a transesterification process. Several countries have made considerable investments in the production and quality control of biodiesel [4,5]. According to Ferreira *et al.*, addition of biodiesel in fossil diesel can significantly reduce the emission of pollutant gases, such as benzene, toluene, ethylbenzene, o-xylene, m-xylene and p-xylene [6].

Inorganic analysis of biodiesel is important to ensure the quality of this fuel since metals can promote deposition in engines that can degrade the performance of biodiesel-fueled engines and lead to problems [3,7]. Sodium and K are commonly found in biodiesel because both are used as catalysts in the transesterification processes. Calcium and Mg can be incorporated into biodiesel during the washing step [8].

Maximum contents of Ca, K, Mg, Na, P, and S in biodiesel have been established around the world, and European standard methods recommend the use of flame atomic absorption spectrometry (FAAS) (EN 14108 and EN 14109) or inductively coupled plasma optical emission spectrometry (ICP OES) (EN 14538), with samples diluted using an organic solvent, such as xylene or kerosene [9].

Sample preparation alternatives such as decomposition, microwave-assisted digestion and preparation of emulsions and microemulsions have been proposed to minimize problems related with fuel analysis, e. g. the toxicity of organic solvents, the need for preparing standard solutions in an organic medium and the relatively low stability of such standards [10,11].

Dry decomposition and microwave-assisted digestion provide a reduction of residual carbon content and sample viscosity and, especially when employing closed-vessel microwave ovens, losses of volatile elements and contamination can be avoided [10-12]. Oliveira *et al.* proposed a new procedure for Na determination using flame atomic emission spectrometry (F AES) and dry decomposition for sample

preparation. The developed procedure is simple and relatively inexpensive [13]. Lobo et al evaluated wet digestion using a focused-microwave oven and preparation of a microemulsion of biodiesel for Cd, Cu, Ni and Pb determination by graphite furnace atomic absorption spectrometry (GF AAS) with electrothermal atomization. Experimental design was used as a tool to optimize pyrolysis, and atomization temperatures and sample preparation procedures. According to the authors, for Cu and Pb determinations, sample preparation procedure was the most important step [14].

Emulsions and microemulsions have been proposed for the direct analysis of fuels, especially because both allow calibration using inorganic calibration standards [15,16]. An emulsion is defined as a heterogeneous system containing two liquid phases, one of them dispersed in the other by means of a mechanical process [17]. Elemental analysis of biodiesel emulsions was carried out by ICP OES with axial and radial configurations with yttrium employed as internal standard [18]. In this work, biodiesel samples were emulsified using Triton X-100 (polyoxyethylene (10) octylphenyl ether), nitric acid and water.

Alternatively, a microemulsion is a transparent, homogeneous and thermodynamically stable solution, probably due to the small dimensions of its droplets (between 5 and 100 nm) [17]. Jesus *et al.* also investigated a water-in-oil microemulsion preparation for the determination of Ca and Mg by FAAS. The main advantages, compared to the dilution method, were the use of inorganic calibration standards, high stability of analytes, and no need of carcinogenic organic solvents [19]. Recently, a microemulsion preparation using Triton X-100, 20 % v v^{-1} HNO_3 and n-propanol was employed to perform biodiesel trace analysis by inductively coupled plasma mass spectrometry (ICP-MS). Accuracy was achieved by adding oxygen in to the composition of the auxiliary gas However, sensitivity losses were observed due to the formation of oxides [20]. The addition of oxygen to plasma gases is useful for the analysis of organic samples for avoiding carbon deposits on the torch and pre-optical system, to reduce the background emission, the so called Swan bands, and to improve the stability of the plasma [20-22].

The goal of this work was to evaluate the feasibility of Ca, K, Mg, Na, P and S determinations using microemulsion as sample preparation and addition of oxygen in the composition of the auxiliary gas of the plasma when using an ICP OES with axial configuration.

2. Experimental

2.1 Instrumental

An ICP OES (Vista AX, Varian, Mulgrave, Australia) with axially viewed configuration and coupled charge device solid-state detector cooled at -35 °C by a Peltier system was employed to determine the metals, P and S. The thermostated echelle polychromator allowed spectral measurements in the 167 to 785 nm range. Argon (99.999 %) and oxygen (99.99 %) gases (White Martins, Sertãozinho, SP, Brazil) were used. The ICP OES instrumental parameters are presented in Table I. All measurements were carried out by monitoring four high intensity emission lines for each analyte (Table I). The microemulsions were homogenized by using a vortex mixer (Thermolyne type 37600 mixer, Dubuque, IA, USA).

Table I. Characteristics and instrument parameters used in ICP OES

Characteristics	Instruments conditions
Generator frequency (MHz)	40
Torch inner diameter (mm)	2.3
Sample introduction system	
Spray chamber	Cyclonic
Nebulizer	Concentric
Parameters	
RF applied power (kW)	1.4
Signal integration time (s)	1.0
Plasma gas-flow rate (L min^{-1})	15.0
Auxiliary gas-flow rate (L min^{-1})	1.5
Nebulizer gas-flow rate (L min^{-1})	0.9
Sample flow rate (L min^{-1})	0.8

Table II. Elements and emission lines monitored.

Element	λ (nm)	Element	λ (nm)
Ca II	317.933	Na I	568.263
Ca II	393.366	Na I	568.821
Ca II	396.847	Na I	588.995
Ca I	422.673	Na I	589.592
K I	404.414	P I	177.434
K I	404.721	P I	178.222
K I	766.491	P I	213.618
K I	769.897	P I	214.914
Mg II	279.553	S I	178.165
Mg II	279.800	S I	180.669
Mg II	280.270	S I	181.972
Mg I	285.213	S I	182.562

2.2 Chemicals and Reagents

All glassware and polypropylene flasks were washed, soaked in 10% v v^{-1} HNO_3 and rinsed with deionized water prior to use. All solutions were prepared using analytical-grade reagents. Water was distilled and deionized with a Milli-Q system (Millipore Corp., Bedford, MA, USA).

Microemulsions were prepared using HNO_3 (Merck, Darmstadt, Germany), previously purified using a sub-boiling system (Milestone, Sorisole, Italy). Triton X-100 (Acros, Somerville, NJ, USA), n-propanol and light mineral oil (Tedia, Rio de Janeiro, RJ, Brazil) were used without further purification. Inorganic reference solutions of Ca, K, Mg, Na, P and S for calibration were prepared by appropriate dilutions of mo-

noelemental stock solutions containing 1000 mg L^{-1} of each analyte (Tec-Lab, Hexis, São Paulo, SP, Brazil).

Biodiesel samples B100 obtained from different vegetable sources (African palm, castor beans, mix of palm and soybeans) were provided by the Center for Characterization and Development of Materials (CCDM), UFSCar and UNESP, São Carlos, SP, Brazil. Biodiesel B100 is considered pure, without any fossil diesel addition.

