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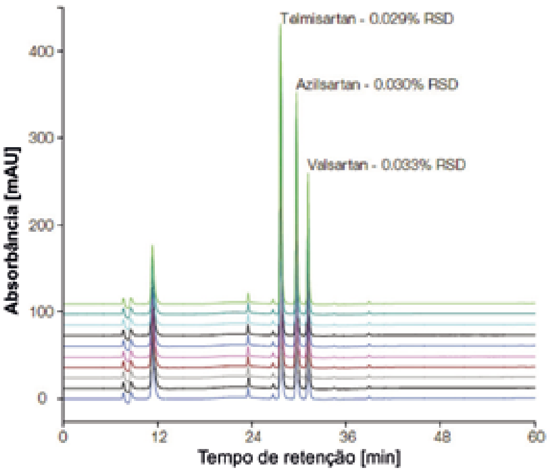

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EDITORIAL

The Brazilian Meeting on Analytical Chemistry (ENQA 2013) reached its 17th edition as a well-established event for discussion of a diversity of multidisciplinary subjects. These are characterized to be of interest of not only the analytical community members but also to researchers of areas as diverse as Biology, Physics, Mathematics and Engineering. The 17th ENQA, held in Belo Horizonte-MG and for which the theme "Analytical Chemistry and Quality of Life" was chosen upon a careful scrutiny, reunited 1038 participants from 22 Brazilian States and from nine other countries. During the event, discussions regarding the future directions of Brazilian Analytical Chemistry were widely carried out. Highlighted topics were the development of new instruments and analytical protocols applied to complex matrixes. The increasing participation of the analytical chemistry community on fields such as food safety and environmental preservation was also noteworthy. In summary, the 17th ENQA was a fruitful and successful event which certainly hit the high expectations of all participants.

The special issue of the Brazilian Journal of Analytical Chemistry (BrJAC) dedicated to the 17th ENQA brings four selected papers and an exciting interview with Professor Wilson de Figueiredo Jardim and viewpoint regarding the current stage of the Brazilian Analytical Chemistry written by the eminent Professor Érico Marlon de Moraes Flores. An overview about the 17th ENQA was prepared by one of the members of the Organizing Committee, Professor Zenilda de Lourdes Cardeal (Departamento de Química, Universidade Federal de Minas Gerais). Moreover, Professor Daniel L. G. Borges (Departamento de Química, Universidade Federal de Santa Catarina) presents his personal impressions about the 17th ENQA and expectations about the 18th ENQA, which will be held in Florianopolis in 2016.

Finally, I also would like to acknowledge Professors Lauro T. Kubota and Marco Aurélio Zezzi Arruda as well as to the other members of the BrJAC editorial staff for their invaluable assistance during the preparation of this Special Issue. I hope all of you can enjoy the readings!!!

Rodinei Augusti
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SUMMARY

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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!

17TH BRAZILIAN MEETING ON ANALYTICAL CHEMISTRY (ENQA): ANALYTICAL CHEMISTRY AND LIFE QUALITY.

The Brazilian Meeting on Analytical Chemistry involves significant scientific discussions of analytical chemistry research and education in Brazil as well as an assessment of the area scenarios in the world.

Analytical research in Brazil started in 1940 in Rio de Janeiro and São Paulo and has spread to other places in Brazil since 1950. Scientific discussions of the analytical area, together with all other branches of chemistry, were performed within the Annual Meeting of the Brazilian Society for Progress in Science (SBPC) until 1977. From 1978 until 1982 the discussions were held during the annual meetings of the Brazilian Chemical Society (SBQ). In 1982 the First National Meeting on Analytical Chemistry (ENQA) was held in Rio de Janeiro and after 1983 it has become biennial. The significant growth of analytical chemistry in Brazil is evident at each ENQA conference. In 1982 there were only 6 graduate programs in Analytical Chemistry accredited by the Brazilian Federal Agency CAPES, however currently 28 programs are accredited.

In 1995 the 8th ENQA was held in Belo Horizonte with about 400 posters presented. For the 17th Brazilian Meeting on Analytical Chemistry (17th ENQA), also held in Belo Horizonte from October 6th to 9th, 2013, a remarkable number of more than 800 posters were presented. The 17th ENQA envolved people from 22 Brazilian States in addition to participants from nine other countries (mainly as lecturers). Of the 1038 participants, 38% were graduate students, 33% university teachers/researchers, 18% undergraduate students, 9% specialists of companies and 2% high school teachers.

The theme of the meeting, chemistry and the quality of life, was discussed in 7 plenary sessions and 15 coordinated sessions and showed advanced works in different areas of analytical chemistry. It was requested that the slides used should be written in English and this allowed a significant participation of foreign visitors. There were significant scientific communication accompanied by very good cheese bread and fraternal conversations, besides instructive and relaxed presentations that made the audience laugh on many occasions.

The idea that Brazilian research on analytical chemistry must move outside academic borders was emphasized, taking into account the responsible intervention of the academic communityy regarding food safety and environmental preservation.

Finally, the high quality of all posters made the task of the Organizing Committee to reward only twenty among them very difficult.

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ANALYTICAL CHEMISTRY IN BRAZIL: WORKING AT THE FRONTIER

Analytical Chemistry is, in its essence, a field of chemical research that interfaces Chemistry and other sciences such as Biology, Physics, Mathematics and Engineering, among many others. This close contact with other fields of research has become so intimate in the past decades that chemists with solid analytical formation have become increasingly integrated to departments and research institutes that are not necessarily associated to the name "Analytical Chemistry". But is Analytical Chemistry, as an individual research area, on the verge of vanishing? If not, which themes can be regarded as "frontiers" in analytical research?

The answer to the first question is fairly straight. Analytical Chemistry will probably never vanish as a scientific field, since science depends directly on its developments. This is quite evident in Brazil – the latest edition of the Brazilian Meeting on Analytical Chemistry (or *ENQA*, from its Portuguese denomination), held in Belo Horizonte, was able to reunite over 1200 participants from all regions of Brazil, in addition to researchers from other countries. The numbers are similar to those from the 2011 event, which means that the Brazilian Analytical Chemistry community is stable and solid. But are we working at the frontiers of Analytical Chemistry? Although a current definition of "frontier" is somewhat arguable, the fact is that some of the most prominent results in analytical sciences in the past few years have been concentrated in themes such as the development of analytical protocols applied to complex biological and biochemical systems, the development of new instruments and sensors and the application of newly developed analytical procedures aiming at environmental and toxicological analysis. The 17th ENQA has shown that Brazilian Analytical Chemistry is adapted to what can be regarded as the 'state-of-the-art' in terms of analytical investigation. Topics such as developments in mass spectrometry, new insights into metallomics and matabollomics speciation analysis, novel sample preparation protocols and equipments and new sensorial devices were widely discussed, suggesting that we are heading in the right direction regarding advanced analytical research. The 17th ENQA was a successful event and it excelled in maintaining the high quality that has been historically associated to the ENQAs.

Now the challenge of maintaining the high level of scientific discussion will be passed on to the 18th ENQA. The event will be held in the coastal city of Florianópolis-SC, in September 2016, and it will be planned to promote a broad discussion on Analytical Chemistry and all its sub-specialties. The organizers of the 18th ENQA will embrace the challenge of planning a high-level event, with a scientific program that is compatible with the advanced degree of evolution of Analytical Chemistry in Brazil. Let's all meet in Floripa!

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Wilson de Figueiredo Jardim

One of the most exciting and important fields of analytical chemistry is environmental chemistry. Currently, this area of investigation has been on the spot due to the concerns with water availability and quality for human consumption. Professor Wilson de Figueiredo Jardim, from the Universidade Estadual de Campinas (Unicamp) is one of the pioneer scientists that did not overlook the social and political role of chemists that are involved with environmental chemistry. He talked to us for one hour about the history of this field of investigation in Brazil, of which he is a very important representative. Professor Jardim expressed his concerns about water, energy, sanitation, "green dogmas" and many other issues that are interesting for the experienced chemists – many of whom might have never thought about them – and also those in the beggining of the career.

For the beginners, professor Wilson guarantees: with a mosquito repellent, a sun blocker and a good idea, any responsible and ethic scientist can explore the outdoor laboratories available in Brazil: Rain Forest, Pantanal, Cerrado, Caatinga, and many other ecosystems in our rich country. Chemists can leave the traditional labs and build their own in the open field. There is a lot to study.

Patricia Logullo

How come a chemist did become an environmental scientist? Could you tell us about the milestones in the history of your career?

After graduating, I was hired as a teacher in a technical school in Campinas, and then I became a professor at the Federal University of Paraíba, working in the area of sanitation. Soon after, I joined the United Nations Development Programme (UNDP), working in Africa. In the return to Brazil, I was hired by the Rhodia Research Center, in Paulínia.

In 1980, I left the country again for a PhD program in the University of Liverpool, with a scholarship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). In my thesis, I explored the aspects related to copper toxicity in cyanophyceae. Late in 1983, I came back to Brazil and in 1984, I started my career at Unicamp (Universidade Estadual de Campinas), at the Department of Analytical Chemistry. In 1987, I engaged in a post-doctorate program at the Center for Environmental Studies, Drexel University, in Philadelphia, and in another in 1993, at the Program of Environmental Engineering, University of Delaware, both in the United States.

At the beginning of my activities here in Campinas, I worked with the chemical speciation of metals in aquatic environments. However, the scientific community was not very mature to discuss this topic in the 80s. So I started a project on mercury in the Amazon, where I worked for over 10 years, in a total of 12 scientific expeditions. Numerous students were trained in this area: chemistry in the fieldwork. Together, we have helped to clarify several aspects that still remained obscure in the chemistry of mercury in the tropics.

> “I have always felt that white-coat chemists should take a look outside, through their lab windows, where challenges really are.”

Around the same time (early 1990s) I started working with advanced oxidation processes, and, in 1992, the first Masters dissertation in this issue was presented in Brazil. We began exploring the subject applied to confined atmospheres, water, soil and waste, and various groups in Brazil now develop research on this area. We also created the Meeting on Environmental Applications of Advanced Oxidation Processes (EPOA), which today is already established as the biggest event of the area in Brazil and Latin America. Furthermore, we designed the first Meeting of Environmental Chemistry (EnQAMB), in the LQA (Laboratory of Environmental Chemistry), which was to be sated as the largest event of its kind in Brazil.

Over the past five years, I have devoted myself to the study of emerging contaminants in water, especially in drinking water. We have brought the discussion of this subject to the Ministry of Health, the water treatment companies and suppliers and the civil society, and I think it was of great value to society. In fact, we have just published a book about the presence of caffeine in water in the main capitals of the country.

During my career, I coordinated two thematic projects from Fapesp (Fundação de Amparo à Pesquisa do Estado de São Paulo), I am also the vice-coordinator of an INCTAA (National Institute of Science and Advanced Analytical Technologies) and I have coordinated more than 20 national and cooperative research projects. Well, I have six patents, including one licensed, and I have supervised masters and doctorate students, and I won awards for my work too. I have never left Unicamp since 1984, and I have just retired from the university, in February 2013.

It seems that you have engaged in the environmental cause very early in your career, but Chemistry has been traditionally a research field to feed the industry. How did you manage to work in this context?

I have always felt that white-coat chemists should take a look outside, through their lab windows, where challenges really are. However, in fact Chemistry has not been very present in the environmental field... Since the publication of Silent Spring, by Rachel Carson, in the 60s, civil society has been much more concerned about the risks of endangering nature. Still, more than 50 years have passed and we do not have many of the answers we need yet. Please note that only recently we started to consider water as a limited resource.

Unfortunately, accidents called the attention of chemists and engineers to the environmental aspects of the industrial production. After the Seveso disaster, in Italy, in 1976 (releasing dioxins in the athmosphere by a small chemical plant) and the Bophal gas tragedy in India (when a gas leak from a pesticide plant exposed more than 500 thousand people to methyl isocyanate and other toxic substances), in 1984, the industry began to watch for risky situations. Therefore, the “green chemistry" was born inside the industry, and "responsible care" emerged as an issue. The Responsible Care Program was created for pollution prevention, a series of codes, principles and checklists adopted by industries in many countries to prevent accidents and reduce risks. Therefore, we see that the chemistry that fed the industry had to search for methods to avoid the risks involved in the chemical production. This is, ironically, how the issue of environment protection has emerged in the chemical industry.

The Responsible Care Program dates from 1985. Has it been possible to introduce the environment care concepts in the university since then?

When I started to teach, in the 80s, students saw the environmental study with cepticism. There was a lot of misinformation about it at that time, and we lacked the habit of joining with other professions to study: there was no place for multidisciplinarity. There was no tradition on that field. We did not have textbooks to study environmental chemistry, and the available information was very disjointed.

When did it start to change?

In the beginning of the 2000s. Back then, the discipline of environmental chemistry dealt only with phenomena involving human health. Since then, the students, much to the influence of society, started to bring to the classroom everyday affairs: what they listened on TV or the radio was inspiration for research questioning. The concern with the environment had spread from the academia to the whole society, and this made our job easier. Although still with a lot of misinformation, we were managing someway to make students think about concepts of environmental chemistry and about how they applied to real world. Today, it is more natural to teach environmental chemistry, because society demands it harder.

Is disinformation over? Reduced?

No. While the concern with the environment is positive and necessary, there are still many wrong concepts widely spread by the midia. I will give you an example: some regions of Brazil are now suffering from floods, while others, like the state of São Paulo, go through a serious lack of water, and the reservoirs are dry. The midia urges everybody to save water: close the taps while brushing your teeth, take short showers and so on. This is very good: to involve every individual with the responsibility. However, what is the real result of these procedures, if we consider that no less than 70% of the available water is used in agricultural irrigation? What is the real meaning to teach a boy how many years a cigarette butt takes to break down, if the rock below it is 2 billion years old? Maybe it is more important for him to know that a OH-radical is very short-lived (the in vivo half-life is about 10^{-9} seconds), but still very toxic and dangerous.

How do you fight misinformation?

We can do that by bringing their scientific disquiets to the real world. In a globalized world it is quite easy to do that: why don't you ask the bus driver that took you to the university today about the amount of diesel that was spent in the way? What would be the consequences of changing one type of fuel for other? Why not searching the Internet for some of the answers in the scientific literature? Why don't you calculate how much electricity you can save in your house with simple measures? When chemistry students can see the impact of knowledge on their lives, they can engage more easily in scientific initiation projects. Their interest in the field has been growing, and they come with a desire to save the world. They are passionate for searching responses.

What they do not know is that sometimes there is not a single answer in the real world... Environmental chemistry is not that Cartesian, and many times the result of a research in analytical, environmen-

> **"We have built our chemistry based on the German School, you know. Dirty and wet feet of mud are not proper things for that kind of laboratory. This would be considered an inferior kind of science in the old times. The chemistry schools had no chemists working in the open field at that time: nature was for geologists and biologists. (...) We are still fighting the remnants of the stigma."**

tal chemistry depends on the strategy of analysis you make. Besides, there are many uncertainties, many gaps of information that prevent us from finding solutions for everything. It is a fascinating field, and at the same time, a challenging field.

Is it easier to go to the real world today? You dived into the Amazon region, to study mercury in the water. This demanded 12 scientific expeditions in the years 80-90 and still render publications. What technical, operational, logistical difficulties did you face back then and what facilities the young researcher can count on today? Is it easier to investigate chemistry in the environment, in loco?

We have built our chemistry based on the German School, you know. Dirty and wet feet of mud are not proper things for that kind of laboratory. This would be considered an inferior kind of science in the old times. The chemistry schools had no chemists working in the open field at that time: nature was for geologists and biologists.

The major contribution I believe we gave for Chemistry in Brazil has been training students for fieldwork. It is very important for graduation students being trained about how to extract data in adverse conditions, and we did not have it in Brazil. And yet there are few today. We are still fighting remnants of the stigma.

My Amazon journey was the result of my willing to leave the lab and to see the largest chemical reactor in the world. Very little was known of it then, and all research about the Amazon had been published by foreigners: the forest atracted researchers from abroad — and this happens often: large research programs are designed outside Brazil. I am not xenophobic, but I believe we, Brazilians, should get more interested in our environment.

Mercury was a shortcut to study the Amazon: it is a ubiquitous, naturally occurring metallic element that can be used in the study of the water and organic matter cycles. Controlling the anthropogenic releases of mercury throughout its lifecycle has been the key issue in the Minamata Convention, recently signed, since this element can be highly toxic to humans and animals.

We studied the mercury cycle in the Negro River Basin, and its different seasonal and region-based behaviors. We have shown, for example, that speciation varies according to gender, age and body constitution of the person examined for intoxication.This is something you examine *in loco*, considering the surroundings, the soil, the bottles you use for sample storage, and the site from which you take water samples. Considering the Amazon, you have to plan the loggistics to exhaustion: everything is more difficult and more expensive than in the normal lab. For example: you need a boat with a reliable source of electric power, because the staff will need to spend 10 days onboard, and working. A guiding bushman is essential: the "mateiro", even at these times of Google Earth and GPS, is the only way to avoid getting lost in the forest. Because of that, much time has to be devoted to the planning of expeditions: this can avoid crucial losses. This created a bond with the region, and today I go to the Amazon as a tourist, to visit the places and friends I made there. I have a lot of satisfaction there.

When you were a young graduation student, did you plan or dream with those adventures?

I am 60 years old now. Looking back, I must admit that my dream back then was to go abroad, to study in other countries. And in fact I did. Today, studying abroad is much easier, especially with the "Ciência sem Fronteiras" program. It is almost trivial to study outside Brazil now. The challenge is to study the issues in Brazil and to understand the politics involved in scientific reasoning...

What do you mean? What is the link between the academy and the politics?

For a long time, the university remained away from public policies, especially regarding the environment, because of a lack of: space, interest, dialogue and mechanisms to participate. Knowledge was generated, but kept witheld inside

> **“For a long time, the university remained away from public policies, especially regarding the environment, because of a lack of: space, interest, dialogue and mechanisms to participate. Knowledge was generated, but kept witheld inside the university, because researchers did not have to report the accountability of the work. In the last 20 years, society has been asking for information about the use of public money, and the capability of the science to generate real benefits, satisfaction to the communities. The university is now engaged in public policies.”**

the university, because researchers did not have to report the accountability of the work. In the last 20 years, society has been asking for information about the use of public money, and the capability of the science to generate real benefits, satisfaction to the communities. The university is now engaged in public policies. An example of that is the Portaria 2914, issued in 2011, which sets forth the procedures for control and monitoring of water quality for human consumption and its potability standards in Brazil. Every utility company in Brazil has to fulfill the quality requirements imposed by this law, in order to safeguard human health. And the parameters for the 50 compounds listed were set by scientific research.

What about the water crisis in São Paulo region?

The water shortage we are going through was predicted some time ago. I am certain that the governors and the utility company were aware of the crisis coming: technical information was available. The problem is that scientific knowledge is not always taken into consideration by politics. And when the scientist is not able to communicate directly with society, this gap shows in politics.

The Cantareira reservoir levels will not recover to the next year, when the crisis is going to be worse. In this situation, we have to use the "dead volume", i.e., the deep reservoirs. And the quality of the water from the deeper layers of the dam is lower.

What tools you can use today to analyze the quality of water for human use?

As I said, Brazilian law regulates about 50 compounds present in the drinking water. However, we are investigating more than new 1,000 substances per year, and we are exposed somehow to more than 40,000 per day. At the same time, we know the toxicity of less than 1,000 compounds. Therefore, tap water, that contains more than 1,000 compounds, even when complying with the law — that regulates only 50 —, can potentially bring adverse effects to human health. Even when the water companies are complying with the limits imposed by law. However, we cannot possibly legislate about 1,000 compounds. When population density grows uncontrolled, sanitation decreases, as more and more unknown substances are discarded without treatment. How could we possibly analyze and regulate thousands of substances in a scenario like this?

We believe that the answer for this is in trying to forget individual values and starting to deal with the interaction between them, and with markers that would work as a proxy for pollution. With this idea in mind, we came to caffeine.

Caffeine is a stimulant substance naturally found in many seeds and plants, and widely used by humans, because its toxic dose is very high, and therefore it is allowed everywhere. However, only 5% of the caffeine that you find in rivers, lakes and other water sources are really from natural sources: the other 95% is from wastewater, untreated sewage. Caffeine is present in soft drinks, energetics, drugs and medications that are discarded in wastewater and contaminated soils. Therefore, when you find caffeine in water, you know it is contaminated with untreated sewage. The amount of caffeine found in tap water is not harmful to human health. But, once found, it can tell us about the quality of the water, i.e. other compounds that were released to sewage can also be present in this water. It is a chemical indicator, a fingerprint of contamination. A good desinfection system eliminates pathogens, but not caffeine. When you treat water with chloride, for example, you kill the pathogens by breaking cell membranes, eliminating pathogenicity, but you do not remove some chemical compounds that can also cause toxicity or diseases.

Even if we consider caffeine as non-toxic for humans, we must remember that recent research has found an association between caffeine and estrogenicity in drinking waters. Therefore, water with caffeine could potentially have some hormonal activity. Furthermore, if you find caffeine in water, you can consider it as contaminated water — and that water

> **“Caffeine is present in soft drinks, energetics, drugs and medications that are discarded in wastewater and contaminated soils. Therefore, when you find caffeine in water, you know it is contaminated with untreated sewage. The amount of caffeine found in tap water is not harmful to human health. But, once found, it can tell us about the quality of the water, i.e. other compounds that were released to sewage can also be present in this water. It is a chemical indicator, a fingerprint of contamination.”**

“The environmental chemistry has grown impressively and quality; but we still need to encourage more field research, *in situ* investigations, measurements in real time. But these studies are really expensive and there are many bureaucratic difficulties in finding financing: how to justify that you need to pay a bushman? That you need to buy a good GPS device? In the Amazon, if you suddenly need 10 thousand bucks to make a payment, there is no cash dispenser machine in the corner to take the money. Details that can hamper the work.”

can be contaminated with other compounds too, such as antibiotics. If you release antibiotics in water sources, bacteria can become resistant to it, which is a very dangerous situation for humans. These markers open new possibilities for analytical chemistry, because they are simple and fast to analyze.

It seems that this application of analytical chemistry makes the field much closer to the applied sciences than to basic sciences. Is that right? Are there possibilities for patent requirements in this area?

Yes, it is certainly an applied field of science. And patents are a distant consequence of our work. Unicamp has created a very useful office for the support of scientists in patent requirements. The office protects the university about the misuse of the products, and it takes care not only of the numbers of patents, but also the exploitation, including underuse of the patents and intellectual property. The office makes audits of licensed products and monitoring of contracts.

Brazil has increased the number of patents, but we do not know about the quality of production. The “numerology” imposed by development agencies, requiring researchers to publish studies with high numbers of citations, has made Brazilian researchers produce more, but that does not mean the products generated by research are monitored regarding the use in real world (in the industry, in the environment). We really have to rethink the indicators of research production.

When working with applied research, we have to think not only in the solution of the problem, but in the problem itself: in nature there are always numerous factors involved. The analytical chemist who works with environmental chemistry is required to visit the petrochemical or hydroelectric power plant to see what are the problems there that need solving. These are the solutions that the field really needs now, and consequently, solving a real problem with innovative approach brings the patent naturally.

The environmental chemistry has grown impressively and quality; but we still need to encourage more field research, *in situ* investigations, measurements in real time. But these studies are really expensive and there are many bureaucratic difficulties in finding financing: how to justify that you need to pay a bushman? That you need to buy a good GPS device? In the Amazon, if you suddenly need 10 thousand bucks to make a payment, there is no cash dispenser machine in the corner to take the money. Details that can hamper the work.

Even so, we are going well and I think that by having more people especialized in the area, we will succeed. You need to create the expectation, encourage the students and show them that the field is an outdoor laboratory. Insect repellent, sun screen and a good an idea is all we need.

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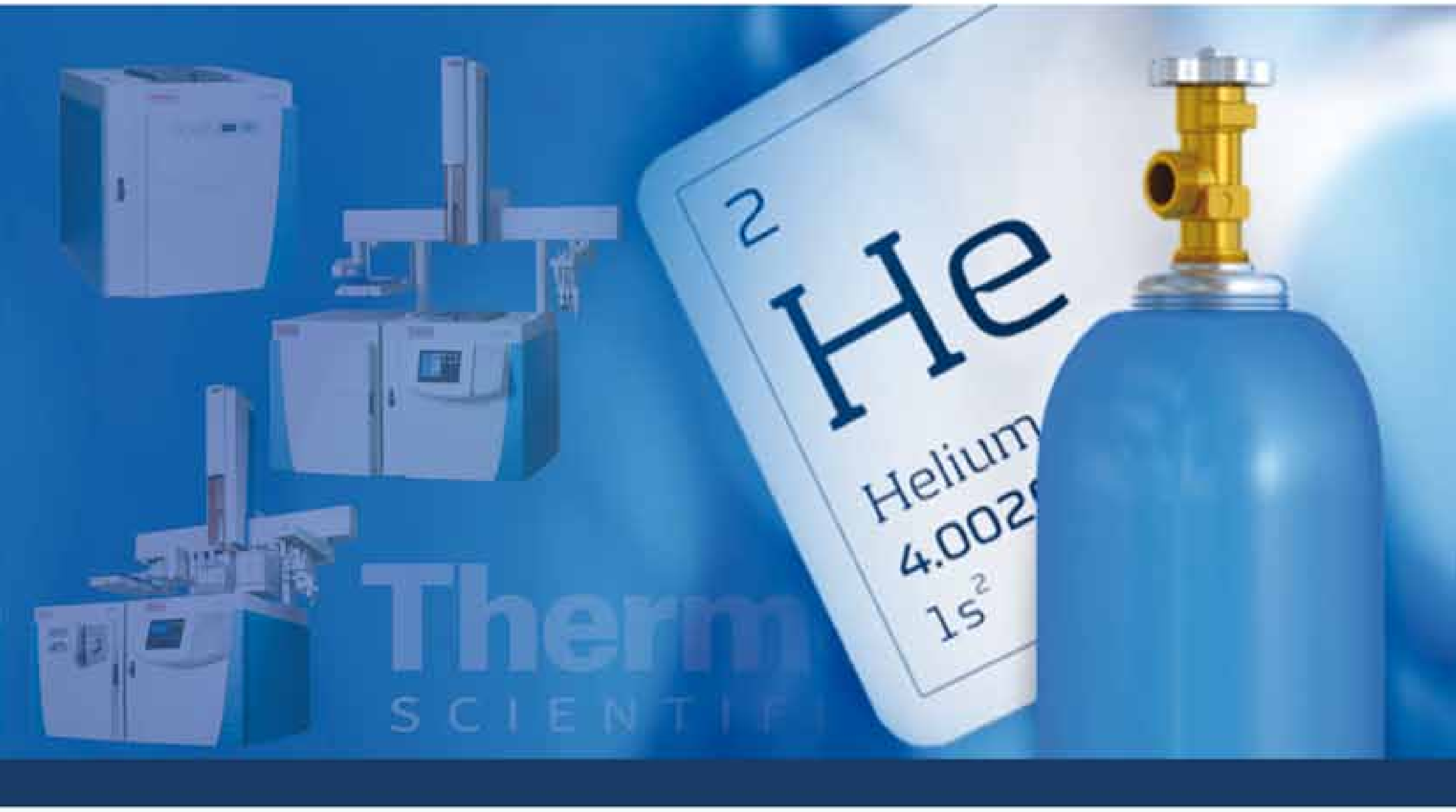
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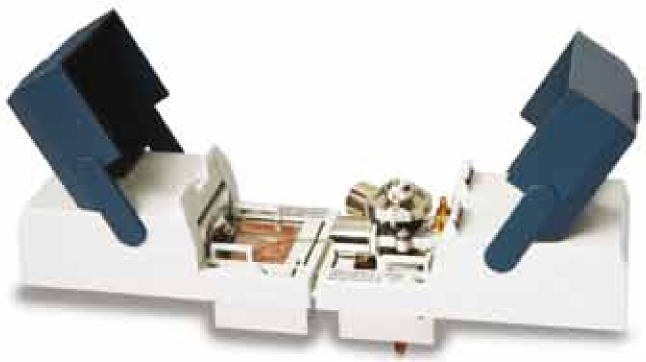

Azithromycin drug determination in pharmaceutical formulations by UV spectrophotometry and HPLC-UV

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Abstract

The UV spectrophotometric and HPLC-UV methods for determining azithromycin in pharmaceutical formulations have been developed and validated. Linearity was established in the range of 0 - 183 mg L^{-1} for UV spectrophotometric and 100-1000 mg L^{-1} for HPLC. The correlation coefficients were equal to 0.999 and the F-tests for analysis variance were 1.53 and 4.36 for each methods, respectively, both lower than the critical values (2.96 and 4.52). Relative standard deviations of samples were lower than 1.50% and recovery percentages were within the range of 95-105%. The contents determined and their expanded measurement uncertainties were 501 ± 24 mg for tablets by UV espectrophotometry, and 501 ± 15 mg for tablets and 953 ± 27 mg for oral suspension by HPLC.

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Keywords: Azithromycin, UV spectrophotometric, HPLC-UV, Pharmaceutical formulations.

1. Introduction

Azithromycin is an azalide antibiotic derived from erythromycin by inserting a methyl-substituted nitrogen atom in the lactone ring. The expansion of the lactone ring provided improvements in the spectrum of action against Gram-positive microorganisms and oral bioavailability in comparison to erythromycin. Azithromycin's action mechanism inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, thereby preventing the translocation of mRNA. Azithromycin is effective against a variety of Gram-positive and Gram-negative bacteria being used in the treatment of respiratory infections and sexually transmitted diseases, among others [1].

The Brazilian Pharmacopoeia's official method for azithromycin determination is microbiological [2]. However, microbiological methods are not specific; they can detect both the active metabolite and the ingredients. Instrumental methods capable of overcoming the limitations of the microbiological methods have been highlighted. A further technique that has been used for the determination of this drug was HPLC with UV detection [3-10]. Since most of these methods used a long C18 colunm (250 x 4.6 mm, 5 μm), a flow rate larger than 1 mL min^{-1} was required. The mobile phase used was a mixture of organic solvent and phosphate buffer, but due to the low solubility of this salt in the organic solvent, there is a limited variation of these proportions and successive filtrations are often required. The elution times ranged between 4.8 and 11 min. The U.S. Pharmacopeia (USP) describes two chromatographic methods for azithromycin determination: amperometric electrochemical detector and UV detection at 210 nm. In the second method, a mobile phase is used containing a mixture of acetonitrile and phosphate buffer (pH 8.2) (6:4, v/v), C18 column (150 x 4.60 mm; 5 μm) and flow rate of 0.9 mL min^{-1} [11].

There are also reports in the literature using spectrophotometry in the visible region [12-14], however, a derivatization reaction is required. Ashour & Bayram [12] developed a spectrophotometric method for the assay of azithromycin and erythromycin in pure and pharmaceutical formulations. The proposed method was based on the reaction of the drugs with sodium 1,2-naphthoquinone-4-sulphonate in alkaline medium at 25 °C to form an orange-colored product of maximum absorption peak at 452 nm. Jayanna *et al.* [13] described a visible spectrophotometric method for the estimation of azithromycin from tablet. The method is based on the reduction of potassium permanganate in an alkaline medium with azithromycin. The decrease in absorbance of potassium permanganate at 547 nm was measured, as it decolourised upon reduction by azithromycin. De Paula *et al.* [14] also proposed a spectrophotometric determination of azithromycin in pharmaceutical formulations. The method is based on the charge transfer reaction between the drug and quinalizarin (50 mg L^{-1}) in a methanol medium. The radical anion (absorbing species) was formed in the medium immediately after mixing the reagents and showed maximum absorption at 564 nm.

This paper presents a new and simple method for azithromycin determination by UV spectrophotometry, without derivatization steps, as well as a novel approach to the HPCL-UV method. The use of acetonitrile and 50 mmol L^{-1} ammonium hydroxide/ ammonium acetate buffer solution (80:20 v/v) as mobile phase and a shorter C18 column (100 x 3 mm; 2.2 μm) allowed azithromycin isocratic elution at 7 min with a flow rate of 0.200 mL min^{-1}. The optimized methodology used a salt with good solubility in organic solvents, a column of smaller dimensions, a significantly lower mobile phase flow rate with a similar analysis time, when compared with the time reported in the literature. Therefore, the optimized chromatographic method presented lower solvent consumption and, consequently, lower waste production. Both methods were validated and special consideration was given to estimate measurement uncertainty, since this is a very important parameter to evaluate the reliability of a method, but has been scarcely reported in the literature. The spectrophotometric method was applied to azithromycin determination in tablets and the chromatographic method to tablets and an oral suspension.

2. Experimental

2.1. Chemicals and reagents

Standard azithromycin was purchased from the Sigma-Aldrich (St. Louis, Missouri, USA). Acetonitrile (HPLC grade), ethanol (HPLC grade), acetic acid, phosphoric acid and ammonium hydroxide were obtained from J.T. Baker (Xalostoc, Tlaxcala, Mexico). All other chemicals were of analytical grade.

2.2 Apparatus

The measurements of the absorption spectra were performed in a double beam scanning UV/Visible spectrophotometer (model Cary 100 Bio, Varian; Mulgrave, Vic., Australia) using quartz cells of optical path equal to 1.0 cm.

A Finnigan™ Surveyor Plus™ HPLC System, Thermo Scientific, (West Palm Beach, FL, USA) provided with an automatic injector, a diode-array detector and a column oven was employed.

2.3 Chromatographic conditions

Chromatographic analysis was performed on Shimadzu Shim-pack XR-ODS column (100 x 3 mm; 2.2 μm) (Columbia, MD, USA) and kept at 40 ºC. An isocratic elution was employed with 80% acetonitrile and 20% ammonium hydroxide/ammonium acetate buffer (pH 9) for 10 min. Buffer (50 mmol L^{-1}) was prepared by adding 290 μL of glacial acetic acid and 1.30 mL of ammonium hydroxide to 200.0 mL of ultrapure water. Flow rate was 0.200 mL min^{-1} and injection volume was 10 μL. UV detection was performed at 217 nm. Under these conditions azithromycin retention time was roughly 6 min.

2.4 Standard preparation

Stock solutions of azithromycin were prepared using 131 and 400 mg azithromycin made up to 50.00 mL in a volumetric flask using ethanol and acetonitrile for spectrophotometry and HPLC, respectively.

2.5 Analytical curves

Standard addition curves at six concentration levels (0, 37, 73, 110, 147 and 183 mg L^{-1}) were made in authentic quadruplicates (n=4) for azithromycin determination by spectrophotometry. At all points of the standard addition curves aliquots of 1000 μL of sample solution were added.

External calibration curves at five concentration levels (100, 250, 500, 750 and 1000 mg L^{-1}) were made in authentic triplicates (n=3) for chromatographic determination of azithromycin.

Different working ranges were used to adjust the sensitivity of the methodologies, since the optical path length of the spectrophotometer was greater than the DAD.

2.6 Sample preparation

2.6.1 UV spectrophotometry

Five tablets containing 500 mg of azithromycin (as given by the manufacturer) were weighed and finely powdered. 12.5 mg of powder was transferred to a 10.00 mL volumetric flask to which ethanol was added. After 20 min of sonication, the solutions were centrifuged at 1073 G for 15 min. All procedures described were made in authentic quadruplicates.

2.6.2 HPLC

Five tablets containing 500 mg of azithromycin (as given by the manufacturer) were weighed and finely powdered. 42 mg of the powder was transferred to a 10.00 mL volumetric flask to which acetonitrile was added. After 20 min of sonication, the solutions were centrifuged at 1073 G for 15 min. The supernatants were diluted five times with acetonitrile before HPLC injection. For recovery studies, samples were prepared with standard addition. Sample solutions were prepared by adding 1000 μL of supernatant and 320 μL of stock solution of 4000 mg L^{-1} in 5.00 mL of acetonitrile. All procedures described were made in authentic quadruplicates.