2.3 Microemulsion preparation

Microemulsions were prepared in polypropylene graduated flasks using 1.0 mL of biodiesel to which were added 0.5 mL of 20 % v v^{-1} HNO_3 plus 0.5 mL of Triton X-100. The volume was made up to 10.0 mL with n-propanol and the flash was homogenized using a vortex mixer during 2 min. Mineral oil was used for preparing calibration solutions. However, only a volume of 200 μL was added due to viscosity differences between biodiesel and mineral oil. Low volumes of inorganic stock solutions were added before forming the microemulsion, because the addition of a large volume of aqueous solution may destabilize it.

The volume of mineral oil added was based on previous tests using FAAS and it was observed that a volume of mineral oil 5-fold lower than the biodiesel volume could suitably simulate the biodiesel matrix medium. Probably, the major influence is caused by the effect of viscosity on sample nebulization, however effects caused by carbon emission should not be excluded. The preparation of biodiesel microemulsions using ethanol as co-solvent was also investigated, but a phase separation was observed in the microemulsion containing high concentrations of metals after resting for 15 min, probably due to polarity differences.

3. Results and discussion

3.1 Optimization of oxygen gas and nebulization gas flow rates

The effect of the oxygen gas flow rate added to the auxiliary argon gas was evaluated. It was observed that without using this gas and employing a cyclonic spray chamber with a concentric nebulizer, the emission of the Swan bands was increased, but the plasma was not extinguished. It should be emphasized that the use of a Sturman-Masters spray chamber with a V-groove nebulizer without adding oxygen to the plasma caused plasma extinction. This behavior could be explained due to the greater size of the drops produced with the V-groove nebulizer, since the inner diameters of the nebulizers used were between 150-400 μm for concentric and 500-800 μm for V-groove [23].

Oxygen gas was added to the auxiliary gas of the plasma from 4.5 to 205 mL min^{-1} and the results were evaluated by monitoring signal intensity and SBR (signal-to-background ratio) values. These experiments were carried out employing 1.4 kW of applied power and 0.7 L min^{-1} nebulizer gas flow rate since these conditions were suitable for complex samples considering plasma stability.

Although signal intensity and SBR values for each element presented no significant variations, 165 mL min^{-1} was adopted for the oxygen gas flow rate as a compromise condition due to diminution of the Swan bands. The Swan band emission could be visually observed by forming a green colored region in the plasma and it can cause high background signals and fluctuation of signal intensities; consequently, there is a deterioration of sensitivities and limits of detection.

The effect of nebulizer gas flow rate on signal intensities and SBR values was also evaluated in the range from 0.7 to 1.0 L min^{-1}. In these experiments, the oxygen gas flow rate was kept at 165 mL min^{-1}. Losses of signal intensities around 48, 51, 37, 15 and 30% for Ca, K, Mg, P and S, respectively, were observed when the nebulizer gas flow rate was increased. Probably, this behavior is a consequence of the low residence time of atoms in the plasma. The only exception was observed for Na, which presented a slight increase of signal. However, SBR values did not present significant variations. Therefore, 0.7 L min^{-1} was chosen for further experiments considering that it improved the sensitivity and neither perturbations of the plasma nor high Swan band emissions were observed.

3.2 Analytical figures of merit

Figures of merit for biodiesel microemulsion analysis employing ICP OES using an argon-oxygen plasma were evaluated under the optimized conditions in the microemulsion medium. Sensitivity, defined as the slope of the calibration curve, and limits of detection (LOD) are used to characterize an analytical procedure and these results are summarized in Table III.

Table III. Figures of merit of biodiesel microemulsion analysis.

Element / λ (nm)	Sensitivity (intensity L mg^{-1})	R	LOD[a] (mg L^{-1})
Ca 317.933	25247.8	0.9987	0.04
Ca 393.366	1156087.9	0.9927	0.04
Ca 396.847	1629670.2	0.9962	0.03
K 766.491	69371.5	0.9995	0.02
Mg 279.553	440518.5	0.9999	0.23 μg L^{-1}
Mg 279.800	2198.3	0.9988	0.20
Mg 285.213	17959.0	0.9990	0.04
Na 588.995	449254.1	0.9962	2.58
Na 589.592	268673.7	0.9975	2.17
P 178.222	77.6	0.9990	0.21
P 213.618	641.1	0.9993	0.03
P 214.914	118.9	0.9994	0.11
P 253.561	236.0	0.9994	0.06
S 180.669	132.0	0.9994	0.60
S 181.972	113.8	0.9997	0.21
S 182.562	42.9	0.9990	0.80

(a) Instrumental limits of detection

Limits of detection were calculated according to Thomsen *et al.* [24] using the background equivalent concentra-

tion (BEC). The BEC and LOD were calculated according to the following equations:

$$BEC = \frac{C_s}{SBR} \quad SBR = \frac{I_s - I_{blank}}{I_{blank}} \quad LOD = \frac{3 \times BEC \times R.S.D.}{100}$$

where, C_{rs} is the concentration of the multielement reference solution; *SBR* the signal-to-background ratio, I_{rs} and I_{blank}, the signal intensities for multielement reference and blank solutions, respectively, and *R.S.D.* the relative standard deviation for 10 measurements of the blank solution.

The analytical performance of the proposed method was evaluated for Ca (317.933, 393.366 and 396.847 nm), K (766.491 nm), Mg (279.553 and 279.213 nm), Na (588.995 and 589.592 nm), P (178.222, 213.618, 214.914 and 253.561 nm) and S (180.689, 181.972 and 182.562 nm). Some emission lines initially monitored were excluded due to the distorted profiles of the analytical signals and/or high background signals. Figure 1 (a,b) shows the emission spectra of Na and K, 568.263 and 769.897 nm, respectively.

Linear calibration curves, with correlation coefficients better than 0.99, were obtained for concentration ranges from the LOQ to 50 mg L^{-1}. The repeatability, calculated as the relative standard desviation (R.S.D.) for 10 consecutive measurements, varied from 1.53 to 6.76 %. Considering Brazilian legislation requirements (Resolução ANP no. 7, 19.03.2008, ANP – Agência Nacional do Petróleo, Gás Natural e Biocombustíveis), maximum allowed values in biodiesel samples is Na + K 5.0 mg kg^{-1}, Ca + Mg 5.0 mg kg^{-1}, P 10 mg kg^{-1}, and S 50 mg kg^{-1}. According to Table III, LODs were suitable for all elements, except for Na (588.995 and 589.592 nm).

3.3 Evaluation of accuracy

Since there is no adequate certified reference material for the present investigation, the accuracy of the procedure developed for inorganic analysis of biodiesel microemulsions prepared using n-propanol by ICP OES was assessed by addition and recovery tests. Recoveries for biodiesel samples produced from palm oil, castor oil, mixtures of palm and soybean oil, plus a sample of unknown source, are shown in Table IV.