Powder for an oral suspension containing 900 mg/22.5 mL (as given by the manufacturer) was weighed and stored. 442 mg of the powder was transferred to a 10.00 mL volumetric flask to which acetonitrile was added (n=4). Other procedures were the same as described for the tablets.

3. Results and discussion

3.1. Optimization of the UV spectrophotometric methodology

Initially, 201 nm was determined as the wavelength of maximum azithromycin absorbance in ethanol. Then, azithromycin solutions were read at concentrations of 150 and 300 mg L^{-1}, which showed absorbance equal to 0.43 and 0.86, respectively. Next, the range of 0 to 183 mg L^{-1} for the standard addition curves was defined; so that the sixth point (150 mg L^{-1} of sample + 183 mg L^{-1} of standard) of the curve did not exceed absorbance range.

3.2. Optimization of the HPLC methodology

In order to select a pH buffer in which azithromycin was mostly present in its neutral form, hence its considerable interaction with the C18 phase, a distribution diagram was made (Figure 1) from its pK_{as} values: 8.6 and 13.3. The selected buffer was ammonium hydroxide/ ammonium acetate (pH 9), since at this pH approximately 70% of azithromycin presents itself in its neutral form and the mobile phase is in the working range of the column (2-10). The mobile phase polarity was varied and the optimized composition was acetonitrile and 50 mmol L^{-1} ammonium hydroxide/ ammonium acetate buffer solution at a ratio of 80:20 (v/v), using a C18 column (100 x 3 mm; 2.2 μm) and isocratic elution for 6 min at a flow rate of 0.200 mL min^{-1}.

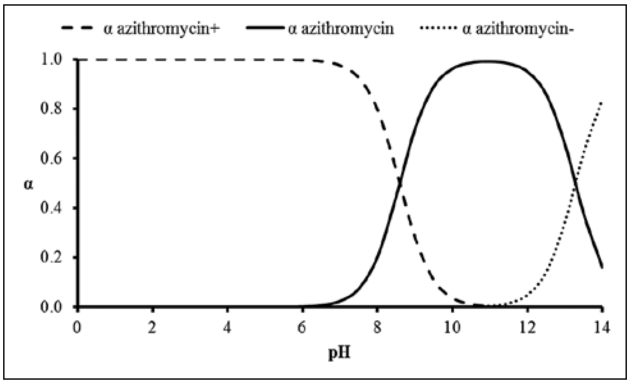

Figure 1. Azithromycin distribution diagram.

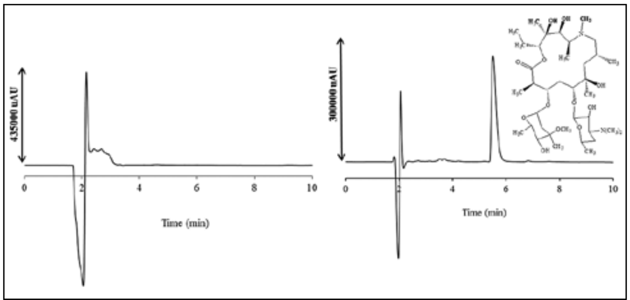

Figure 2.Chromatograms of 500 mg L^{-1} azithromycin under conditions: (A) similar to UPS's method; (B) optimized experimental.

Subsequently, in order to compare the results obtained by optimized methodologies, the mobile phase according to USP using a C18 column, however with smaller dimensions (100 x 3 mm, 2.2 μm) and a lower flow of 0.200 mL min^{-1}, was reproduced. Analyzing the chromatogram (Figure 2), it could be seen that under similar conditions as those of USP, azithromycin did not provide a satisfactory interaction with the stationary phase. This can be explained by the fact that at pH 8.2 azithromycin is 70% in its protonated form. Therefore, comparison of measurements obtained by the optimized methodologies and by a method similar to USP was not performed. The optimal mobile phase also had the advantage of using an organic salt (acetate) which is highly soluble in organic solvents, such as acetonitrile. Phosphate must be used with caution in the mobile phase composition, as it may precipitate in a mixture with organic solvents; this reduces variations in the mobile phase composition and often requires successive filtrations.

3.3. Single-laboratory validation

After optimization of the chromatographic and spectrophotometric methods, some validation parameters were evaluated: linearity, measurement uncertainty, repeatability, trueness, detection and quantification limits.

3.3.1. Linearity

Initially, an F-test was performed to evaluate the homogeneity of variances, through the ratio of the larger variance by the smaller variance of each calibration curve level [15]. The values calculated for standard addition and external standard curves were 44.51 and 71243, respectively. The critical values were 9.28 for $F_{(0.05,3,3)}$ and 19.00 for $F_{(0.05,2,2)}$, thus, the variances of both analytical curves were heterogeneous. It is noteworthy that the F-test can only be applied if responses from each level are normally distributed. However, the number of statistics samples of the levels was very small (n=3 and n=4), and, therefore, hardly any hypothesis test will be capable of rejecting the normality approach.

The mathematical procedure most commonly used for regression in analytical chemistry is the ordinary least squares method (OLSM). This method requires two basic conditions: a) the uncertainties of the independent variable must be zero or negligible, b) the uncertainties of the different levels of the dependent variable must be statistically equal. There is a restriction in the second condition which results in heteroscedasticity variances in both curves. Therefore, it is necessary to apply the weighted least squares method (WLSM). It is designed in the same way as OLSM but includes weights for each level of the curve to be inversely proportional to the level of dispersion values so that the distance between the function and the experimental data is primarily reduced to smaller variance levels. The weight used was the inverse of the variance estimated for the data of the replicates [15].

In this work, the slope (a) and intercept (b) of the curves were calculated according to equations 1 and 2 (Table I). The analytical curves are shown in Figure 3.

$$a = \frac{(\sum w_i \times \sum w_i x_i y_i) - (\sum w_i x_i \times \sum w_i y_i)}{(\sum w_i \times \sum w_i x_i^2) - \sum (w_i x_i)^2} \quad (1)$$

$$b = \frac{\sum w_i y_i}{\sum w_i} - a \times \frac{\sum w_i x_i}{\sum w_i} \quad (2)$$

where:

$w_i = \dfrac{1}{s_i^2}$: weight

x_i: azithromycin concentration

y_i: analytical response

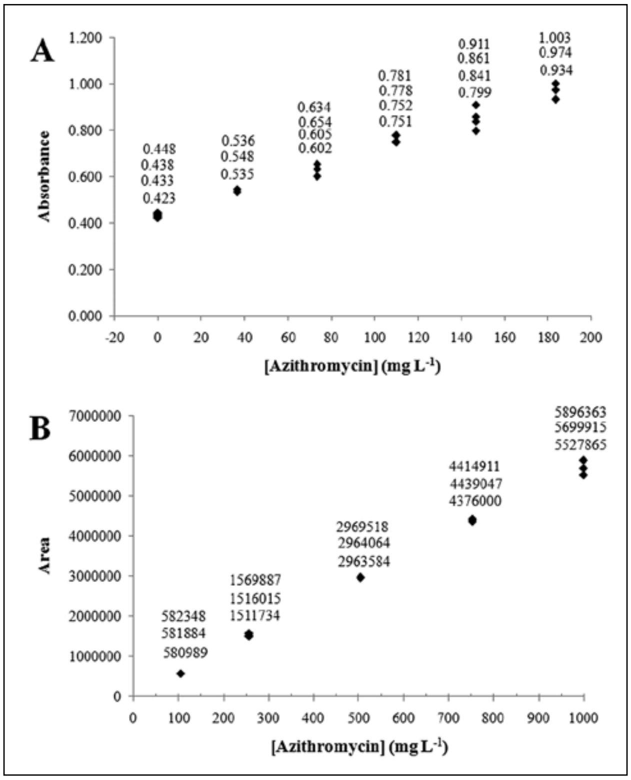

Figure 3. Spectrophotometric standard addition curves (A) and chromatographic external calibration curves (B) for azithromycin determination.

The fit models were evaluated by the correlation coefficient (r_w) (Equation 3). The r-values obtained for the spectrophotometric and chromatographic curves indicated that the linear fit was suitable (Table I).

$$r_w = \frac{S_{xyw}}{\sqrt{S_{xxw}S_{yyw}}} = \frac{\sum_{i=1}^{i}\sum_{j=1}^{j} w_i(x_i-\bar{x})(\bar{y}_{ij}-\bar{y})}{\left[\sum_{i=1}^{i}\sum_{j=1}^{j} w_i\,(x_i-\bar{x})^2 \times \sum_{i=1}^{i}\sum_{j=1}^{j} w_i(\bar{y}_{ij}-\bar{y})^2\right]^{1/2}} \qquad (3)$$

where:
w_i: weight of each level
j: number of replicates
i: number of levels

ANOVA was also performed to evaluate lack of fit. The F-statistic was the ratio of mean squares for lack of fit and pure experimental error (Equation 4) [16]. If the calculated F is less than the critical value, there is no evidence of lack of fit in the model. The F values were 1.53 and 4.36 for the spectrophotometric and chromatographic curves, respectively. The spectrophotometric model showed no evidence of lack of fit at 95% confidence, since $F_{(0.05,4,17)}$ equals 2.96. For the chromatographic model, at 97% confidence ($F_{(0.03,4,10)}$ = 4.52), no evidence of lack of fit was verified.

$$F = \frac{\sum w_i n_i(\hat{y}_i - y_{im})^2 / m-p}{\sum\sum w_i(y_{ij}-y_{im})^2 / nm-m} \qquad (4)$$

where:
w_i: weight of each level
n_i: number of replicates of each level
m: number of levels
p: number of model parameters
nm: total number of measures

3.3.2. Measurement uncertainty

Measurement uncertainty is a non-negative parameter that characterizes the dispersion of the values attributed to a measurand, based on the information used [17]. The estimated uncertainty is fundamental to ensure reliability and comparability of the results of analyses, supporting the decision on the conformity of the product.

The procedure for estimating measurement uncertainty defines the measurand, identifies the sources and quantifies the uncertainties of the input quantities, estimates the combined uncertainty, and declares the expanded uncertainty for a given coverage probability [18].

In these analyzes, six primary sources for both procedures were considered: concentration given by the curve (c), measured sample weight ($w_{measured}$), total sample weight (w_{total}), volume of the volumetric flask in which the sample solution (V_1) was prepared, aliquot of the sample solution

Table I. Results obtained for the validation of the methodologies and quantification of the samples

Method	Sample	Linearity				Content found (mg)	Measurement uncertainty		RSD (%)	LOD (mg L^{-1})	LOQ (mg L^{-1})	R_{mean} (%)
		Slope ± s	Intercept ±s	r_w	$F_{calculated}$		$U_{95\%}$	EVC (%)				
UV	Tablet	$(433 \pm 4) \times 10^{-3}$	$(29 \pm 1) \times 10^{-4}$	0.999	1.53	501	24	4.77	1.00	41	61	100.2
HPLC	Tablet	$(-379 \pm 7) \times 10^{2}$	5958 ± 5	0.999	4.36	501	15	1.05	0.98	103	151	98.4
	Oral suspension					953	27	2.86	1.41			95.8

$F_{(0.05,4,17)} = 2.96$; $F_{(0.03,3,10)} = 4.52$

used for dilution (V_2) and the final dilution volume (V_3). Equations 5 and 6 show the functions of measurement of azithromycin concentrations.

$$c_{UV} = \frac{\left(\frac{-b_0}{b_1}\right).V_1.V_2.w_{total}}{V_3\ .w_{\mathrm{measured}}} \qquad (5)$$

$$c_{HPLC} = \frac{\left(\frac{y-b_0}{b_1}\right).V_1\ .V_2.w_{total}}{V_3\ .w_{\mathrm{measured}}} \qquad (6)$$

where:
C_{UV}: analyte concentration by UV
C_{HPLC}: analyte concentration by HPLC
y: reading of the test sample
b_0: intercept
b_1: slope
V_1, V_2, V_3, w_{total} and $w_{measured}$ were defined previously.

Note that the input quantity (C) is composed of regression parameters b_0 and b_1, the covariance between them, and for the HPLC procedure, the reading of sample *y*. The development of the calculations will justify the option to unify these sources in quantity (c), which is represented in the equations of the measurand by the factor in brackets.

The combination of the uncertainties of these sources by the Law of Propagation results in the combined standard uncertainty. If the effective degree of freedom of the output quantity is sufficiently large or infinite, a multiplicative factor of 1.96 may be used to achieve a coverage probability of 95%. This means that the declared value is approximately the mean of a normal distribution, and the expanded uncertainty covers a symmetric range below 95%. It is recommended that this approximation of the multiplicative factor is done when uncertainties of type B represent the largest contribution to the combined uncertainty, because these functions come from probability densities chosen *a priori* and included in the calculation with infinite degree of freedom. Otherwise, it would be necessary to calculate the effective degrees of freedom of the output quantity by the Welch-Satterthwaite equation [18].

One way to facilitate the propagation of the uncertainty, making it more clear and didactic, is to separate each equation of the measurand, highlighting the factor in brackets, that represents the estimated concentration for each calibration curve (C_{cal}). Thus, the uncertainty of C_{cal} (u_{cal}) can be estimated, to subsequently combine u_{cal} with other sources. Equations 7 and 8 show how to estimate C_{cal}, and equations 9 and 10 to calculate u_{cal}.

$$C_{calUV} = \left(\frac{-b_0}{b_1}\right) \qquad (7)$$

$$C_{calHPLC} = \left(\frac{y-b_0}{b_1}\right) \qquad (8)$$

$$u^2_{C_{calUV}} = (u_{b_0})^2\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_0}}\right)^2 + (u_{b_1})^2\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_1}}\right)^2 + 2cov(b_0,b_1)\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_0}}\right)\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_1}}\right) \qquad (9)$$

$$u^2_{C_{calHPLC}} = (u_y)^2\left(\frac{\partial u_{C_{cal}}}{\partial u_y}\right)^2 + (u_{b0})^2\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_0}}\right)^2 + (u_{b_1})^2\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_1}}\right)^2 + 2cov(b_0,b_1)\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_0}}\right)\left(\frac{\partial u_{C_{cal}}}{\partial u_{b_1}}\right) \qquad (10)$$

Once these estimates are made, the combination of u_{cal} with other sources can be shown in a single equation that fits both procedures (Equation 11).

$$u_C^2 = (u_{C_{cal}})^2\left(\frac{\partial u_C}{\partial u_{C_{cal}}}\right)^2 + (u_{V_1})^2\left(\frac{\partial u_C}{\partial u_{V_1}}\right)^2 + (u_{V_2})^2\left(\frac{\partial u_C}{\partial u_{V_2}}\right)^2 + (u_{w_{total}})^2\left(\frac{\partial u_C}{\partial u_{w_{total}}}\right)^2 + (u_{w_{\mathrm{weight}}})^2\left(\frac{\partial u_C}{\partial u_{w_{\mathrm{weight}}}}\right)^2 + (u_{V_3})^2\left(\frac{\partial u_C}{\partial u_{V_3}}\right)^2 \qquad (11)$$

Propagation from the following equation of the simplified measurand (Equation 12) is then obtained.

$$C = \frac{\mathrm{C}_{cal}.V_1.V_2.w_{total}}{V_3\ .\mathrm{w}_{\mathrm{measured}}} \qquad (12)$$

The values of the expanded uncertainty for the coverage probability of 95% ($U_{95\%}$) are shown in Table I.

The expanded variation coefficient (EVC) shows the percentage of measurement uncertainty in relation to the concentration calculated (Equation 13). The present study considered the uncertainties arising from the calibration curve and the steps of sample preparation. The EVC values were lower than 5% in agreement with the ones commonly reported in the literature [19] (Table I).

$$EVC = \frac{U_C}{C} \times 100 \qquad (13)$$

where:
U_C: expanded uncertainty of the analyte concentration
C: concentration of analyte

3.3.3 Repeatability

Repeatability was evaluated by the relative standard deviation (RSD) of the content found for the samples analyzed under the same conditions and on the same day [20]. The RSD was less than 1.5% by HPLC and equal to 1% by spectrophotometry (Table I).

3.3.4. Trueness

Trueness evaluates the degree of agreement between the values obtained by the standard method and the expected values [15, 17]. In this study, trueness was evaluated by recovery assays (R). The R values (Table I) were within the range of 95-105% as recommended for pharmaceutical formulations. Trueness was studied only as a criterion for quality control and validation parameter, not being inserted in the measurement uncertainty estimate.

3.3.5. Detection and quantification limits

The estimates of the detection (LOD) and quantification (LOQ) limits were performed by the method based on the confidence interval of the calibration curve. The estimate of the analytical signal from the regression equation has a standard error and the product of this error by the appropriate value of Student's t distribution allows calculating the confidence interval of the calibration curve, which has the form of two hyperbolic lines around the curve. The intercept of the upper limit of the confidence interval is known as critical y (y_c) (Equation 14), and its projection at the lower limit provides the LOD (Equation 15) of the method [21]. For LOQ, x_c (Equation 16) is the value of concentration (x) at the point where the linear coefficient of the calibration curve (b_0) equation intersects the curve of the lower limit of the confidence interval, and the projection of x_c at the upper control limit gives the value of y_h (Equation 17). The intersection of y_h with the curve of the lower limit and the projection of this point on the x axis provides the LOQ (Equation 18) [21]. The values of LOD and LOQ obtained (Table I) were adequate for analysis of the pharmaceutical formulations, since the sample solution concentration could be adjusted according to measured sample weight and final solution volume.

$$y_c = b_0 + \left(\sqrt{\frac{\sum y_i - \hat{y}_i}{nm-2}}\right) \times t_{(0.05,nm-2)} \times \sqrt{\left(\frac{1}{nm}\right) + 1 + \frac{\bar{x}^2}{\sum(x_i - \bar{x})^2}} \quad (14)$$

$$LOD = \frac{2}{b_1} \times \left(\sqrt{\frac{\sum y_i - \hat{y}_i}{nm-2}}\right) \times t_{(0.05,nm-2)} \times \sqrt{\left(\frac{1}{nm}\right) + 1 + \frac{(y_c - \bar{y})^2}{b_1^2 \times \sum(x_i - \bar{x})^2}} \quad (15)$$

$$y_h = b_0 + 2 \times \left(\sqrt{\frac{\sum y_i - \hat{y}_i}{nm-2}}\right) \times t_{(0.05,nm-2)} \times \sqrt{\left(\frac{1}{nm}\right) + 1 + \frac{(x_c - \bar{x})^2}{\sum(x_i - \bar{x})^2}} \quad (16)$$

$$x_c = \left(\sqrt{\frac{\sum y_i - \hat{y}_i}{nm-2}}\right) \times \frac{t_{(0.05,nm-2)}}{b_1} \times \sqrt{\left(\frac{1}{nm}\right) + 1 + \frac{\bar{x}^2}{\sum(x_i - \bar{x})^2}} \quad (17)$$

$$LOQ = \left(\frac{y_h - b_0}{b_1}\right) + \left(\sqrt{\frac{\sum y_i - \hat{y}_i}{nm-2}}\right) \times \frac{t_{nm-2}}{b_1} \times \sqrt{\left(\frac{1}{nm}\right) + 1 + \frac{(y_h - \bar{y})^2}{b_1^2 \times \sum(x_i - \bar{x})^2}} \quad (18)$$

3.4. Pharmaceutical formulation analysis

The spectrophotometric methodology allowed azithromycin quantification in tablets with precision and trueness. However, it was not possible to determine azithromycin contents in the oral suspension, due to the high absorption of soluble excipients in ethanol (Figure 4). An experiment using acetonitrile was also performed; however, the problem persisted. It was possible to determine azithromycin in tablets and oral suspension by the chromatographic method. The excipients did not interfere with quantification since they did not co-elute with azithromycin. This could be confirmed by comparing the chromatograms of the oral suspension, standard and oral suspension with addition of standard (Figure 5).