According to Table IV, recoveries ranged from 103.3 to 117.5, 93.5 to 113.5, 76.3 to 92.5 and 88.3 to 116.1% for Ca, Mg, P and S determinations, respectively. Considering that the biodiesel microemulsion is a complex sample and that it contains high carbon concentrations, these results demonstrate the applicability of the developed procedure for direct biodiesel sample analysis.

For all other wavelengths monitored, positive and negative errors and recoveries as low as 20 or as high as 210 % were observed, indicating that these wavelengths were inadequate for monitoring analytical signals. Therefore, accuracy was not demonstrated for Na and K determinations.

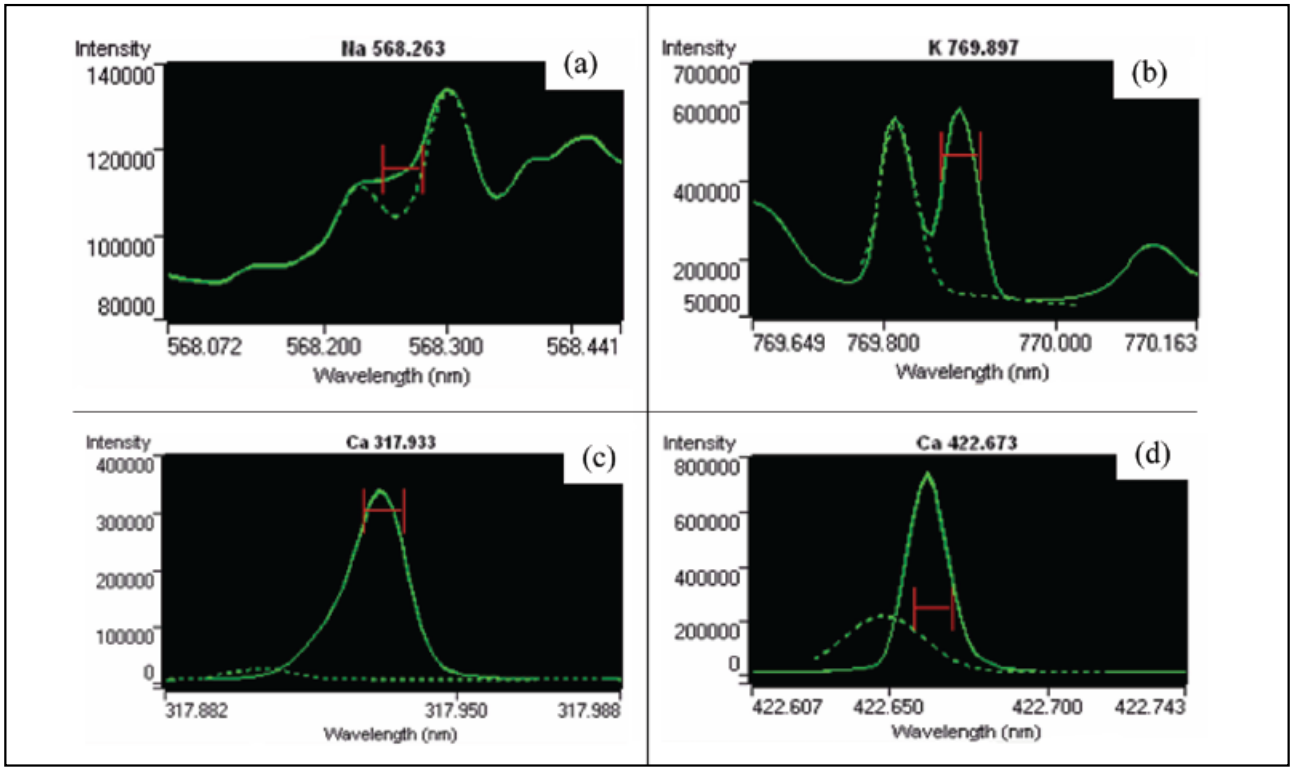

Figure 1. Emission spectra of Na (568.263 nm) (a), K (769.897 nm) (b), Ca (317.933 nm) (c) and Ca (422.673 nm) (d). The solid line represents the analyte signal emission and the dotted line the background signal.

Figure 1 (c,d) shows the spectrum of Ca at two wavelengths and, probably, the accuracy was not obtained at 422.673 nm due to background effects. The same behavior was observed for other tested emission lines.

Table IV. Addition-recovery experiments for biodiesel samples.

Element / λ (nm)	Added (mg L^{-1})	Recovery (%)				
		African palm oil	Castor beans	Mix of palm	Soybean	Unknown
Ca 317.933	1.0	109.8	103.3	117.5	105.4	113.1
Mg 279.553	1.0	107.9	102.5	113.5	104.0	112.6
Mg 280.270	1.0	98.5	93.6	103.1	95.4	102.1
Mg 285.213	1.0	98.7	93.5	95.0	97.5	98.1
P 214.914	2.0	88.2	87.9	95.2	85.7	87.7
P 253.561	2.0	92.5	76.3	91.0	82.8	91.3
S 181.972	5.0	116.1	102.4	112.7	93.3	110.8
S 180.669	5.0	108.0	97.5	106.3	104.9	88.3

The samples analyzed in this study presented Ca, Mg, P and S concentrations lower than the LODs established by ICP OES with axial configuration. The same biodiesel samples were also analyzed following the standard method indicated by Associação Brasileira de Norma Técnicas (ABNT NBR 15553), which recommends sample dilution in organic solvent, and Ca, K, Mg and Na were not detected. Calcium and Mg were also determined by FAAS using the same microemulsion sample preparation procedure and again concentration values were below the limits of detection for this method [25].

4. Conclusions

The procedure developed is an attractive alternative for Ca, Mg, P and S determinations due to simple and fast microemulsion formation and it avoids the use of organic solvents. It can be applied for determining these elements in biodiesel samples and external calibration may be performed using mineral oil and inorganic standards. However, addition of oxygen to the plasma is fundamental to reduce emission caused by the Swan bands and sufficient accuracy was not achieved for Na and K determinations by the proposed procedure.

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Determination of elements constituints of button cells

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Abstract
This work deals with the development of a method for determination of elements constituints of Zn-air, Li-MnO_2 and Zn-MnO_2 button cells. Samples of these button cells were analyzed with respect to As, Be, Cd, Co, Cr, Hg, Li, Mn, Ni, Pb, Tl and Zn concentrations. The concentrations of the elements were determined using inductively coupled plasma optical emission spectrometry (ICP OES). Pneumatic nebulization was used to introduce the sample solution in the ICP, except for As in all button cells and Hg in Li-MnO_2 button cells. Hydride generation (HG) and ICP OES were used for As determination whereas CV (cold vapour) and ICP OES were used for Hg determination. The button cell samples were decomposed with *aqua regia* under heating in closed PTFE flasks. Analyte recovery tests were used to evaluate precision and matrix effects. The concentrations of Cd, Hg and Pb found in the analyzed button cell samples were in accordance with the recommendations of the Brazilian Environmental Council (CONAMA). High levels of Hg were found in Zn-air and Zn-MnO_2 button cells. Toxic elements (As and Tl) not controlled by CONAMA were also found, mainly in Li-MnO_2 button cells.