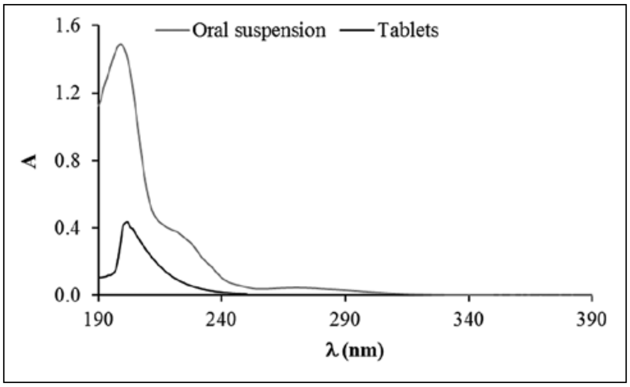

Figure 4. Azithromycin UV spectrum in tablets and oral suspension at the theoretical concentration of 150 mg L^{-1}.

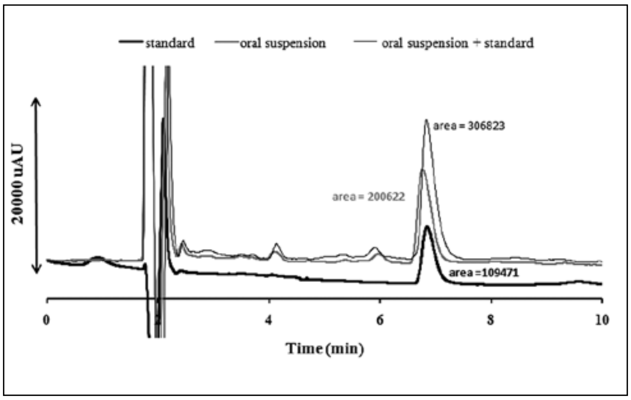

Figure 5. Chromatograms of 250 mg L^{-1} azithromycin standard solution, 500 mg L^{-1} azithromycin in oral suspension, and oral suspension with addition of the same standard solution.

An unpaired t-test with pooled variance was performed to compare the results of azithromycin quantification in tablets by both optimized methodologies, because the variances were considered homogeneous by the F-test. The t value was 0.071, at 95% confidence, and the critical value, $t_{(0.05,6)}$, was 2.45. Therefore, there was no evidence of significant differences between the two methods for azithromycin tablet quantification.

4. Conclusion

In this work, two methods for azithromycin determination in pharmaceutical formulations were developed and validated. The advantages of using the spectrophotometric methodology are its low cost, low maintenance requirements, easy operation and low solvent consumption. However, it presented spectral overlap with excipients in the sample. The chromatographic method allowed azithromycin quantification in tablets and oral suspension, and the excipients did not interfere in the analysis. In addition, an external calibration curve was used for chromatographic determination; this required fewer tests when compared with the standard addition, hence, it is possible to obtain a higher analytical frequency rate. Both methods could be used in routine analyses of azithromycinin pharmaceutical formulations.

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6. References

1. Drew, R.H.; Gallis, H.A., *Pharmacotherapy*, 1992, 12, 161.
2. *Farmacopeia Brasileira*, 2, 5th ed., Brasília, Agência Nacional de Vigilância Sanitária, 2010
3. Shaikh, K.A.; Patil, S.D.; Devkhile, A.B., *J. Pharm. Biomed. Anal.*, 2008, 48, 1481.
4. Yang, Z.Y.; Wang, L.; Tang, X., *J. Pharm. Biomed. Anal.*, 2009, 49, 811.
5. Ghari, T.; Kobarfard, F.; Mortazavi, S.A., *Iran. J. Pharm. Res.*, 2013, 12, 55.
6. Odendaal, R.W.; Liebenberg, W.; Aucamp, M.E., *Pharmazie*, 2012, 67, 984.
7. Gaudin, K.; Kauss, T.; Gaubert, A., *Anal. Lett.*, 2011, 44, 2732.
8. Venkatesh, V.; Prabahar, A.E.; Suresh, P.V.; Maheswar, C.U.; Rao, N.R., *Asian J. Chem.*, 2011, 23, 312.
9. Yanamandra, R.; Chaudhary, A.; Bandaru, S.R.; Patro, B.; Murthy, Y.L.N.; Ramaiah, P.A.; Sastry, C.S.P., *E-J. Chem.*, 2010, 7, S363.
10. Al-Rimawi, F.; Kharoaf, M. , *J. Chromatogr. Sci.*, 2010, 48, 86.
11. http://www.uspbpep.com/usp32/pub/data/v32270/usp32nf27s0_m6740.html, accessed December 2013.
12. Ashour, S.; Bayram, R., *Spectrochim. Acta, Part A,* 2012, 99, 74.
13. Jayanna, B.K.; Nagendrappa, G.; Arunkumar; Gowda, N., *Indian J. Pharm. Sci*., 2012, 74, 365.
14. De Paula, C.E.R.; Almeida, V.G.K.; Cassella, R.J., *J. Braz. Chem. Soc.*, 2010, 21, 1664.
15. *Manual de Garantia da Qualidade Analítica. Ministério da Agricultura, Pecuária e Abastecimento.* Secretaria de Defesa Agropecuária (SDA), 2011. http://www.agricultura.gov.br/arq_editor/file/Manual_Garantia_Analitica.pdf, accessed December 2013.
16. Neto, B.B.; Scarminio, I.S.; Bruns, R.E., *Como Fazer Experimentos*; Campinas, SP; Editora da Unicamp, 2007.
17. International vocabulary of metrology – Basic and general concepts and associated terms (VIM). Joint Committee for Guidelines in Metrology, 2012, nº *JCGM* 200.
18. Evaluation of measurement data – Guide to the expression of uncertainty in measurement. Joint Committee for Guidelines in Metrology, 2008, nº *JCGM* 100.
19. Hund, E.; Luc Massart, D.; Smeyers-Verbeke, J.. *Anal. Chim. Acta*, 2003, 480, 39.
20. ICH Guideline, Q2(R1), 2005, *Validation of Analytical Procedures: Text and Methodologies.* http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf, accessed December 2013.
21. Ribeiro, F.A.L.; Ferreira, M.M.C.; Morano, S.C.; Silva, L.R.; Schneider, R.P.. *Quim. Nova,* 2008, 31, 164.

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Seasonality influence evaluation of metal concentrations in Ondas River Watershed – BA

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Abstract

The present paper aims to evaluate the seasonality influence on chemical species concentrations in the Ondas River watershed (ORW), located in the western region of Bahia, by applying multivariate statistical techniques. Twenty-three elements and eight physical-chemical variables were evaluated at nine different points along the watershed. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) allowed the identification of two distinct groups of water samples, characterized by collections made during the dry and rainy seasons, indicating the seasonality influence on the concentration variation of the evaluated species. However, no significant metal concentration variation was observed along the watershed. Dry period water samples had higher concentrations of heavy metals when compared to the rainy season; furthermore, rainy season samples were richer in sodium, potassium and chloride and showed higher alkalinity and electrical conductivity values.

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Keywords: Ondas River Watershed, metals, seasonality.

Introduction

Obtaining information on water quality is essential to understand environmental processes and their relationship to changes in anthropogenic and/or natural origins. Natural sources may be related to hydrology, climate, geology and soil types that influence water quality [1-3]. Anthropogenic inputs such as agricultural, urban and industrial activities are also representative of the potential changing in aquatic ecosystems [4-7].

Chemical movements and distributions in aquatic systems are dynamic and their changes can be observed according to the time and space in which monitoring is performed. However, there is a sharp change in the concentration of dissolved substances in river water samples in heavy rainfall periods, which is largely attributed to surface runoff. Rainfall variations and, consequently, the runoff effects, among others, have a strong influence on river flow and consequently on the chemical concentrations in river water [8-12].

A large number of samples and variables (e.g., physical, chemical, biological and/or temporal variables) usually should be considered, in order to understand aquatic system dynamics. The relationships between them are also too large to be understood, and a technique that can summarize this large correlation becomes necessary [13].

Data integration for a complex system such as this can be achieved by multivariate statistical processing, which is widely employed to characterize and evaluate the quality of surface waters. This methodology is useful to demonstrate spatial and temporal variations caused by natural and anthropogenic factors associated with seasonality [8,14].

The Ondas River watershed (ORW) is located in the western region of Bahia, located between the 11º59' and 12º32' south parallels and the 45º00' and 46°20' west meridians, with an area of 5157 km ^{2}, and inserted in the regional area of the São Francisco river watershed. The watershed drains two municipalities of this region, which are represented by Barreiras and Luis Eduardo Magalhães cities, respectively, with populations of 137,427 and 60,105 inhabitants [15]. Major ORW tributaries are the Pedras, Borá and Vereda das Lages rivers [16].

From these considerations, a multivariate statistical technique was applied to identify the variable similarities that determine the water quality in the perennial section of the watercourses of the Ondas River watershed during dry and rainy seasons.

Experimental

Study area

The ORW crosses the territorial area of Barreiras and Luiz Eduardo Magalhães cities, located in western Bahia State, Brazil. The main stream (Ondas River) is about 730 km long and its tributaries, the Borá and Pedras rivers are, respectively, 121 km and 344 km long [17]. Many activities related to agricultural (irrigation and a small hydroelectric facility center) and urban activities (towns/cities) are encoun-

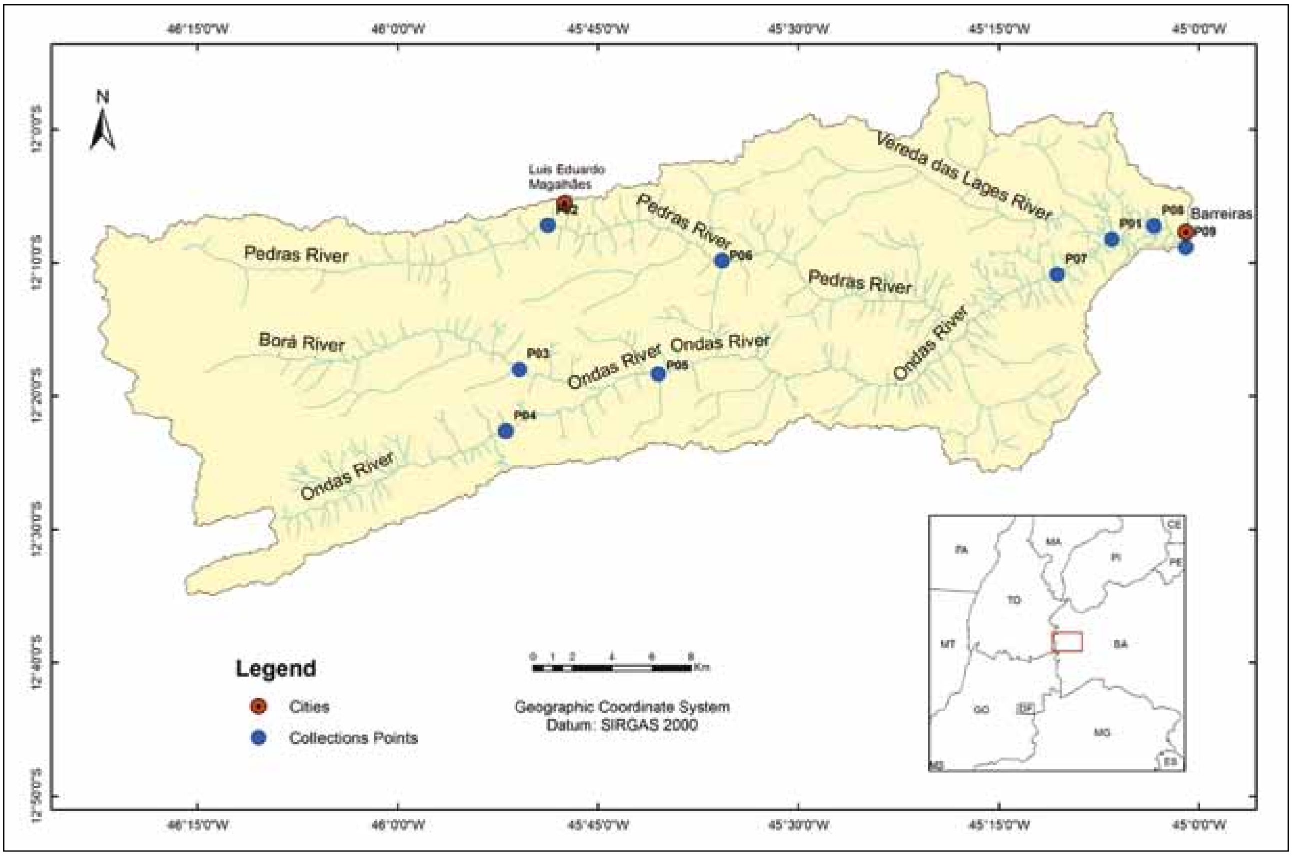

Figure 1: Geographic location of the Ondas River watershed.

tered along its course that, combined with climatic conditions (rain and drought), lead to river pollution. There are few mineral and industrial activities in the region, although recently in the watershed area, a large deposit of thallium associated with cobalt and manganese has been discovered, still under study for extraction [18].

ORW presents an exoreic drainage pattern, draining to the northeast with channels hierarchy ranging from first to fourth order, with 459 (four hundred fifty-nine) channels, from which 372 (three hundred seventy-two) are first order [19]. The amount of first-order channels indicates that the groundwater is not too deep, favoring irrigated farming, livestock and other activities.

The **regional** geology shows that about 97%, of the area corresponds to the Urucuia Group [20,21] (upper-cretaceous of the San Franciscana Basin), and only 3% of the area is covered by the Bambuí Group slates and carbonate sediments.

The Bambuí Group slates and carbonate rocks occupy just small areas at the easternmost portion of the basin and the few outcrops show the dominance of argillaceous rocks over limestone or other carbonates facies.

The Urucuia Group is represented by eolian and fluvial mature sandstone and conglomerate. The sandstone and the minor conglomerates show only siliceous cement and, in the majority of the cases, low contents of clay matrix or pseudo matrix. Locally iron or manganese oxide minerals can be observed as well developed cements. These types of cement occur in shallow sandstone beds, showing a recent diagenetic phase.

The top soil is developed by the decomposition of sandstone, resulting predominantly in sandy covers of different colors [19]. Deep wells for groundwater production (up to 300 meters) show the presence of sandstone, argillite and fine conglomerates associated with the Lower Cretaceous Areado Formation. The Areado Group rocks commonly show carbonate cement as a digenetic mineral phase.

According to CPRM [22] (Brazilian Geological Survey) the largest watercourse channels are filled by alluvial deposits, and sediment composed by sand and gravel [20].

According to studies by Soares Neto [16], six soil types have been identified in this region: **Reddish-yellow Oxisol**, developed by changes in the Urucuia group sandstone (covering most of the area of ORW); **Sandy Andisol** (consisting predominantly of quartz) derived from the Urucuia group sandstone; **Spododol** with predominant sandy texture, formed by colluvial/alluvial deposition of transported materials from the slopes; **Reddish-yellow Alfisol** (less representative), found only in depression parts; **Fluvic Entisol**, formed by recent sediment depositions of different particle size and **Incepsol**, shallow and poorly developed, being sometimes rocky and gravelly, often associated with rock outcrops.

The location of the study area, as well the details of the sampling sites are shown in Figure 1 and Table I, respectively.

The point P01 is characterized as an area with the river's starting point, which also forms the Vereda das Lages River. There is the presence of several small farms and domestic wastewater release into the river.

Points P02, P03 and P04 refer to the Pedras River, the Borá River and the Ondas River respectively, which cross the BR 020 going towards Brasilia-DF. These points are located in areas of extensive irrigated agriculture.

Table I. Sampling points and their characteristics in the study area

Points	Coordinates	Use and Occupancy Area
P01 – Vereda das Lages River	12°8′14.14″S 45°06′32.57″W	Headwaters and presence of small farms with domestic wastewater release.
P02 – Pedras River	12°7′10.98″S 45°48′45.61″W	Next to large areas occupied for grain cultivation.
P03 – Borá River	12°17′58.99″S 45°50′54.63″W	Next to large areas occupied for grain cultivation.
P04 – Ondas River	12°22′36.30″S 45°51′55.12″W	Next to large areas occupied for grain cultivation.
P05 – Ondas River	12°18′20.32″S 45°40′30.79″W	Close to large areas for grain cultivation with operation of a small hydropower plant (SHP).
P06 – Pedras River	12°9′49.61″S 45°35′44.56″W	Close to villages and populated areas; also grain cultivation.
P07 – Ondas River	12°10′51.60″S 45°10′37.92″W	Close to villages and presence of small farms with discharge of domestic sewage.
P08 – Ondas River	12°7′13.29″S 45°3′23.00″W	Close to villages and presence of small farms with discharge of domestic sewage.
P09 – Ondas River	12°8′49.45″S 45°0′58.55″W	Near the urban area of Barreiras.

The P05 point is located after the confluence of the Ondas and Borá Rivers and is close to a village and a small hydroelectric plant (SHP) which provides irrigation. The P06 point is located in Pedras River near a village and also an area of grain production/irrigation.

P07 point is located in an areas of small farms, but with little presence of people. At this point there are feasibility studies for thallium mining.

P08 and P09 points are located near the Ondas river outlet and are characterized by the existence of several farms and proximity to the Barreiras urban area. People living in this region do not have basic sanitation or public drinking water supplies.

Sampling plan

Land use and current occupation status were analyzed from Google Earth Professional software images. The image interpretation followed certain patterns of colors, textures and shapes, which indicated land use and occupation. For example, smooth aspects in the band that best reflects the green, corresponds to canopy uniformity, thus reforestation areas have more smooth and homogeneous appearances than nature reserves areas, native Cerrado vegetation areas are less rough when compared to forests [23]. Irrigated areas show, besides a uniform appearance, bright green color and irrigation systems and irrigation inputs can be easily identified by a circle. The differences in shades of green indicate changes in floristic composition in native areas and vigor in agricultural areas. Urban areas, deforestation and areas prepared for planting or at early stage of cultivation (***e.g.,*** exposed soil) show colors ranging from pink to reddish tones. These bands best reflect red, and usually have more regular shapes.

Analytical data for the selected indicators were compared with the types of land use and occupation identified in the images, considering the region around each sampling point. The same information was observed in the field during the step of sampling.

Sampling and analytical methods

Sample collection was seasonal in order to describe water quality throughout periods of drought and rain. The dry season covers the months from April to September and the rainy months are from October to March, according to data from the available historical series of rainfall stations in the study area.

Water samples were collected in inert plastic bottles (PVC), previously washed with a 10% HNO_3 aqueous solution (v/v), and packed in a Styrofoam box. These samples were filtered through a 0.45 mm membrane and part of the filtered samples were preserved by adding dilute nitric acid to pH 1.5.

Water sample pH, temperature, conductivity and salinity were analyzed, during sampling, using a WTW Multi 340i multiparameter probe. Total solids and turbidity parameters were determined in the laboratory. Alkalinity was determined with an automatic titrator (Schott Trito Easy Line) using 0.02 mol L^{-1} H_2SO_4 standardized with 0.05 mol L^{-1} NaOH.

After filtration, the chemical elements (Ca^{2+}, Fe, Mn, Ba, Cd, Mg, Zr, Cu and Cr) were determined by an atomic emission spectrometer with inductively coupled plasma (ICP-AES) using a Spectroflame FVM03 from Spectro Analytical Instruments. Na^+ and K^+ determinations were performed with an atomic absorption spectrophotometer (AAS) using an Analyst 200 Atomic Absorption Spectrometer from Perkin Elmer. Samples without acidification were used for anion determinations (F^-, Cl^-, NO_3^-, SO_4^{2-} and PO_4^{2-}) by ion chromatography (IC) with suppressed conductivity detection (Dionex, model ICS-Ion Chromatography System 90). All chemical analyses were performed using recommended methods from *Standard Methods for the Examination of Water and Wastewater* [24].

Statistical analysis and data interpretation

Identification of possible factors or sources that affect aquatic system quality during dry and rainy periods was performed by application of multivariate statistical techniques using principal component analysis (PCA). In these multivariate procedures, means and principal components were applied to the values found for the elements using STATISTIC Version 7.0 software for statistical analysis [25].

Results and discussion

Water metals distribution

The metal determination results and physicochemical variables for the two seasonal differences, between dry and wet periods, are provided in Tables II and III, respectively. Brazilian maximum values parameters for water quality evaluation are established by CONAMA Resolution 357/05 [26]. Concentrations of Al, Co, Cr, Cu, Fe and Ni exceeded the limits prescribed by CONAMA Resolution 357/05 in both periods. It was also observed that for the rainy months the samples showed higher conductivity, as well as higher concentrations of Ca, K, Mg and Na. This indicates the influence of species leached from the soil as well as domestic sewage, since other mineral concentrations in the rainy season are lower [27]. This may be attributed to the high evaporation and intense anthropogenic activities (increase of agriculture) in summer [27,28]. The diluting effect due to heavy rainfall resulted in the consequent reductions of the total concentrations of the metals. Variations up to 80% annual precipitation in the rainy season subsequently dilute the river pollutants, especially in the agricultural area. Thus, precipitation and seasonal anthropogenic activities played important roles in influencing the concentration of heavy metals in the surface water of the reservoirs [29]. During the study period, the analyzed metals showed no significant spatial variations, except for the P01 point (rainy season). This point showed higher concentrations for all studied variables in relation to the other points.

The overland washout during the heavy rains period is considered the main explanation for element distributions, since the strong discrepancy of the metal content is linked to the rain regime. The possible geogenic or pedogenic origins of the metals or their temporal incongruity cannot be considered for the watershed study. The cretaceous rocks are mainly composed of siliceous mineral phases (quartz and silica cement) which are poorly reactive and show low solubility in the weathering environment. The small amount of oxide and clay minerals in rock and soil are represented by kaolinite, gibbsite, hematite and goethite, all stable minerals with low contents of Na, Ca, Mg, K, and other elements studied.

Table II. Metal concentrations - Dry periods

	Unit	Point 1	Point 2	Point 3	Point 4	Point 5	Point 6	Point 7	Point 8	Point 9	CONAMA 357/05
pH	-	5.1	5.2	5.3	5.4	5.8	5.3	6.5	6.8	6.8	6.0 – 9.0 (*)
Temp	ºC	21.5	24.4	23.7	23.9	24.7	24.4	26.6	27.1	27	-
Electrical Cond.	µS/cm	12	4	4	0.1	4	3	12	9	7	-
Alkalinity	mg/L	3.82	0.54	1.35	1.46	2.04	1.81	1.27	1.12	2.12	-
Turbidity	uT	0.3	0.12	0	0.1	0.25	0.36	0.16	0.17	0.26	100 (*)
Sodium	mg/L	0.24	0.19	0.19	0.17	0.22	0.28	0.29	0.31	0.31	200 (**)
Potassium	mg/L	0.05	0.04	0.06	0.06	0.15	0.28	0.31	0.32	0.32	-
Chlorides	mg/L	3.25	0.33	0.22	0.21	0.45	0.15	0.48	0.23	0.84	250 (***)
Strontium	mg/L	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	-
Europium	mg/L	0.10	0.09	0.08	0.09	0.09	0.08	0.08	0.08	0.08	-
Titanium	mg/L	0.29	0.26	0.23	0.26	0.26	0.23	0.24	0.23	0.23	-
Calcium	mg/L	0.88	0.60	0.56	0.63	0.86	1.41	1.52	1.58	1.42	-
Vanadium	mg/L	0.43	0.39	0.35	0.38	0.39	0.36	0.37	0.35	0.35	0.1 (*)
Magnesium	mg/L	1.92	1.81	1.57	1.70	1.69	1.66	1.76	1.69	1.60	-
Iron	mg/L	0.34	0.31	0.27	0.27	0.31	1.70	1.32	2.02	0.41	0.3 (***)
Nickel	mg/L	0.53	0.48	0.35	0.42	0.40	0.31	0.38	0.50	0.31	0.025 (*)
Zirconium	mg/L	0.27	0.24	0.21	0.24	0.24	0.22	0.22	0.22	0.21	-
Copper	mg/L	0.25	0.23	0.20	0.22	0.23	0.20	0.22	0.20	0.20	0.009 (*)
Aluminum	mg/L	2.44	2.26	2.07	2.22	2.30	2.17	2.19	2.14	1.97	0.1 (*)
Chromium	mg/L	0.32	0.30	0.26	0.28	0.29	0.27	0.26	0.25	0.25	0.05 (***)
Manganese	mg/L	0.04	0.04	0.03	0.04	0.04	0.05	0.04	0.05	0.04	0.1 (***)
Barium	mg/L	0.14	0.09	0.09	0.09	0.10	0.10	0.09	0.10	0.08	0.7 (***)
Cobalt	mg/L	0.47	0.42	0.29	0.40	0.42	0.38	0.39	0.38	0.35	0.05 (*)
Zinc	mg/L	0.17	0.09	0.09	0.10	0.10	0.10	0.09	0.08	0.05	0.18 (*)

(*) Class 2 CONAMA 357/05; (**) Limits for human consumption, CONAMA 396/08; (***) Maximum Allowable Values for the two CONAMA resolutions.

Table III. Metal concentrations - Rainy periods

	Unit	Point 1	Point 2	Point 3	Point 4	Point 5	Point 6	Point 7	Point 8	Point 9	CONAMA 357/05
pH	-	6.2	5.7	5.6	5.7	5.8	6.1	5.9	5.9	6.0	6.0 – 9.0 (*)
Temp	ºC	27.8	25.1	24.2	24.4	28.7	26.4	28.3	29.1	29.3	-
Electrical Cond.	µS/cm	20	6	6	7	6	7	7	8	7	-
Alkalinity	mg/L	4.10	1.54	1.71	1.46	2.07	2.81	2.27	3.22	3.12	-
Turbidity	uT	9.78	0.17	0.26	1.51	0.64	0.72	3.39	5.56	8.65	100 (*)
Sodium	mg/L	1.49	0.28	0.28	0.20	0.37	0.41	0.42	0.54	0.45	200 (**)
Potassium	mg/L	1.30	0.03	0.05	0.05	0.31	0.33	0.37	0.43	0.43	-
Chlorides	mg/L	2.55	0.53	0.52	0.31	0.55	0.55	0.48	0.45	0.94	250 (***)
Strontium	mg/L	0.01	0.004	0.004	0.002	0.004	0.005	0.004	0.004	0.006	-
Europium	mg/L	0.03	0.02	0.03	0.02	0.03	0.03	0.01	0.01	0.02	-
Titanium	mg/L	0.07	0.07	0.09	0.06	0.07	0.07	0.04	0.03	0.06	-
Calcium	mg/L	2.24	1.33	1.31	1.22	1.65	1.63	1.93	1.73	1.81	-
Vanadium	mg/L	0.11	0.12	0.12	0.08	0.10	0.12	0.07	0.04	0.09	0.1 (*)
Magnesium	mg/L	1.92	1.81	1.57	1.70	1.69	1.66	1.76	1.69	1.60	-
Iron	mg/L	0.11	0.21	0.22	0.20	0.20	0.26	0.20	0.20	0.22	0.3 (***)
Nickel	mg/L	0.59	0.49	0.46	0.47	0.51	0.51	0.47	0.52	0.42	0.025 (*)
Zirconium	mg/L	0.08	0.08	0.08	0.06	0.07	0.08	0.05	0.04	0.06	-
Copper	mg/L	0.10	0.07	0.07	0.05	0.06	0.06	0.03	0.02	0.04	0.009 (*)
Aluminum	mg/L	0.89	0.85	0.96	0.83	0.93	0.93	0.68	0.58	0.76	0.1 (*)
Chromium	mg/L	0.09	0.07	0.10	0.06	0.08	0.08	0.04	0.04	0.07	0.05 (***)
Manganese	mg/L	0.04	0.04	0.03	0.04	0.04	0.05	0.04	0.05	0.04	0.1 (***)
Barium	mg/L	0.04	0.04	0.03	0.02	0.04	0.04	0.04	0.05	0.03	0.7 (***)
Cobalt	mg/L	0.32	0.31	0.31	0.27	0.32	0.30	0.27	0.26	0.29	0.05 (*)
Zinc	mg/L	0.11	0	0	0.01	0.02	0.04	0	0.04	0	0.18 (*)

(*) Class 2 CONAMA 357/05; (**) Limits for human consumption, CONAMA 396/08; (***) Maximum Allowable Values for the two CONAMA resolutions.

In this sense, the seasonal distribution of the major elements and also of the minor metal content is strongly related to the surface washing by superficial water from different land uses. Intensive agricultural use is considered one of the most important, because of the high volumes of agrochemical and chemical fertilizers necessary for the annual crops. Nitrogen-potassium-phosphate soluble fertilizers are intensively launched by year on a regular basis, as well as herbicides and insecticides. All these products may show metal or organic radicals in their formulae and can easily explain the occurrence of the studied elements in the surface water resources and in the groundwater.

The iron and manganese can be at least in part attributed to geogenic and/pedogenic origins. These metals are quite abundant in the oxides and hydroxides present even in the soil cover (mainly the Oxisol) and in the sandstone (that may be locally cemented by oxide minerals).

Principal component analysis (PCA)

The experimental work involved analyses of the metals and of physical-chemical parameters for seasonal differences between two different dry and wet periods; all samples were analyzed in triplicate. Thus, a total of 27 experiments were performed for the determination of Al, Ba, Ca, Cl, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Sr, Ti, V, Y, Zn and Zr in river samples. Moreover, physicochemical variables were evaluated: alkalinity, conductivity, pH, temperature and turbidity. PCA was adopted to assist in the interpretation of elemental data. This powerful method allows identifying the different groups of metals that correlate and thus can be considered as having a similar behavior and common origin.

The data obtained in these fifty-four analyses were evaluated using PCA. The 19 elements and five physical-chemical variables were established as columns and the river water samples as rows. The evaluation was performed on autoscaled data due to the different orders of magnitude in element concentrations.

Since the decision of how many components to extract is based on Eigen values, screen plots and component interpretability, subsequent analyses were done with both more and fewer components than the default to determine which number of factors was optimum [30].

The loadings of original variables for the first and second principal component are showed in Table IV, they represent 62.25% and 17.96% of the total variance, respectively. Therefore, the first two principal components can model the data because they describe 80.21% of the total variance. Figure 2 presents the PC1 X PC2 scores plot resulting from the PCA analysis.

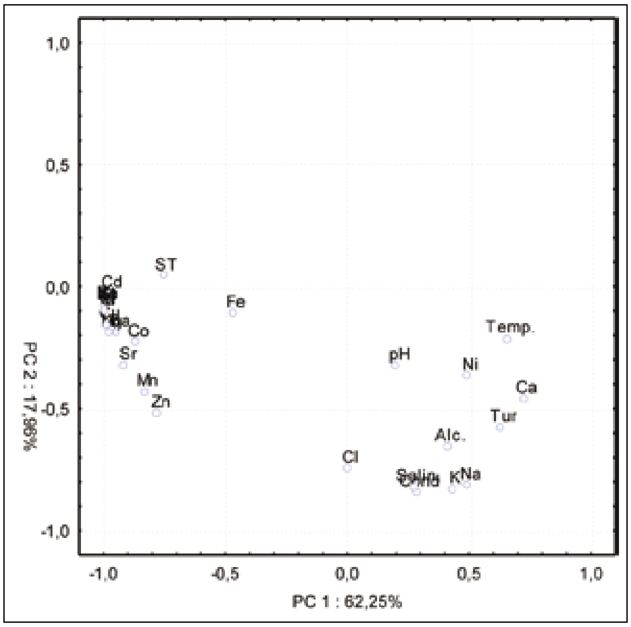

Figure 2. Loading of the variables of the first two principal components

Table IV. Loading matrix from principal component analysis

	PC 1	PC 2
pH	0.205467	-0.326176
Cond	0.287064	-0.846236
Temp.	0.659463	-0.215036
Alc.	0.413634	-0.658610
Tur	0.628601	-0.579216
Na	0.496313	-0817191
K	0.433641	-0831650
Cl	0.004547	-0.748826
Sr	-0.909647	-0.324671
Y	-0.991015	-0.101875
Ti	-0.993322	-0.086442
Ca	0.726214	-0.462352
V	-0.993980	-0.086066
Mg	-0.974980	-0.190222
Fe	-0.466274	-0.111315
Ni	0.495510	-0.368349
Zr	-0.992095	-0.103585
Cu	-0.983081	-0.156770
Al	-0.994307	-0.080103
Cr	-0.991336	-0.099385
Mn	-0.828051	-0.432871
Ba	-0.941828	-0.188539
Co	-0.865033	-0.231042
Zn	-0.772286	-0.521749

The first component (PC1) was associated with Al, Ba, Cr and Mn. These components may be associated to manganese concentration present in this regions [31]. The second factor (PC2) can be in part associated to the geological formation of the watershed. The second component was comprised of high Na and K loadings and must be related to an anthropogenic origin.

As can be seen in Figure 3, two clusters of the analyzed samples are formed, one with the rainy period samples and another with the dry period samples. A complete separation between samples of rainy and dry periods can also be observed. PCA also shows that dry period samples are richer in heavy metals than the rainy period ones, whose samples are richer in Na, K and Cl but have lower alkalinity, conductivity, and salinity parameters. The separation occurs because the first PC has 21 parameters with large loadings including 18 with large negative loadings. Samples from the dry period have higher concentrations of these mentioned parameters than samples of the rainy season.

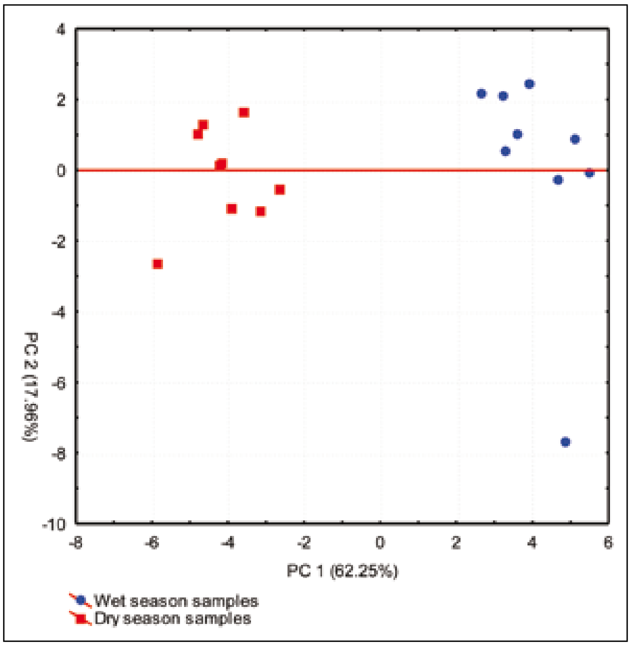

Figure 3. Scatter plot of the scores on the first two principal components

Hierarchical cluster analysis (HCA)

HCA was carried out by Ward's procedure, applied on the Euclidean distances and the resulting dendrogram is depicted in Figure 4.