Keywords: button cells, elements constituints, ICP OES, sample preparation

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1. Introduction

The growing production of smaller and cheaper electronic devices such as cameras, computers, calculators, watches, alarms and security systems has greatly increased the demand for small batteries (a set of cells grouped in series or parallel is nominated battery). According to data reported by the European Portable Battery Association (EPBA) [1], about 450 million of batteries were sold in 2009 in Europe. Accurate information about the number of batteries and button cells sold in Brazil does not exist, but around three billion of them are produced for domestic use per year [2].

In Europe, the recycling of batteries was about 40 tons in 2002, 70 tons in 2006 and more than 160 tons in 2008 [1]. The control of the disposal and destinations of batteries in Europe is regulated by EU directives [3] since 2008, which establish that all used batteries must be collected separately and recycled [1,3,4]. In Brazil, unfortunately, most batteries have been discharged in household waste and then in landfills [5]. Contact with water and other substances [6] will contribute to battery decomposition and to the leaking of toxic substances as a consequence.

A great number of illegally manufactured batteries are sold in Brazil [7], which do not follow standards in manufacturing and are not submitted to quality control. These batteries may contain much higher concentrations of toxic elements, which can be ten times higher than those found in batteries produced legally.

In 1999, the Brazilian Environmental Council (CONAMA) established maximum levels of Hg, Cd and Pb in batteries [8], provided guidelines for the disposal and destinations of used batteries and recognized their toxic potential to the environment (according to leaching tests already realized) [5,9]. A new resolution [10] was approved by CONAMA in 2008, which establishes that Hg, Cd and Pb concentrations in button cells and batteries must be less than 2.0%, 0.002% and 0.1%, in mass, respectively.

A variety of button cells are produced and comercialized, including Zn-air, Zn-Ag, Zn-HgO, Zn-MnO_2 (alkaline) and Li-MnO_2. Non-aqueous solvents are used in Li-MnO_2 button cells (due to the high reactivity of Li with water) whereas aqueous-alkaline media (NaOH/KOH) are used in the others [11]. Zinc used in button cells may be contaminated with toxic elements such as Cd, As and Hg at $\mu g\ g^{-1}$ level [12]. In addition, Hg is added to button cells in order to prevent leakage of hydrogen produced by electrochemical reaction. About 88% of the Hg found in municipal landfills may come from used batteries and electronic devices that contain Hg [11]. On the other hand, rare and valuable elements are used in new types of batteries. For example, indium salts are used in Hg-free batteries, increasing the demand for In (a rare and valuable metal) [13].

Methods dealing with determination of elements constituints of batteries and button cells are scarce. A method for determination of Hg, Cd and Pb in alkaline batteries was published by EPBA [14] in 1998. Guo and O'Hara [15]

adapted this method for Hg determination in button cells. According to this method, the button cells are mechanically opened and the different parts digested separately. Richter *et al.*[13] proposed digestion of the whole button cell in *aqua regia* with heating in microwave oven. The analysis is easier and faster in comparison to that corresponding to the EPBA method. Cold vapor (CV) in conjunction with atomic absorption spectrometry (AAS) or in conjuction with inductively coupled plasma optical emission spectrometry (ICP OES) [14,15], and ICP OES [13] were employed for the determination of Hg, while inductively coupled plasma mass spectrometry (ICP-MS) [13] and ICP OES [14,15] were for the determination of Cd and Pb.

Inductively coupled plasma optical emission spectrometry, due to its high sensitivity and multielemental features, is appropriate for determination of trace, minor and major elements in batteries. However, the respective matrices are quite complex and the solution obtained from sample decomposition has a high content of dissolved salts. Therefore, appropriate nebulizers for solutions with high contents of dissolved solids are required. Besides, spectral and non spectral interferences need to be evaluated for each type of battery analyzed. Chemical vapor generation (that encompasses CV and hydride generation (HG) is recommended for Hg and hydride forming elements. It not only reduces interferences because of matrix separation, but increases sensitivity due to higher sample transport efficiency to the ICP, in comparison to nebulization systems.

In the present work, As, Be, Cd, Co, Cr, Hg, Li, Mn, Ni, Pb, Tl and Zn are measured in button cells commercialized in Brazil. Chemical vapor generation and ICP OES are used for As and Hg determination, whereas ICP OES and pneumatic nebulization are used for the other elements. Different sample decomposition procedures are investigated. The main purpose is to investigate if commercialized and discharged button cells follow the Brazilian Legislation (CONAMA) [10] regarding to Hg, Cd and Pb concentrations. Additional elements (Tl, As and Be) are investigated in view of their toxicities. Elements such as Mn, Li, Co, Ni, Cr and Zn are determined in order to check the button cells type and respective matrix, which may interfere in trace elements determination.

2. Experimental

2.1. Instrumentation

All elements were determined using an ICP OES spectrometer (Optima 2000 DV™) from PerkinElmer. The parameters used are shown in Table I.

A chemical vapour generation system (described elsewhere [16]) was employed for As and Hg determination. Conventional pneumatic nebulization was used for the other elements (including Hg in Zn-air and Zn-MnO_2 button cells, where the element concentration was high).

Table I. ICP OES parameters and accessories employed

Parameters and accessories	ICP OES
Plasma power	1500 W
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	0.2 L min^{-1}
Nebulizer gas	0.6 - 0.8 L min^{-1}
Purge gas	2.5 mL min^{-1}
Spray Chamber	Cyclonic
Nebulizer	GemCone®
Injector tube	Alumina (2 mm i.d.)
Resolution	High
Signal processing	Peak area (7 points per peak)
Sample flow rate	2.0 mL min^{-1}
Replicates	2
Plasma view	Axial
Emission line[a] (nm)	As(I)[b], 193.696; Be(II), 313.107; Cd(I), 228.802; Co(II), 228.616; Cr(II), 267.716; Hg(II)[c], 194.168; Li(I), 670.784; Mn(II), 257.610; Ni(II), 231.604; Pb(II), 220.353; Tl(I), 276.787 and Zn(II), 206.200

(a): (I) and (II) refers to atomic and ionic lines, respectively; (b): chemical vapor generation was used; (C): chemical vapour generation and pneumatic nebulization were used (the chemical vapour generation system used is described elsewhere [16]).