HCA was applied to Al, Ba, Ca, Cl, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Sr, Ti, V, Y, Zn, Zr, alkalinity, conductivity, pH, temperature, turbidity physicochemical variables in an attempt to comprehend how they are associated and its seasonality relationship. This analysis produced two variable clusters, namely dry (A) and rainy (B) seasons, which can be seen at linkage distance 10 (Figure 4). The observed results in the dendogram are in agreement with results achieved by Carvalho [32], increased river water flows, triggered by rains, can contribute to dilute the metals concentration in the aquatic environment.

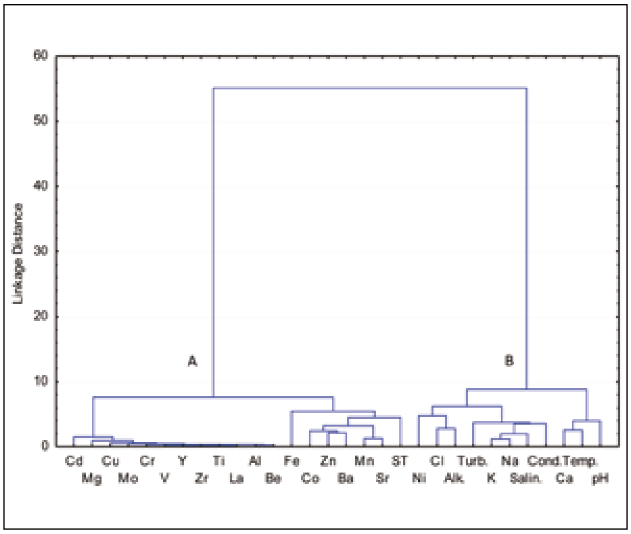

Figure 4. Variables Dendrogram. A = Dry period and B = Rainy period

Conclusions

The low total dissolved solids of the studied waters (6 to 20 µS/cm in the rainy season and 3 to 12 µS/cm in the dry period) are compatible with the geology and main soil types of the watershed. Mature sandstone with silica cement functions as a poorly reactive material and cannot be an important source of metals or other elements. The reddish-yellow Oxisol that is widespread in the principal study area, in the same way, shows low contents of soluble phases and will contribute little to the mineralization of the surface water and the shallow groundwater.

The PCA and HCA techniques allowed the identification of two distinct groups formed by dry and rainy period samples, indicating that water quality, in the watershed, is influenced by seasonality. During the study period, the heavy metals studied did not show significant spatial variations. PCA showed that the dry period samples are richer in heavy metals than the rainy period samples and that the rainy season samples have higher sodium, potassium, chloride, alkalinity, and conductivity parameters. This behavior is compatible to the overland washing by rainwater that put in solution part of the metals and other substances applied in the different land uses in the watershed studied.

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References

1. Mcmahon, G.; Harned D. A.; *Environ. Manage.*, 1998, *22,* 887.
2. Libos, M.; Rotunno Filho, O. C.; Zeilhofer, P.; *Rev. Bras. Recursos Hídricos*, 2003, *8*, 115.
3. Xie, X.; Norra, S.; Berner, Z.; Uben, D. A. St.; *Water, Air, Soil Pollut.*, 2005, *167,* 39.
4. Pan, Y.; Herlihy, A.; Kaufmann, P.; Wigington, J.; Van Sickle, J.; *Hydrobiologia*, 2004, *515,* 59.
5. Wantzen, K. M.; Siqueira, A.; Cunha, C. N.; Sá, M. F. P.; *Ecosyst.*, 2006, *16* 713.
6. Azrina, M. Z.; Yap, C. K.; Ismail, A. R.; TAN, S. G.; *Ecotoxicol. Environ. Saf.*, 2006, *64,* 337.
7. Marques, M. N.; Cotrim, M. B.; Pires, M. A. F.; Beltrame Filho, O.; *Quim. Nova*, 2007, *30,* 1171.
8. Vega, M.; Pardo, R.; Barrado, E.; Deban, L.; *Water Res*. 1998, *32,* 3581.
9. Malik, R. N.; Nadeem. M.; *Environ. Geochem. Health*, 2011, *33,* 525.
10. De Andrade, E. M.; Araújo, L. De F. P.; Rosa, M. F.; Disney, W.; Alves, A. B.; *Eng. Agríc. Jaboticabal*, 2007, *27,* 683.
11. Santos, I. N.; Horbe, A. M. C.; Silva, M. S. R.; Miranda, S. A. F.; *Acta Amazonica*, 2006, *36,* 229.
12. Silva Jr., R. O.; Dall'Agnol, R.; Oliveira, E. P.; *Geoch. Bras.*, 1999, *13(2),* 163.
13. Dillon, W. R.; Goldstein, M.; *Multivariate Analysis. Methods and Applications*, Wiley, New York, 1984.
14. Moura, L. H. A.; Boaventura, G. R.; Pinelli, M. P.; *Quim. Nova*, 2010, *33,* 97.
15. <http://www.ibge.gov.br/cidadesat/topwindow.htm>. accessed in May 2012.
16. Soares Neto, J.P.; Souza, N. M.; *Magistra*, 2007, *19*, 104.
17. Amorim Junior, V.; Lima, O. A. L; *R. Bras. Geogr.* 2007, *25*, *117*.
18. Morais, W. C. J. & Muraro, C. C. In: *Âmbito Jurídico, Rio Grande*, XVI, n. 117, october, 2013. Disponível em: <http://www.ambito-juridico.com.br/site/?n_link=revista_artigos_leitura&artigo_id=13723>, accessed in June 2014.
19. CPRM. 2008. Carta geológica do Brasil ao milionésimo. Disponível em: <http://www.cprm.gov.br> Acesso em 10 de junho de 2013.
20. Campos, J.E.G. & Dardenne, M.A. *Rev. Bras. de Geociênc*. 1997, 27(3): 269-283.
21. Campos, J.E.G. & Dardenne, M.A. *Geocienc*. 1999,18(2): 481-499.
22. Castro, K. B.; Martins, E. S.; Gomes, M. P.; Reatto, A.; Lopes, C. A.; Passo, D. P.; Lima, L. A. S.; Cardoso, W. S.; Carvalho Junior, O. A.; Gomes, R. A. T.; *Bol. Pesqui. Desenvolv./ Embrapa Cerrado*. 2010. *288. 32p.*
23. Miranda, H. Sato, M. N. In:*Efeitos do Fogo na Vegetação Lenhosa do Cerrado*; Miranda, H.; Sato, M., Scario, A.; Souza-Silva, J.C.; Felfili, J.M. eds.;) Ministério do Meio Ambiente, Brasília, 2005.
24. Clesceri, L. S.; Greenberg, A. E.; Eaton, A. D.; *Standard Methods for the Examination of Water and Waste Water*. 20th ed.; APHA, AWWA, WEF, Washington, 1998.
25. Stafsoft, Inc., STATISTICA for Windows (Computer Program Manual), Tulsa, 2005.
26. http://www.in.gov.br/visualiza/index.jsp?data=18/03/2005&jornal=1&pagina= 58&totalArquivos=192, accessed in April 2012.
27. Soma, G.; Abhay Kumar, S.; *J. Hazard. Mater.*, Doi.org/10.1016/j.jhazmat.2013.09.067.
28. Olias, M.; Nieto, J. M.; Sarmiento, A. M.; Ceron, J. C.; Canovas, C. R.; *Sci. Total Environ*. 2004, *333*, 267.
29. Li, S.; Zhang, Q.; *J. Hazard. Mater*. 2010, *181,* 1051.
30. Hair Jr. J. F.; Anderson, R. E.; Tatham, R. L.; Black, W. C.*; Multivariate Data Analysis*, 5th ed.;. Macmillan, New York, 1998.
31. http://www.ufrgs.br/sbmqanaisq04qapresenta4junho.pdf, accessed in April 2014.
32. Carvalho, C. E. V.; *Environ. Geol.*1999, *37*, 297.

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Multi-product calibration: preliminary studies to determine quality parameters in industrialized juices based on ultraviolet spectroscopy

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Abstract

Fast determination of constituents such as carbohydrates and vitamin C in industrialized juices was obtained through the development of a multivariate calibration model by relating the ultraviolet spectra and the standard method to its determination. Knowing that the maintenance of the multivariate calibration models can be laborious, in order to keep the models updated it is worthwhile to investigate if multi-product models can be developed. In this study five different flavored industrialized juices with known carbohydrates and vitamin C contents were analyzed by ultraviolet spectroscopy. In total, 50 samples were analyzed covering all five products and a carbohydrates and vitamin C content range of 0.10 to 0.15 g/mL and 0.03 to 0.60 g/mL, respectively. A linear model based on partial least squares regression was evaluated. Thirty-three samples were used for model development and seventeen samples were used for the independent validation set. Feasible results were obtained for carbohydrates and vitamin C with RMSEP values of 1.26×10^{-4} g/mL and 0.0099 g/mL of juice, respectively. For both carbohydrates and vitamin C the fit presented correlation coefficients on the order of 0.99. These results indicate that the models developed for carbohydrates and vitamin C quantification can be used as an alternative to the standard procedures used nowadays in the juice industry, providing advantages such as low waste generation and fast determinations.

Keywords: vitamin C, carbohydrates, spectroscopy, quantification, PLS.

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1. Introduction

Spectrometric techniques can be used together with multivariate calibration to determine various compounds. For the determination of liquid samples, UV-Vis spectroscopy can be used [1] since the region contains information about the chemical structures of the compounds due to chromophore absorptions. There are several papers combining UV-Vis and multivariate calibration to quantify various constituents in samples such as pharmaceuticals [1, 2], petroleum [3] and beverages [4].

Maintenance of the multivariate calibration models can be laborious. Thus, to save time on updates, it is worthwhile to investigate if multi-product models can be developed [5]. The disadvantage of using a local procedure (one-product calibration) is that a large number of computations are required for each prediction in routine analysis, each specific calibration equation can only be used for a small population of samples and each sample must be clearly identified to be able to select the best prediction equation [6].

To deal with those disadvantages an alternative option that has not been much explored to date could be multi-product calibrations. The early studies came from 1992 [7] and 1994 [8] and were done on relatively homogeneous data sets, having as the primary goals evaluations of the performance of new algorithms. Berzaghi et al. [6] worked with a large and non homogeneous data set, but their research focused only on the performance of their weighted regression method, called LOCAL by the authors. In 2006, Micklander et al. [5] introduced multi-product calibration. This study included five different linear and non-linear calibration methods (partial least squares regression, neural networks and three local regression techniques). Different pre-processing methods were tested by the authors (multiplicative scatter correction, first and second order derivatives, extended inverted signal correction, standard normal variate transformation and second order derivatives combined with multiplicative scatter correction). The authors' conclusions were that the local regression models gave the smallest root mean square error of prediction, followed by neural networks, whereas partial least squares regression (PLS) presented the worst results. These methods are not easily implemented in routine analysis in industry because even nowadays many industries are resistant to implementing spectroscopy combined with chemometrics in its quality control systems due to problems related to the acquisition and/or maintenance of the calibration models. Moreover, when using spectroscopy and chemometrics, industries usually buy previously built models from another company, often not providing confident results due to the fact that the models were developed using different conditions compared to the new ones that will be used. Even with PLS, which has the ability to model interferences, the constructed model can make confident predictions only in samples containing interferences that were modeled [9]. Regression methods mathematically more complex than PLS and the use of many types of preprocessing can negatively compromise the implementation of these methods in practical applications, such as in industry. Recently, an application of multi-product multivariate calibration based on PLS was proposed in the literature to quantify some constituents of coffee and banana residues [10], but the authors did not denominate these as multi-product. From these observations, the objective of the present study was to evaluate a PLS model using multi-product calibration and UV spectroscopy to determine quality parameters in the juice industry.

2. Experimental

2.1. Samples

The industrialized juices were acquired in Paraná State (Brazil). The commercially-available juices presented flavors of peach, passion fruit, mango, orange and pineapple.

2.2. Experimental parameters and software

Ultraviolet spectra were acquired with a PG Instrument Ltd spectrometer, model T80+, in the range 200 to 400 nm (steps of 1 nm) using a 1 mm quartz cuvette. Calibration models were developed using the PLS-Toolbox version 5.22 from Eigen_Vector Technology for Matlab R2007b (The Mathworks Inc., Natick, MA, USA), based on a PLS1 method. The models were evaluated using the values of the fit correlation coefficient (R), obtained from the fit between *Yi versus ŷ,* and accuracy that, in multivariate calibration, is generally expressed as the Root Mean Squares Error of Prediction (RMSEP), calibration (RMSEC) and cross-validation (RMSECV) (equations (equations 1-3):

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{nv}(y_i - \hat{y}_i)^2}{nv}} \qquad (1)$$

$$RMSEC = \sqrt{\frac{\sum_{i=1}^{nv}(y_i - \hat{y}_i)^2}{nc}} \qquad (2)$$

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{nv}(y_i - \hat{y}_i)^2}{ncv}} \qquad (3)$$

where *nv* is the number of samples in the validation set, y_i is the reference value of the sample *i* and *ŷ* is the predicted value of the sample *i*, *nc* is the number of samples in the calibration set, and *ncv* is the number of samples in the cross-validation set. The accuracy expressed by equations 1 to 3 assumes that the error in the reference values is negligible. In applications where this assumption cannot be made this error should be taken into account, as discussed by Faber and Kowalski [11].

The occurrence of systematic errors in the model was investigated by a *t*-test described in ASTM E1655-05 [12]. First, an average bias is calculated for the validation set:

$$bias = \frac{\sum_{i}^{nv}(y_i - \hat{y}_i)}{nv} \qquad (4)$$

Then the standard deviation of validation (*SDV*) is obtained by:

$$SDV = \sqrt{\frac{\sum[(y_i - \hat{y}_i) - bias]^2}{nv - 1}} \qquad (5)$$

Finally, the t value is given by:

$$t_{bias} = \frac{|bias|\sqrt{nv}}{SDV} \qquad (6)$$

If the t_{bias} calculated is greater than the critical *t* value at the critical confidence level, there is evidence that the bias included in the multivariate model is significant.

A total of 50 samples of juices were used in this work. Each sample was submitted to conventional analysis, according to the official methods for carbohydrates [13] and vitamin C [14] and the results were used as reference values (*y*) for model development.

3. Results and Discussion

Figure 1 shows the spectra of the five juices, each with a different flavor (A to E) and of all 50 samples together (F). These samples, for calculations purposes, were split into 33 calibration samples and 17 validation ones, by using the Kennard-Stone algorithm [15].

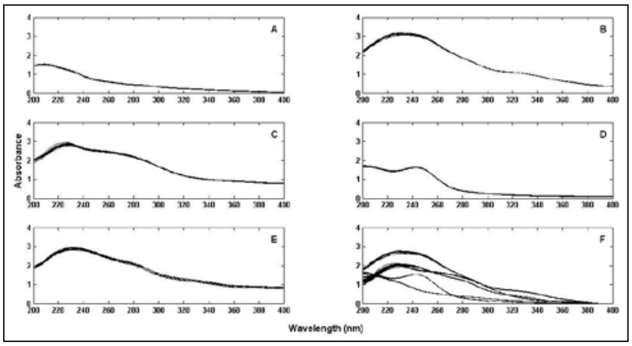

Figure 1. Ultraviolet spectra of industrialized juices. (A) pineapple; (B) orange; (C) mango; (D) passion fruit; (E) peach; (F) all 50 juices plotted together.

The optimum model dimension was determined by the minimum RMSECV for the calibration samples, obtained by leave-one-out cross-validation. Four latent variables for both carbohydrates and vitamin C, were necessary to retain a significant variance in the data and to avoid a significant bias in the model. The presence of relevant bias was tested with the prediction results for the validation samples by the *t*-test suggested by ASTM E1655-05 [11]. The results showed that the bias included in the model was not significant, since the *t* values obtained were 0.526 and 2.222 for carbohydrates and vitamin C, respectively. These are lower that the critical value of 2.921 with 99% of confidence.

The figures of merit for multivariate calibration were described in earlier papers [2, 16-21] and are not described in detail here. The results for the figures of merit obtained from PLS models are shown in Table I.

Table I. Figures of merit for the PLS multi-product calibration models.

Figures of Merit		Carbohydrate model	Vitamin C model
Accuracy (g/mL)	**RMSEP**	1.26×10^{-4}	0.0099
	RMSEC	4.14×10^{-4}	0.0100
	RMSECV	4.45×10^{-4}	0.0112
Fit	**Slope**	1.00	1.0001
	Intercept	6.60×10^{-5}	0.0006
	R	0.9994	0.9977
Analytical sensitivity^{-1}		1.24×10^{-4}	0.0018
LD		4.09×10^{-4}	0.0060
LQ		1.20×10^{-3}	0.0183

An ideal multivariate calibration model will show close values of RMSEP, RMSEC and RMSECV due to the random errors fit in the model. According to Burn and Ciurczak [22] the RMSEC always decreases along with increases in the number of latent variables (LV). As more LV are included in the calibration model, the model begins to fit the random errors imbedded in the spectra and concentrations. Therefore, the RMSEC will always decrease as more LV are added. In contrast, the RMSECV and RMSEP occasionally increase when more LV are included. However, new samples not included in the calibration set will have a different realization of random errors. Therefore, the calibration model will not fit these errors to the same degree as the errors in the calibration set. When extra LV that mostly describe random errors are included in the calibration model, these factors will not fit the errors in future samples and the RMSECV and RMSEP may increase. In practice, obtaining the same values for these parameters is not an easy task and so it is better that RMSEC present slightly higher values than RMSEP. A RMSEP value higher than the RMSEC value indicates that the model is overfitted and fewer latent variables are necessary for that model [22]. In this proposal the observations mentioned above are satisfied, indicating that the number of latent variables used in the model was properly chosen.