2.2 Materials and reagents

Commercial argon (White Martins/Praxair, Brazil) was used as nebulizer gas, plasma gas and auxiliary gas in ICP OES. Argon was also used as carrier gas in the chemical vapour generation system. Nitrogen (99.996%) (from White Martins/Praxair) was used as purge gas in the optical system of the ICP OES spectrometer. High purity water (resistivity of 18.2 MΩ cm), obtained from a Milli-Q system (Millipore Corp., Bedford, MA, USA) was used for preparation of samples and solutions. All glassware and plasticware used were decontaminated by contact with 10% (v/v) HNO_3 for 48 h and subsequent washing with high purity water.

For preparation of samples and calibration solutions, HNO_3 (65% in mass) and HCl (37% in mass) (both from Merck, Darmstadt, Germany) were used. A 0.5% (m/v) $NaBH_4$ (Vetec, Rio de Janeiro, Brazil) solution in 0.03% (m/v) NaOH (Merck) and 20% (v/v) HCl (Merck) were employed for As determination using HG and-ICP OES. For Hg determination using CV, the $NaBH_4$ solution concentration was 0.05% (m/v), established according to previous papers [17,18]. This solution was prepared fresh daily. The flow rate of sample, HCl and $NaBH_4$ solutions used in the chemical vapour generation system were 1.0, 1.5 and 1.8 mL min^{-1}, respectively.

Calibration solutions of As, Cr, Hg, Mn, Ni and Zn were prepared from appropriate dilution of 1000 mg L^{-1} monoelement standard solutions of the elements (Titrisol/Merck). Calibration solutions of Be, Cd, Co, Li,

Pb and Tl were prepared by serial dilution of a multielement stock solution (Plasma Cal SCP33MS from SCP Science, Montreal, Canada) containing 10 mg L^{-1} of each element. The concentration range of the calibration curves are shown in Table II. External calibration was used throughout the work.

A metallic block equipped with a temperature controller (model TE-007d from Tecnal, Piracicaba, São Paulo, Brazil) and polytetrafluoroethylene (PTFE) flasks (capacity for 50 mL of solution, with screw caps) was used for button cells decomposition. A hot plate (model 0690003 cells from Presto, Eau Claire, WI, USA) was also employed for that purpose.

2.3 Samples and sample preparation

New or spent button cells were purchased or obtained in establishments that sell and collect button cell batteries (in Porto Alegre, RS, Brazil). The mass of the Zn-air and Zn-MnO_2 button cells ranged from 0.3 to 0.9 g. They were analyzed entirely, without any previous mechanical treatment. The mass of the Li-MnO_2 button cells was aproximately 2.8 g. These button cells were cut symmetrically in 3 or 4 parts in order to obtain pieces with mass around to 0.7 or 0.9 g. The number of button cells analyzed was 45 (24 were Zn-air (samples 1, 2, 3 4, 5, 6, 7 and 8), 2 were Li-MnO_2 (samples 9 and 10) and 15 were Zn-MnO_2 (samples 11, 12, 13, 14 and 15). Each sample corresponded to three button cell of the same batch; those button cells having the same identification given by the manufacturer were considered from the same batch. The type of button cells analyzed, sample identification and respective mass are summarized in Figure 1. In addition, 27 Zn-air button cells were used to evaluate decomposition procedures (Table II).

Three procedures (A, B and C) of decomposition were investigated by varying the amount of acid added and the type of system employed (open or closed vessel). Figure 2 summarizes the procedures investigated.

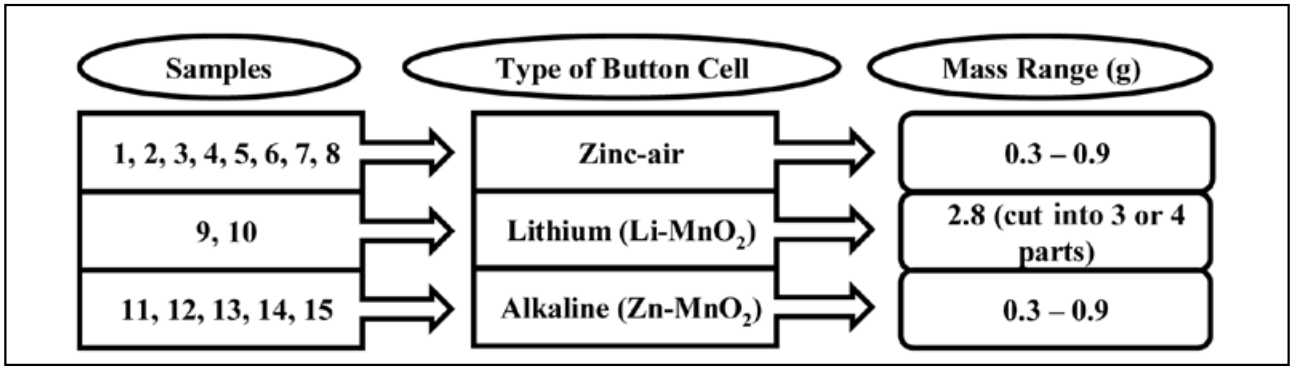

Figure 1. Identification of the button cells analyzed and respective mass range. Each sample corresponds to three button cell of the same batch.