Accuracy values represented by RMSEC, RMSEP and RMSECV showed that the estimated values of the multivariate models presented good agreement with reference methods. The RMSEC and RMSEP are global parameters and they incorporate random and bias errors. Accuracy is better presented, for example, by the slope, the intercept, and the elliptical joint confidence regions [23]. For plots of the elliptical joint confidence region, as shown in Figure 2, the experimental and predicted values (Table II) for both analytes from the validation set were used. It is observed that the ellipse contains the ideal point (1.0) for slope and intercept, respectively, showing that the reference value and the PLS model are not significantly different at the 95% confidence level. It is possible to conclude also, on the basis of the 95% confidence intervals, that no constant or proportional systematic errors were observed in the model, since the intervals contain the expected values of 1 and 0 for the slope and the intercept, respectively.

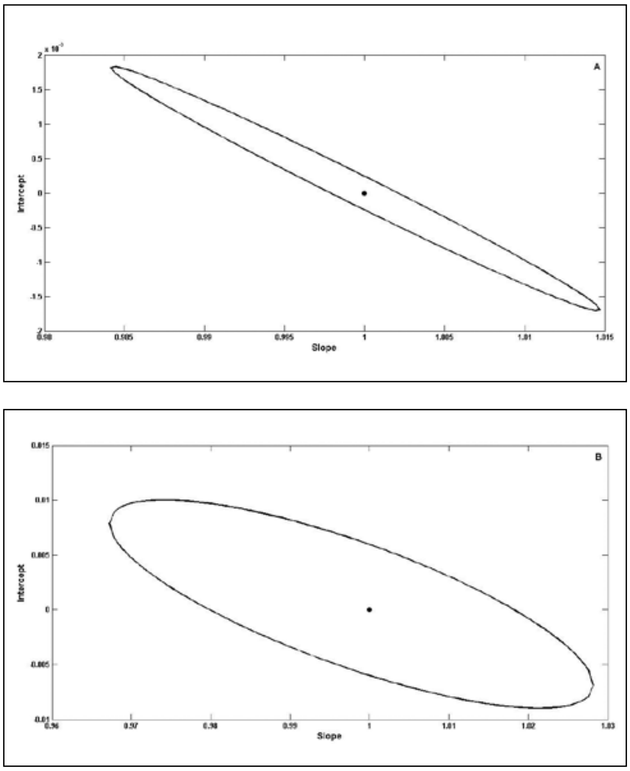

Figure 2. Elliptical joint confidence regions at 95% for the slope and intercept of the regression of predicted concentrations versus reference experimental values using ordinary least squares. (A) Carbohydrates. (B) Vitamin C.

The sensitivity corresponds to the fraction of analytical signal that is due to the increase of the concentration of a particular analyte at unit concentration. In inverse multivariate calibration models (such as PLS), it can be calculated by the inverse of the regression coefficients vector [16]. The results for sensitivity are somehow complex to judge because of the preprocessing adopted in PLS. However, the analytical sensitivity is simpler and more informative to compare and to judge the sensitivity of an inverse multivariate calibration method. It is calculated by the relationship between the sensitivity and the instrumental noise. The inverse of this parameter allows one to establish a minimum concentration difference that is discernible by the analytical method in the range of concentrations where it is applied, considering a perfect fit of the model and considering the spectral noise representing the large source of error [2]. Thus, based on the inverse of the analytical sensitivity, it is possible to distinguish samples with concentrations of 1.24x10^{-4} and 0.0018 for carbohydrates and vitamin C, respectively.

Table II. Experimental and predicted values of vitamin C and carbohydrates.

Sample number	Experimental values for Carbohydrates	Predicted values for Carbohydrates	Experimental values for Vitamin C	Predicted values for Vitamin C
1	**0.1500**	0.1500	0.1400	0.1427
2	**0.1000**	0.0998	0.1700	0.1566
3	**0.1400**	0.1399	0.0300	0.0303
4	**0.1100**	0.1100	0.2400	0.2512
5	**0.1400**	0.1401	0.2400	0.2510
6	**0.1400**	0.1402	0.0300	0.0295
7	**0.1500**	0.1501	0.1400	0.1441
8	**0.1500**	0.1500	0.6000	0.6136
9	**0.1400**	0.1401	0.0300	0.0420
10	**0.1000**	0.0999	0.1700	0.1559
11	**0.1500**	0.1500	0.1400	0.1428
12	**0.1500**	0.1499	0.6000	0.6076
13	**0.1400**	0.1402	0.0300	0.0353
14	**0.1100**	0.1102	0.2400	0.2549
15	**0.1500**	0.1499	0.6000	0.6155
16	**0.1400**	0.1400	0.2400	0.2506
17	**0.1500**	0.1499	0.1700	0.1678

Limits of detection (LD) and quantification (LQ) for the models show result coherent with the measured quantities. Therefore, the PLS models are appropriate to quantify carbohydrates and vitamin C, since their ranges are between 0.10 – 0.15 g/mL and 0.03 – 0.60 g/mL, respectively. These models presented similar fit with R of 0.9994 and 0.9977, respectively. The slope and intercept for these models are shown in Table I. This result suggests that, even being a small non-homogenous dataset, the multi-product calibration models can be fit as a linear behavior and can be constructed by a regression method mathematically less complex than locally weighted regression and the neural network.

4. Conclusion

Determinations of carbohydrates and vitamin C were accessed by PLS multi-product calibration models based on ultraviolet spectroscopy. The models presented feasible and acceptable results. The UV-PLS procedure presented the advantage of no sample preparation and other advantages such as simultaneous determination of carbohydrates and vitamin C using the same UV spectra, beyond the possibility for on-line monitoring. Even being a preliminary study, the results show that it is possible to built multi-product calibration models by the PLS regression method. As future prospects of this study, samples of juices of other flavors will be included in the model.

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6. References

1. Wiberg, K.; Hagman, A.; Jacobsson, S.P.; *J. Pharm. Biomed. Anal.*, 2003, 30, 1575.
2. Valderrama, P.; Romero, A.L.; Imamura, P.M.; Magalhães, I.R.S.; Bonato, P.S.; Poppi, R.J.; *Anal. Bioanal. Chem.*, 2010, 397, 181.
3. Hilgemann, M.; Nascimento, P.C.; Dias, D.; Guterres, M.V.; Stringhini, F.M.; Carvalho, L.M.; Bohrer, D.; *J. Petroleum Sci. Technol.*, 2011, 78, 283.
4. Martelo-Vidal, M.J.; Vázquez, M.; *Food Chem.*, 2014, 158, 28.
5. Micklander, E.; Kjeldahl, K.; Egebo, M.; NØrgaard, L.; *J. Near Infrared Spectrosc.*, 2006, 14, 395.
6. Berzaghi, P.; Shenk, J.S.; Westerhaus, M.O.; *J. Near Infrared Spectrosc.*, 2000, 8, 1.
7. Naes, T.; Isaksson, T.; *Appl. Spectrosc.*, 1992, 46, 34.
8. Wang, Z.; Isaksson, T.; Kowalski, B.R.; *Anal. Chem.*, 1994, 66, 249.
9. Brereton, R. G.; *Analyst.* 2000, 125, 2125.
10. Rambo, M.K.D.; Amorim, E.P.; Ferreira, M.M.C.; *Anal. Chim. Acta*, 2013, 775, 41.
11. Faber, K.; Kowalski, B. R.; *Appl. Spectrosc.* 1997, 51, 660.
12. *Annual Book of ASTM Standards*, Standard Practices for Infrared Multivariate Quantitative Analysis - E1655-05, ASTM International, West Conshohocken, PA, USA, 2005.
13. Cecchi, H. M.; *Fundamentos Teóricos e Práticos em Análise de Alimentos*, 2th ed.; Editora da Unicamp: Campinas-SP, p. 75, 2003.
14. *Official Methods of Analysis of the Association of Official Analytical Chemists*. 15th ed., v. 2. Arlington: A.O.A.C., 1990. p. 1058-1059 (method 967.21).
15. Kennard, R. W.; Stone, L. A.; *Technom.* 1969, 11, 137.
16. Valderrama, P.; Braga, J.W.B.; Poppi, R.J.; *Quim. Nova*, 2009, 32, 1278.
17. Valderrama, P.; Braga, J.W.B.; Poppi, R.J.; *J. Agric. Food Chem.*, 2007, 55, 8331.
18. Silva, M.A.M.; Ferreira, M.H.; Braga, J.W.B.; Sena, M.M.; *Talanta*, 2012, 89, 342.
19. Ferreira, M.H.; Braga, J.W.B.; Sena, M.M.; *Microchem. J.*, 2013, 109, 158.
20. Botelho, B.G.; Mendes, B.A.P.; Sena, M.M.; *Food Anal. Meth.*, 2013, 6, 881.
21. Valderrama, P.; Braga, J.W.B.; Poppi, R.J.; *J. Braz. Chem. Soc.*, 2007, 18, 259.
22. Burns, D.A.; Ciurczak, E.W.; *Handbook of Near-Infrared Analysis*, Marcel Dekker: New York, p. 221, 2001.
23. Riu, J.; Rius, F.X., *Anal. Chem.*, 1996, 68, 1851.

Comparison of procedures for selenium species extraction from selenized yeast and evaluation of associated uncertainties

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Abstract

Several extraction procedures have been investigated in order to obtain the highest selenium extraction efficiency in yeast samples. In this work, 10 different procedures using enzymatic and non-enzymatic extracting solutions were studied for this purpose. Selenium extraction yields were estimated based on the results found by ICP OES determination of the total selenium content on the extract and on the whole digested sample from a high pressure asher (HPA-S). Extraction using the solution with only protease led to appropriate recoveries of Se (88 ± 6 %) while selenomethionine (SeMet) was the major observed species. The accuracy of total Se and SeMet determinations were evaluated by analysis of the certified reference material CRM SELM-1. Speciation studies of the extracts were carried out by ion-pairing reversed phase liquid chromatography. This separation mechanism was suitable to isolate the main extractable Se species. The detection limit (LOD) for SeMet was 36 µg kg^{-1}, the linearity was satisfactory in the studied range (0.5 – 3.0 mg kg^{-1}; $y = 134685x + 1411$; $R^2 = 0.99586$; for SeMet) and the extraction efficiency ranged from 82 % to 92 % for different proteolytic extractions and from 12 % to 22 % for non-proteolytic extractions. Recovery obtained for total selenium in CRM SELM-1 was 99.2 %, confirming the reliability of the method. The uncertainty sources were described in each step of the analytical procedure, according to the Guide to the Expression of Uncertainty in Measurement (GUM). The uncertainty sources were discerned in each step of the analytical procedure, employing the rules of propagation of errors. The main uncertainty sources were instrumental repeatability and SeMet concentration in the analytical solution, their sum representing 58 % of the total uncertainty value for SeMet (protease extraction).

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1 Introduction

Selenium is an essential trace element in human nutrition, present in selenoproteins as aminoacid selenocysteine. In humans, more than two dozen of selenoproteins that play critical roles in reproduction, thyroid hormone metabolism, DNA synthesis, and protection from oxidative damage and infection have been identified [1].

Selenium is found in food in two forms: inorganic (selenate and selenite) and organic (selenomethionine and selenocysteine). Both forms can be good dietary sources of selenium. Recently, selenium supplements have been used as chemopreventive to reduce the risk of prostate, lung and colon cancers [2]. On the other hand, when Se ingestion is higher than the organism's absorption and excretion capacity this element can present toxic effects. A serious Se acute intoxication was identified in the United States of American (USA) in 2008 due to the ingestion of a dietary supplement that contained 200 times the labeled content of selenium, affecting 201 people in 10 states [3]. This incident highlights the importance of quality control of nutritional supplements and development of analytical methods able to provide accurate, precise and reliable results for this kind of samples.

The beneficial effects of selenium supplementation have encouraged the search for supplement food. Yeast is a preferred source for supplementation due to the presence of high levels of selenium, as selenomethionine, a less toxic form with more bioavailability and to its low cost [4,5].

Nowadays it is well known that bioavailability, toxicity, environmental impact, etc., of selenium are closely related to its chemical form. Therefore selenium speciation analysis has been the focus of scientists in recent years. Several analytical approaches based on coupling of HPLC with ICP-MS are well established for the determination of selenospecies.

Speciation analysis of yeast requires the development of robust extraction methods able to extract selenospecies present in yeast and the answers to these questions still represent a challenge to analytical chemists that should guarantee species integrity in order to avoid the loss of chemical information of the analytes of interest.

In fact, sample preparation increases the risks of contamination and analyte losses, since they involve sample manipulation to modify the matrix and to make the species more adequate for the analysis.

In speciation analysis, soft extraction procedures are necessary to avoid species interconvertion during extraction. Furthermore, storage procedures (light protection, temperature control, etc.) of the samples should also preserve the original species. In some studies, the oxidation of the selenomethionine to selenomethionine oxide [6] and the oxidation of Se (IV) to Se (VI) have been observed.

Different extraction methods have been employed in the development of analytical methods aiming at speciation. Among them, enzymatic extraction [7,8], alkaline [9,10] and acid extraction [11,12], and extraction with water [13-15] and with buffer solutions [16,17]. According to Cañas et al. [18], buffer salts are used to obtain the ionic strength necessary for optimal solubilization of protein and Tris-HCl is often used due to liberate the free or weakly bonded inorganic species and aminoacids [19]. Sodium dodecyl sulfate has also been widely used to denature proteins and, by forming ion pairs, to render them water soluble, increasing extraction efficiency [20].

The use of proteolytic enzymes for Se-species extraction is one of the most used methods for the extraction of selenomethionine from yeast, without degradation of selenoaminoacids. However, proteolytic enzymes destroy all the information relating to selenium proteins in the original sample as protease is a non-specific enzyme able to act along the whole protein structure [21,22]. Several enzymatic hydrolyses with protease XIV have been used as extraction techniques to release protein-bound compounds such as selenomethione (SeMet) from food and dietary supplements [23,24]. Enzymatic digestion can also be combined with ultrasonic energy [21,25] and with microwave radiation, reducing hydrolysis time [24].

On the other hand, the sample preparation procedures for determination of total selenium, employing HNO_3 and H_2O_2 as an oxidant mixture, have shown good analytical performance, since they are simple and apparently do not present problems related to the loss of volatile species of selenium.

Another important aspect that should be taken into account in speciation analysis is the rigorous quality control of the results. Although there are a significant number of articles in the literature about Se speciation, few deal with quality assurance issues, including the validation of the methods, estimate of measurement uncertainty and traceability of the results to the SI.

The measurement uncertainty, for example, is an important parameter that enables evaluation of result quality, since this parameter provides the quantification of the measurement quality. During a chemical analysis, several steps are necessary to obtain a measurement result, including sampling, sample preparation, introduction of the sample into the equipment, separations in speciation analysis and evaluation of the results. Also, slight variations in the environmental conditions, instrumental drift and others external effects can occur, resulting in dispersion of the final results and, consequently, the analytical results should not be expressed as unique values but as an average values with a dispersion which characterizes the measurement: its uncertainty.

In this work, different enzymatic (lipase, protease) and non-enzymatic (water, SDS, Tris-HCl) extraction procedures were investigated in order to evaluate the most adequate extracting agent. Quantification of SeMet was performed by reversed phase ion pair HPLC coupled to DRC-ICP-MS. The method developed was optimized, validated and the uncertainty sources were investigated according to the Guide to the Expression of Uncertainty in Measurement (GUM) [26].

2 Experiment

2.1 Reagents and instrumentation

The standard reference material NIST SRM 3149 (Se: 10110 ± 20 mg kg^{-1} in 10 % HNO_3) was used to prepare the calibration curves and a stock solution was used to obtain suitable dilutions.

Standards of Se-DL-methionine and selenocystine (both from Sigma-Aldrich, Dorset-UK) were used to compare retention times in the samples.

Methanol (Chromasolv for HPLC ≥ 99.9 %, Sigma-Aldrich, São Paulo-Brazil), acetonitrile (ACN, HPLC/spectro > 99.9 %, Tedia, OH-USA), tetrabutylammonium hydroxide (TBAOH, ≥ 97 %, Sigma-Aldrich, São Paulo-Brazil) and ammonium phosphate ($NH_4H_2PO_4$, ≥ 99.5 %, Sigma-Aldrich, Steinhein-Germany) were used as mobile phase reagents.

Protease type XIV from *Streptomyces griseus* (Sigma, Japan) and lipase type VII from *Candida rugosa* (Sigma, Japan), sodium dodecyl sulfate (SDS, Merck), tris (hydroxymethyl)aminomethane (ultra-pure, $\geq$ 99.9 %, Sigma-Aldrich, MO-USA), and fuming 37 % hydrochloric acid (Merck, Darmstadt-Germany) were used for selenium extraction procedures.

65 % nitric acid (Merck, Darmstadt-Germany) was further purified by sub-boiling distillation in a quartz cell, model Duo-PUR (Milestone, U.S.A.).

Type I water with resistivity of 18 MΩ cm from a Milli-Q System (Millipore Co., Bedford, MA, U.S.A.) was used to prepare solutions.

Standard solutions of L-selenocystine (3000 mg kg^{-1}) and selenomethionine (10000 mg kg^{-1}) were prepared in 2 % v/v HCl and preserved at -20 °C. Selenite and selenate (10000 mg kg^{-1}) were prepared in hydrochloric (Merck, Germany) and nitric acids, respectively, and preserved at 4 °C in the dark.

The yeast sample was kindly supplied by a national manufacturer. The strain of *Saccharomyces cerevisiae* was enriched in selenium to a target concentration of 2000 mg kg^{-1}.

PVDF syringe filters, pore size of 0.45 µm, were used for filtration of samples (Nova Analítica, Brazil).

A C18 Luna column (Phenomenex, USA) was used with the chromatographic system.

An analytical balance from Sartorius, model ME 235S (Germany) was used to weigh samples and standards.