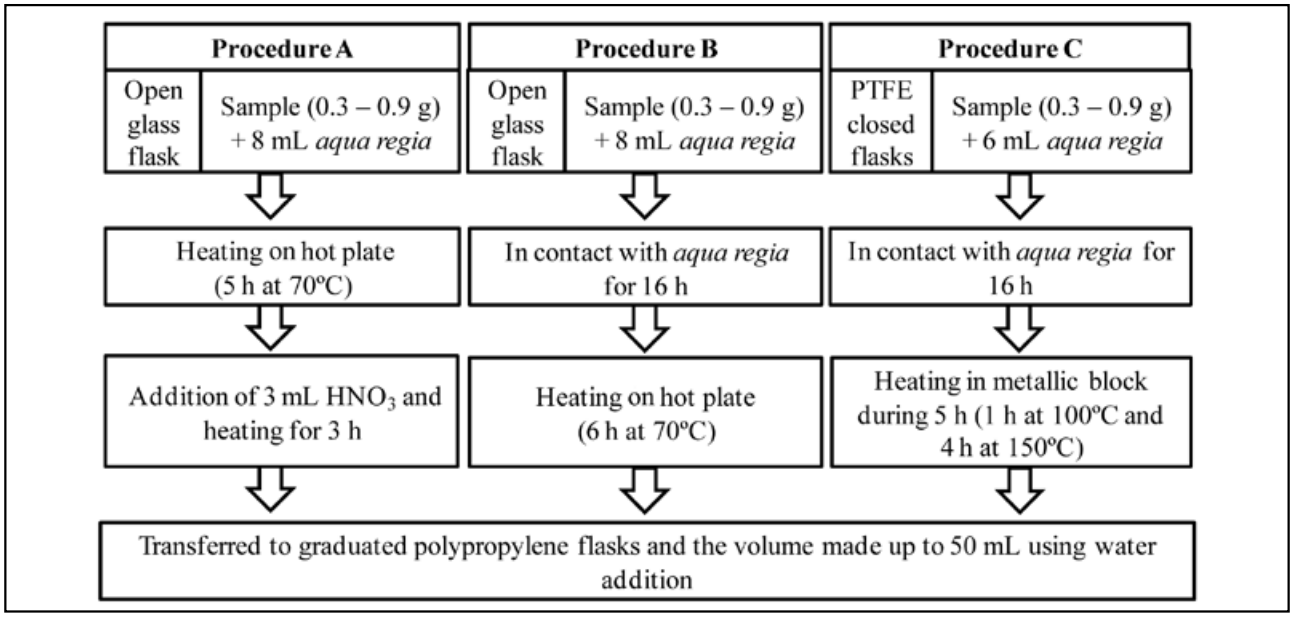

Figure 2. Summary of procedures investigated for button cells decomposition (Zn-air button cells were used).

For decomposition in closed flask (procedure C) the amount of *aqua regia* was reduced in order to avoid overpressure inside the flask. The final sample solution was further diluted for analyte quantification (50-100 times for trace and minor elements and 100-2000 times for major elements).

Recovery tests were carried out in order to evaluate matrix interference and the precision of the results for each type of button cell analyzed. To do so, solutions of the decomposed button cell samples (each sample consisting of three button cells of the same batch, each button cell in 50 mL of solution) were spiked with the analytes, or the solutions were diluted 10, 25, 50, 75 and 100 times before spiking. The amount of spike varied for each analyte, in order to obtain concentrations that were twice that existing in the sample solutions. In the case of a non-detected element, the spike should produce a solution with concentration corresponding to the second point of the calibration curve of the respective element.

3. Results and discussion

3.1 Sample decomposition

With respect to the sample decomposition procedures evaluated, analyte recovery and the presence of insoluble residues were ultimately considered. Best results were obtained using procedure C (Figure 2). In this case, very low amount or no insoluble residues were observed in the solutions of the decomposed samples, in comparison with procedures A and B. This demonstrated that a high oxidizing medium and long period of contact with *aqua regia* were necessary to decompose the button cells. Additionally, analyte recovery in the spiked solutions of samples decomposed by using procedure C was close to 100%. In all sample solutions obtained using procedures A, B and C a small disc of plastic used to separate the anode from the cathode was observed [13]. However, it did not preclude the analyte quantification.

The results obtained for the button cells decomposed under different conditions (Figure 2) are shown in Table II where it can be observed that the element concentrations vary in button cells from different batches that were submitted to the same decomposition treatment. In some cases, the analyte concentration differs in button cells of the same batch, which can be seen through the standard deviation (see Mn, Ni and Pb in A_1). The differences observed for the three button cells corresponding to A_1 could be attributed to inadequate decomposition. However, remarkable differences are not observed for those corresponding to A_2 and A_3, submitted to the same decomposition procedure as those of A_1. It is presumed that the three button cells corresponding to A_1 could not be from the same batch as indicated by the manufacturer or counterfeit button cells may have been mixed in.

Table II. Element concentrations found in Zn-air button cells submitted to different decomposition procedures (A, B and C). The values shown are the mean (in bold) and the standard deviation (in parenthesis) of three determinations (for each sample - A_1 to C_9 - three button cells from the same batch were analyzed in pararell). Each sample corresponds to a different batch.

Method/ Sample Mass	As[a]	Co[a]	Cr[b]	Hg[b]	Mn[b]	Ni[b]	Pb[a]	Zn[b]
A_1 (0.3 g)	**3.25** (0.29)	**185** (22)	**1.45** (0.25)	**0.34** (0.04)	**2.07** (0.91)	**3.66** (1.22)	**25.8** (13.8)	**6.5** (0.5)
A_2 (0.5 g)	**2.96** (0.03)	**194** (3)	**3.65** (0.01)	**1.11** (0.01)	**0.48** (0.05)	**4.83** (0.22)	**123** (7)	**28.8** (1.2)
A_3 (0.8 g)	**1.11** (0.07)	**128** (27)	< LOD	**1.02** (0.04)	**0.40** (0.03)	**3.53** (0.09)	**145** (4)	**32.8** (1.8)
B_4 (0.3 g)	**2.03** (0.01)	**195** (4)	< LOD	**0.79** (0.04)	**0.17** (0.01)	**4.41** (0.14)	**134** (6)	**28.1** (0.3)
B_5 (0.5 g)	**1.87** (0.27)	**195** (8)	< LOD	**1.23** (0.02)	**0.487** (0.005)	**4.65** (0.07)	**124** (1)	**27.2** (0.5)
B_6 (0.8 g)	**0.57** (0.21)	**157** (4)	< LOD	**1.07** (0.10)	**0.122** (0.004)	**2.77** (0.09)	**139** (2)	**32.0** (1.5)
C_7 (0.3 g)	**2.74** (0.19)	**225** (18)	**2.32** (0.35)	**0.625** (0.001)	**0.484** (0.018)	**6.16** (0.41)	**163** (3)	**32.0** (0.5)
C_8 (0.5 g)	**2.50** (0.09)	**252** (10)	**1.34** (0.03)	**0.73** (0.01)	**0.182** (0.012)	**4.84** (0.24)	**167** (6)	**42.6** (0.9)
C_9 (0.8 g)	**2.77** (0.54)	**151** (14)	**1.22** (0.03)	**0.83** (0.20)	**0.155** (0.005)	**4.00** (0.18)	**157** (7)	**44.4** (1.2)

(a): in μg g^{-1}; (b): in % ; LOD: limit of detection (1.40 μg g^{-1} of Cr).

The limit of detection (LOD) and concentration range of calibration curve for each element investigated are shown in Table III. The LOD was obtained from b + 3s; b is the mean concentration of 10 consecutive measurements of the sample blank and *s* is the standard deviation of them. The LOD of the method (in μg g^{-1}) was calculated by considering three times the standard deviation (b + 3s) of ten replicates of the sample blank, 0.3 g of sample mass in 50 mL and the dilution of this solution (50 or 100).

Table III. Limits of detection (LOD) and concentration range of calibration curves

Element	Calibration Curve/(μg L^{-1})[a]	LOD/(μg L^{-1})	LOD/(μg g^{-1})
As	0.5 - 6.0	0.10	1.67
Be	20 - 100	0.06	0.50
Cd	20 - 100	0.19	1.59
Co	20 -100	0.37	3.08
Cr	50 - 1000	0.17	1.40
Hg	10 - 30	3.3[b]; 0.34[c]	27.5[b]; 5.60[c]
Li	20 - 100	0.08	0.70
Mn	30 - 150	0.60	5.00
Ni	100 - 800	0.75	6.23
Pb	20 - 100	2.57	21.4
Tl	20 - 100	2.53	21.1
Zn	1000 - 8000 20 -50	1.32	16.5

(a) The correlation coefficient was typically 0.999; (b): for pneumatic nebulization; (c): for CV

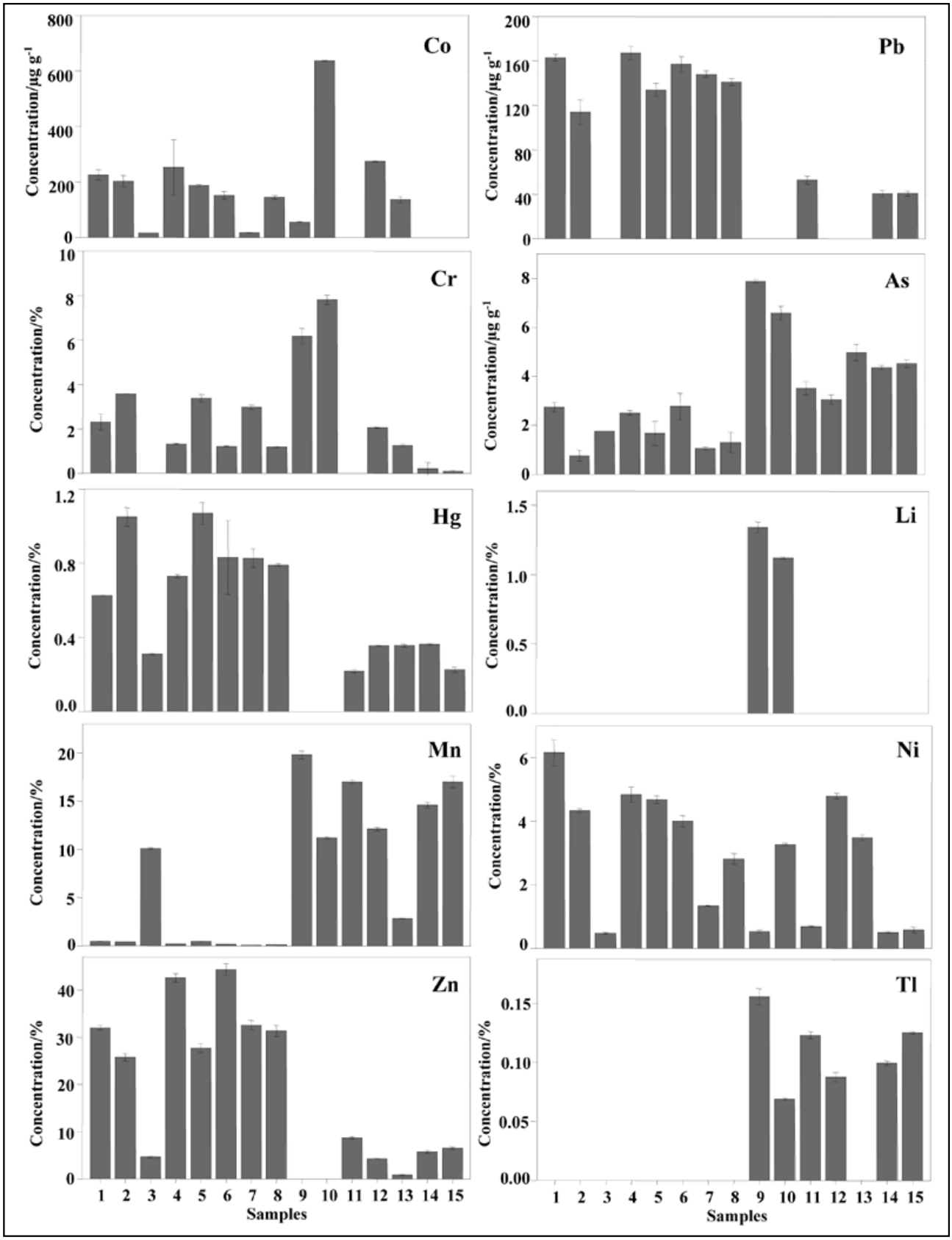

Figure 3. Concentration of elements in button cells; 1-8: Zn-air ; 9 and 10: Li-MnO_2, 11-15: Zn-MnO_2. Error bars represent the standard deviation for three button cells (n = 3). Bars not shown refer to concentrations lower than the respective limits of detection.

3.2 Matrix interference

Small amounts of residue were observed in the solution of some samples, mainly carbon from the electrodes of the button cells [11,19]. Besides, high amounts of ions were present in the sample solutions, demanding their dilution and also employment of a nebulizer for high contents of dissolved solids. Dilution tests revealed that the sample solution needed to be diluted at least 50-fold for Be, Cd, Co, Cr, Li, Mn, Ni, Pb, Tl and Zn and 100-fold for Hg and As to reduce matrix effects. It is worth citing that in the case of As and Hg the sample matrix interferes in the chemical vapor generation step, since the matrix is separated and it is not introduced into the ICP.

Spectral interference [20] was observed for the most sensitive Tl spectral line (190.801 nm) available. In this case, the LOD was relatively high (101 μg g^{-1}) and inappropriate for Tl determination in the analyzed samples. Therefore, another spectral line (276.787 nm) of Tl was monitored and the LOD obtained was 21.2 μg g^{-1}. Spectral lines more sensitive than last one were not found for Tl.

Certified reference materials of the different button cells analyzed do not exist. Therefore, analyte spiking was used to evaluate precision and matrix interference. Satisfactory recoveries were obtained, which ranged from 95 to 110%. However, as already mentioned, it was necessary to dilute the sample solution (at least 50-fold for Be, Cd, Co, Cr, Li, Mn, Ni, Pb, Tl and Zn and 100-fold for Hg and As) to reduce matrix interference and obtain the recoveries cited. Plasma robustness (emission signal of Mg(II) at 280.271 nm/emission signal of Mg(I) at 285.213 nm) was evaluated for the 50-fold diluted sample solution. Robust conditions of the plasma are mainly associated with high power of the generator, and low carrier gas flow rate. The plasma is considered robust if the Mg(II) 280.271 nm/Mg(I) 285.213 nm ratio is 10 or greater. In the present work, the ratio obtained was 12.2, 11.4 and 11.8 for the 50-fold diluted solutions obtained from Zn-air, Li-MnO_2 and Zn-MnO_2 button cells, respectively, at 1500 W and using a nebulizer gas flow rate of 0.8 $Lmin^{-1}$.

3.3 Sample analyses

The concentrations of the investigated elements in the analyzed samples are shown in Figure 3.

Cadmium, Hg and Pb are toxic elements controlled by CONAMA. Fortunately, Cd was not detected in any sample. This element is usually present as an impurity in zinc alloy [13] due to similar chemical properties of Zn (*e.g.*, valence and ionic radius). As a consequence, Cd could be present in Zn-air and Zn-MnO_2 button cells. Lead is also usually present as a contaminant in Zn alloy [13]. In fact, in Figure 3 it can be observed that the Pb concentration increases as the Zn concentration increases in Zn-air button cells. Mercury concentrations in the Zn-air button cells ranged from 0.8 to 1.0% and from 0.2 to 0.4% in Zn-MnO_2 button cells. In contrast, Hg was not detected in Li-MnO_2 button cells. Richter *et al.*[13] analyzed Zn-air button cells sold in Germany and in only one of them did they find Hg whose concentration was higher than the maximum permitted in that country. Button cells are globally produced by the same companies and the levels of the elements should be the same. Therefore, it is necessary to uniform international rules of disposal and destination of button cells.

Beryllium was not detected in any sample but Tl and As were. These results demonstrate the necessity to revise the Brazilian legislation with respect the control of other toxic elements in addition to Cd, Pb and Hg. The presence of Tl in Li button cells has already been reported [6] but not quantified. The concentration of Tl found in Li-MnO_2 button cells correlates with that of As. In Figure 3 it can be observed that the As concentration increases with increasing Tl concentration and it is possible that As is naturally associated with Tl [21]. Chromium is abundant in all analyzed button cells, especially in those of Li-MnO_2 type. In Figure 3 it is important to note that while Cd, Pb and Hg concentrations in Li-MnO_2 button cells are very low or not detected those of As, Tl and Cr are, in general, higher.

As expected, Li is high in Li-MnO_2 button cells, where Co concentration is also higher. This indicates the importance of battery recycling, taking into account the abundance of these elements in the earth crust. Manganese and Zn are major elements found in Li-MnO_2 and Zn-air button cells (Figure 3), respectively.

4. Conclusions

A method for As, Be, Cd, Co, Cr, Hg, Li, Mn, Ni, Pb, Tl and Zn determination in Zn-air, Li-MnO_2 and Zn-MnO_2 button cells was developed and applied. The results obtained demonstrated that the concentration of Cd, Pb and Hg in commercialized button cells were in agreement with the Brazilian legislation. However, Zn-air and Zn-MnO_2 button cells should not be disposed in the environment in virtue of the high Hg content. Toxic elements such as As and Tl should also be controlled, mainly in Li-MnO_2 button cells. The concentrations found for the other elements investigated indicated the importance of battery recycling, not only for protecting the environment, but also for economic reasons.

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Point of View

Analytical Chemistry in Brazil – why should we produce reference materials?

Analytical chemistry is a branch of chemistry that is increasing in Brazil, gaining importance in areas where analytical control is mandatory, such as food safety control of agricultural products, mainly for exportation, which normally requires satisfying phytosanitary and technical barriers. It is important for Brazil to be structured to answer the questions and support the laboratories that carry on these analyses.

The laboratories need to have validated methodologies and to demonstrate their competence when certifying whether trace elements are within the regulatory limits, with knowledge of the uncertainty of their measurements. Proficiency testing (PT) programs are important part of testing laboratory and they have become an integral part of laboratories' quality assurance activities. The samples are prepared by the provider, distributed among the participating laboratories, which perform the analyses and send back the results to the provider for comparison. It is important for the laboratories to demonstrate they are compliant with the limits set for testing errors. These PT programs are also useful to prepare candidate to reference materials (RMs). The certification of RMs is performed on the basis of PT results and statistical evaluation of PT data, with some auxiliary procedures involved.

Reference materials (RMs) are a significant part of quality assurance in analytical chemistry. They play a similar role in chemical measurements as reference standards do in physical ones. So, they should establish metrological traceability when applied for calibration of chemical measurement processes or comparability of the results when used for quality assurance purposes. For a laboratory that is conducting research with an established method or is attempting to validate a newly developed method, RMs are fundamental tools in establishing traceability of results. CRMs are the best available materials that can be used to validate accuracy of results, given that their values can be directly traced by the International System of Units (SI). Since different matrixes can affect instrument response, it is desirable to use RMs of matrixes that are representative of the actual study samples so that comparisons can be drawn between the two during method development and validation. That is, accurate and precise results should be obtained for all matrixes analyzed with a given method. However, achieving such results becomes more difficult when analyzing samples of specific analytes, such as the case of some Brazilian agricultural products.

It is desirable for the reference material to have a high degree of similarity with the matrix to be analyzed, first in order to reflect the same analytical problems that would be observed in real samples. The area of agricultural products and foods is most in need of RMs. This fact is critical in the context of the Brazilian market. Agribusiness in Brazil accounts for about 36% of total exports and 39% of jobs in the country. Despite the existence of some national institutions that begin to produce RMs, these demands are satisfied mainly by importation from countries with high costs. And for a great number of sectors, the materials are not adequate to Brazilian needs, considering they are normally tailored to meet the internal needs of the producing country. It is therefore important and strategic for Brazil to produce its own RMs, allowing the country to furnish materials specific for local needs..

Ana Rita de Araujo Nogueira
Researcher-Embrapa Pecuária Sudeste

Steps of Development

One important difference between developed and in development societies is the cost and speed of creation and flow of knowledge. Knowledge, and all its consequent innovations in science and technology, is a critical step towards an affluent society. Surely we need institutions for research and development, but simultaneously we must improve the information exchange between productive sectors in academy, industry and society. Information must fly smoothly and in a cost effective way.

All this was already known before our digital era, as clearly stated by David S. Landes in "The Wealth and Poverty of Nations" by pointing out: "the invention of invention, that is, the routinization of research and its diffusion". Nowadays, despite it may be seem just as a truism, diffusion still requires improvements in countries in the stage of development that Brazil is going through. No doubts we are evolving to become one of the most powerful world economies during the coming decades, but we need to speed up the process and disseminate it around all active partners and to the well-being of the society.

The launching of Brazilian Journal of Analytical Chemistry (BrJAC) is a milestone with full potential to expand the flow of knowledge. The integration of academy and industry is a must and BrJAC will certainly play a major role in putting them in contact. Why now? Why not before? Because now we have both the combination of economic strength and innovation in academic research in analytical chemistry in Brazil, as recently stated: "… analytical chemistry is healthy and growing, and is ready to support the new role of the country as an emerged power" (J. Braz. Chem. Soc.,20(10):1759,2009).

Great and successful steps were already surpassed, but there is a long and challenging road ahead. We need more engineers and scientists to grow our market competitiveness. We need all professionals connected in well established networks and with clear targets. Bring your knowledge and share it. Let us join forces and work together!

Joaquim A. Nóbrega
Department of Chemistry
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- ***Theses:***

10. Velandia, J. R.; *Doctorate Thesis*, Universidade Federal Rural do Rio de Janeiro, Brazil, 1997.

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