Other equipments, necessary for this work were a pHmeter, MP 230 (Metller Toledo, Switzerland), an AP 56 vortex (Phoenix, Brazil), a shaker thermostat NT 712 (Nova Ética, São Paulo, Brazil), a Z300K centrifuge (Hermle, Germany), an air oven (Nova Ética, São Paulo, Brazil), an ultrasonic bath, model Ultra cleaner 1400A (Unique, Brazil), a Minipuls III peristaltic pump (Gilson, France) and a high pressure asher (HPA-S) (Anton Paar, Austria).

A sequential radial view inductively coupled plasma optical emission spectrometer (ICP OES), model ULTIMA 2 (Jobin Yvon-Horiba, Longjumeau, France), was used to determine the content of total selenium.

An inductively coupled plasma mass spectrometer (ICP-MS), model Elan DRC II (PerkinElmer, Norwalk, CT, USA) was used, coupled to chromatography.

A HPLC system from PerkinElmer, model Flexar (Shelton, USA), was coupled to the Elan DRC II by a EV750-100-S2 switch valve (Cetac, USA). The chromatographic effluent reaches the plasma through a system composed of a cryogenic spray chamber (Isomist Kit, Australia) and a parallel flow nebulizer (Miramist Burgener, Canada).

2.2 Procedures

The HPLC was equipped with a quaternary pump, degasser, autosampler and column oven. The HPLC column was connected to a switching valve via PEEK tubing (0.18 mm id). This valve was coupled to the Elan DRC-ICP-MS. Ammonia was used as reaction gas at 1.0 mL min^{-1} gas flow rate. This system allowed the ICP-MS daily performance check and chromatographic column conditioning at the same time, the column effluent was drained to waste while the daily performance solution was pumped to the ICP-MS. When the valve position is changed, the column chromatographic effluent enters the ICP-MS and the separation of the Se compounds can be detected.

Chromera® speciation software (version 4.0) was used for monitoring both instruments (HPLC and Elan DRC-ICP-MS) and the integration of the chromatographic signal.

Ion-pair reversed phase separation of the selenium compounds was performed on a C18 column. The mobile phase was prepared using TBAOH at 0.05 mmol L^{-1}, $NH_4H_2PO_4$ at 0.5 mmol L^{-1} and ACN at 1 %. The injected volume was 5 µL, using a 200 µL sample loop.

The sample introduction system of the Elan DRC-ICP-MS was set up at 10 °C, minimizing fluctuations and organic solvent losses and increasing the analytical stability of the plasma.

2.3 Moisture content determination

The moisture content of the CRM SELM-1 and yeast sample was determined by removal of water by heating until constant weight at 105 °C.

2.4 Digestion procedures of yeast samples

Before weighing, both CRM SELM-1 and the yeast sample were homogenized manually and approximately 0.25 g were weighed and quantitatively transferred to a quartz digestion vessel using 1.5 mL of concentrated HNO_3 and 1.5 mL H_2O_2. After decomposition, the vessels were cooled until room temperature (21 ± 2 °C) and made up to 15 mL with water. A second dilution of approximately 73 times was realized for analysis.

A high pressure asher (HPA-S) decomposition system was used to digest the samples (240 °C during 60 min) according to an application note supplied with the equipment. This equipment operates in a nitrogen atmosphere, assuring better control of the digestion process.

2.5 Extraction procedures of selenium compounds (Non-enzymatic and enzymatic extraction of Se compounds)

Extraction procedures were investigated for recovery of selenocompounds based on enzymatic and non-enzymatic extraction. Ten sample preparations (solid-liquid extraction, realized in triplicate) were used. For each one, an aliquot of 0.25 g of yeast was weighted followed by the addition of the reagents (listed below). The total volume was 5.0 mL for each procedure. A second dilution of approximately 195 times, for proteolytic extractions, was realized for the analysis.

i. Tris-HCl (30 mmol L^{-1}, pH 7.5);
ii. Tris-HCl + protease (30 mmol L^{-1}, pH 7.5 + 4 mg g^{-1});
iii. SDS (0.2 %);
iv. SDS + Tris-HCl (0.2 % + 30 mmol L^{-1}, pH 7.5);
v. SDS + protease (0.2 % + 4 mg g^{-1});
vi. SDS + protease + Tris-HCl (0.2 % + 4 mg g^{-1} + 30 mmol L^{-1}, pH 7.5);
vii. Protease (4 mg g^{-1});
viii. Lipase (2 mg g^{-1});
ix. Protease + lipase (4 mg g^{-1} + 2 mg g^{-1}) and
x. Water

The yeast samples were mechanically shaken for approximately 30 s in a vortex and incubated in a shaker thermostat, at 200 rpm, during 16 h at 37 °C.

After extraction, the samples were centrifuged for 30 min at approximately 2489 *g* and the supernatant was filtered through a 0.45 µm filter. The resulting solution was stored at -20 °C and diluted appropriately when analyzed by HPLC-DRC-ICP-MS and ICP OES.

2.6 Operational conditions of ICP OES

Total amount of selenium was determined by ICP OES using external calibration. The operational conditions are shown in Table I.

Table I. Operational conditions of ICP OES

Parameters	Values
Radio frequency power (W)	1400
Auxiliary gas flow rate (L min^{-1})	0.4
Nebulizer gas flow rate (L min^{-1})	0.8
Sample flow rate (mL min^{-1})	1.0
Detector voltage (V)	900
Wavelength (nm)	196

2.7 Operational conditions of HPLC-DRC-ICP-MS

Before analysis by HPLC-DRC-ICP-MS, the DRC-ICP-MS was optimized. The optimization of ICP-MS operating in standard mode was carried out by daily performance check keeping the doubly charged (Ba^{++}) and oxide (CeO^+) levels less than 3 % and with higher sensitivity, evaluating the intensities obtained for Mg, In and U.

The speciation analysis was carried out by the coupling HPLC-DRC-ICP-MS. The DRC conditions were previously optimized (NH_3 gas at 1.0 mL min^{-1}, RPq of 0.45 and RPa of 0), see item 3.3.

The separation and elution of the Se compounds (selenomethionine, selenium IV and selenocystine) was achieved in less than15 min using 0.05 mmol L^{-1} of TBAOH, 0.5 mmol L^{-1} of $NH_4H_2PO_4$ and 1 % of ACN as mobile phase (≈ pH 6.3) at 0.25 mL min^{-1}. This mobile phase was compatible with the ICP-MS operating conditions. A solution of 2 % HNO_3, as make-up, was introduced through a "T" piece, increasing the total flow rate into the nebulizer to 1.25 mL min^{-1}. The HNO_3 solution also minimizes the undesirable effects of the mobile phase organic modifier (ACN) as carbon deposits in injector, cones and other parts of the equipment.

The quantification was realized by external calibration, using peak area measurement of the chromatographic signal intensities by monitoring the ^{82}Se, ^{80}Se and ^{77}Se signals.

More experimental information for the HPLC-DRC-ICP-MS system is summarized in Table II.

Table II. Operational conditions of HPLC-DRC-ICP-MS

Parameters	
HPLC	
Column: C18 Luna; Phenomenex	150 mm x 2 mm x 3 µm
Pre-column Security Guard, Phenomenex	4 mm x 2 mm
Mobile phase	TBAOH (0.05 mmol L^{-1}); $NH_4H_2PO_4$ (0.5 mmol L^{-1}); ACN (1 %)
Elution mode	Isocratic
Flow rate	0.25 mL min^{-1}
Injection volume	5 µL
Column temperature	35 °C
DRC-ICP-MS	
RF power	1350 W
Nebulizer gas flow rate	0.96 L min^{-1}
Plasma gas flow rate	15 L min^{-1}
Auxiliary gas flow rate	1 L min^{-1}
Scan mode	Peak-hopping
Sweeps	1
Readings	3183
Replicates	1
Dwell time (ms)	250
Detector operation	Dual
Rpq	0.45
Rpa	0
Cell gas flow rate, NH_3	1.0 mL min^{-1}
Monitored isotopes	^{77}Se, ^{80}Se, ^{82}Se

2.8 Uncertainty sources identifications

In this work, the uncertainty associated with the result was estimated from the specification of the measurand. The measurand is the SeMet concentration, analyzed by HPLC-DRC-ICP-MS, present in selenized yeast, which is defined as Eq. (1).

$$C_{SeMet} = C_0 \times df \times f_{ext} \times f_{rep} \times f_{moist} \quad (1)$$

Where C_{SeMet} is the selenomethionine concentration in the original sample, C_0 is the SeMet concentration in the analytical solution, df is the dilution factor of the sample, f_{ext} is the factor of sample extraction, f_{rep} is the factor of instrumental repeatability and f_{moist} is the factor of moisture.

According to the "Ishikawa diagram" (Figure 1), the uncertainty components include the uncertainty of the selenium concentration in the analytical solution (u_{C0}), the uncertainty of the sample dilution (u_{df}), the uncertainty of the sample extraction (u_{ext}), the uncertainty of the instrumental repeatability (u_{rep}) and the uncertainty associated with moisture (u_{moist}).

3 Results/Discussion

3.1 Optimization of operational conditions of ICP OES

The optimization of the operational conditions of ICP OES was carried out by varying the instrumental parameters of the equipment using a solution of 50 μg kg^{-1} of Se diluted in 2 % HNO_3.

The influence of radiofrequency power (RF) on the Se signal intensity was evaluated in the range 1000 – 1400 W. Selenium has a high ionization potential (9.75 eV) which results in low sensitivity. The intensity of selenium was improved when the RF power was increased from 1000 W to 1400 W. The selenium signal was approximately 3400 counts per second (cps); so, the detector voltage was changed from 750 V to 900 V, increasing the intensity to 13000 cps, approximately. The RF power of 1400 W and a detector voltage of 900 V were then chosen for the subsequent studies.

The auxiliary gas flow rate was evaluated and the intensity of selenium increased when the auxiliary gas flow rate was changed from 0.2 L min^{-1} to 0.4 L min^{-1}. This signal improvement probably occurs due to the changing of the plasma position in the radial view with higher auxiliary flow rates reaching, a good alignment between the analytical zone of the plasma (emission zone) and the entrance slit of spectrometer at 0.4 L min^{-1}.

The sample flow rate on the analytical signal was also investigated. As expected the improvement of the sample flow rate resulted in an increase of the selenium signal due to the higher quantity of sample that reaches the plasma. However, when the quantity of sample in the plasma is too high, a reduction of the energetic conditions can occur, leading to reduction of the population of excited/ionized atoms and ions. This behavior can be observed with sample flow rates greater than 1 mL min^{-1}, thus 1 mL min^{-1} was chosen for subsequent studies.

The Ar nebulizer flow rate was investigated in the range 0.4 – 1.0 mL min^{-1} and the signal intensity increased up to 0.8 L min^{-1}. With higher Ar nebulizer flow rates, the efficiency of the transport of sample to plasma was improved and consequently the signal intensity increased. So, the Ar nebulizer flow rate of 0.8 L min^{-1} was chosen for selenium determination in yeast samples.

3.2 Validation of total Se determination by ICP OES

Once the instrumental conditions were optimized to be more appropriate for total Se determination by ICP OES (item 3.1), the validation parameters repeatability, detection limits, linearity of calibration curve and method accuracy by CRM SELM-1 analysis were determined.

The instrumental repeatability was defined as relative standard deviation (RSD) obtained for analysis of a Se standard

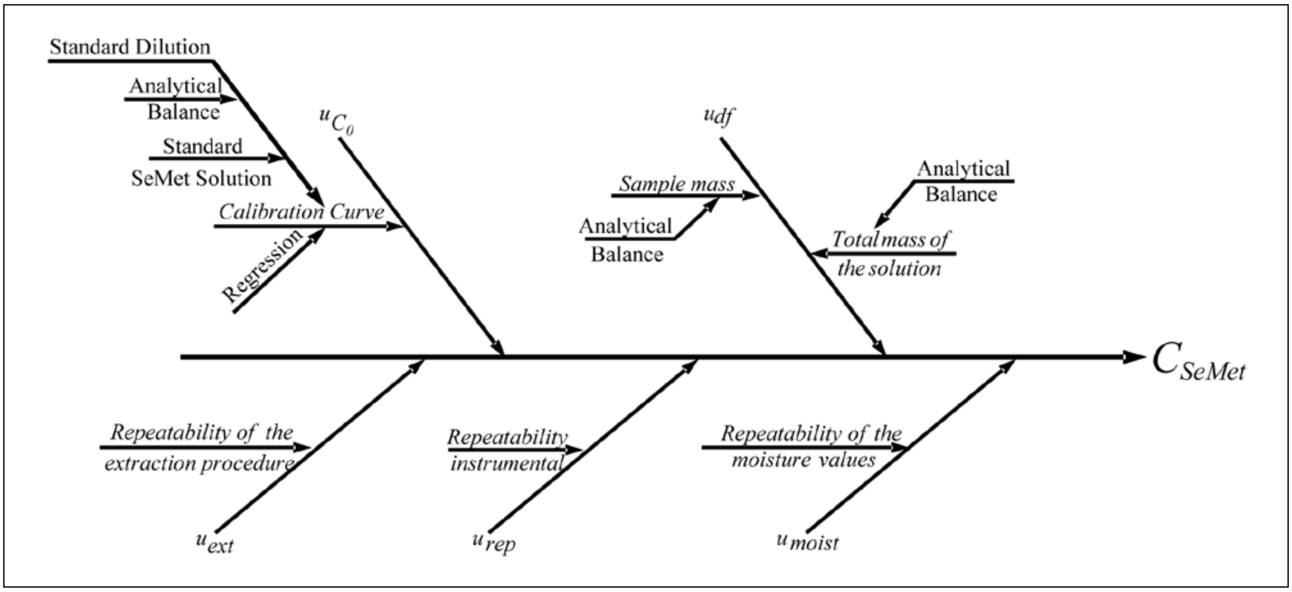

Figure 1. Ishikawa Diagram for determination of SeMet and total selenium concentration.

solution of 0.45 mg kg^{-1}, being 2.0 % the value found for 7 replicates.

The linearity of the calibration curve was considered satisfactory in the range 0.15 - 0.90 mg kg^{-1} with $R^2 = 0.99902$; y = 2556418 x – 427.

The limit of detection (LOD), 5.0 μg kg^{-1}, was obtained by employing the criteria of 3σ of blank concentration.

Method accuracy was evaluated by CRM SELM-1 analysis and good agreement was achieved between the total Se obtained (2048 ± 69 mg kg^{-1}) and the certified value (2059 ± 64 mg kg^{-1}).

3.3 Optimization of operational conditions of the dynamic reaction cell (DRC)

In order to reduce the spectral interference on selenium isotopes in ICP-MS, ammonia was used as a reaction gas, enabling the employment of the most abundant isotope of selenium, ^{80}Se. The optimization of the operational conditions of the DRC was carried out using the mobile phase (TBAOH, $NH_4H_2PO_4$ and ACN), considered as a blank solution, and the yeast sample, 20 μg kg^{-1} diluted in mobile phase. Figure 2 shows the influence of reaction gas flow rate (NH_3) on the m/z 80 intensity.

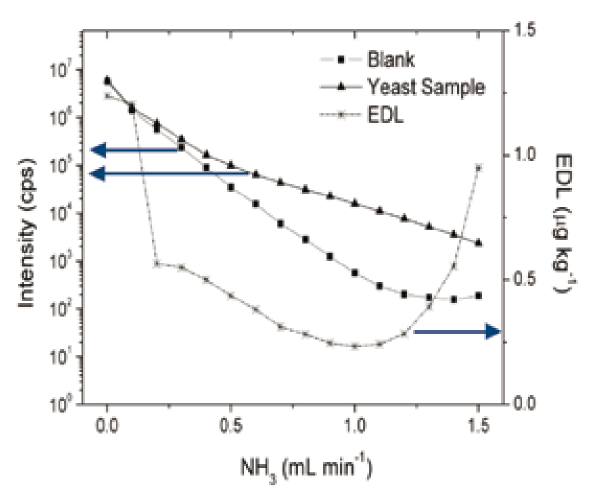

Figure 2. Influence of reaction gas (ammonia) on m/z 80..

The estimated detection limit (EDL) was used as a criterion for dynamic reaction cell optimization, according to Tanner *et al.* [27]. Broadly, the EDL (Eq. 2) is an optimization parameter obtained for each reaction gas flow rate and represents, indirectly, the efficiency of ammonia as reaction gas to solve the problem related with spectral overlap. Although the EDL does not represent strictly the detection limit of the method, it is considered a good estimate.

$$EDL = \frac{3 \times \sqrt{I_{Blank}}}{I_{Sample\ \ Solution} - I_{Blank}} \quad (2)$$

As can be seen in Figure 2, the m/z 80 intensity was reduced from 5x10^6 to around 200 cps and 8000 cps for blank (■) and yeast sample (▲), respectively. The increase of ammonia flow rate reduced the incidence of interfering species, mainly $^{40}Ar^{40}Ar^+$; in this way the ^{80}Se was used for subsequent studies. The most adequate operational conditions of DRC were determined observing the lower estimated detection limit (EDL; ∗) this value being 1 mL min^{-1}.

3.4 Optimization of selenium species separation by HPLC-DRC-ICP-MS

Different percentages of ACN were investigated and the optimal amount was established at 1 %. The employment of higher percentages of the organic modifier can result in carbon deposits in mechanical parts of the equipment (DRC-ICP-MS) while lower values retard the elution of selenium species on the column. In order to avoid the elution of inorganic selenium species in the dead volume of the column, TBAOH was used as ion-pairing reagent and its concentration was selected taking into account chromatographic resolution. The concentration chosen was 0.05 mmol L^{-1}. $NH_4H_2PO_4$ at 0.5 mmol L^{-1} was used to adjust the pH to 6.3.

The samples were prepared in water since differences were not observed in the chromatographic profile between samples prepared in water or in mobile phase.

3.5 Validation of HPLC-DRC-ICP-MS coupling

The repeatability was defined as relative standard deviation (RSD) of the peak area for six injections of ^{80}SeMet, representing 3 % of the precision of the instrumental setup. The calibration curve was built from the SeMet standard solutions using Chromera® speciation software to integrate the chromatographic peak areas. Good linearity was achieved in the concentration range studied (0.5 - 3 mg kg^{-1} as SeMet, n = 3). The Figure 3 shows the calibration curve obtained for different selenium isotopes

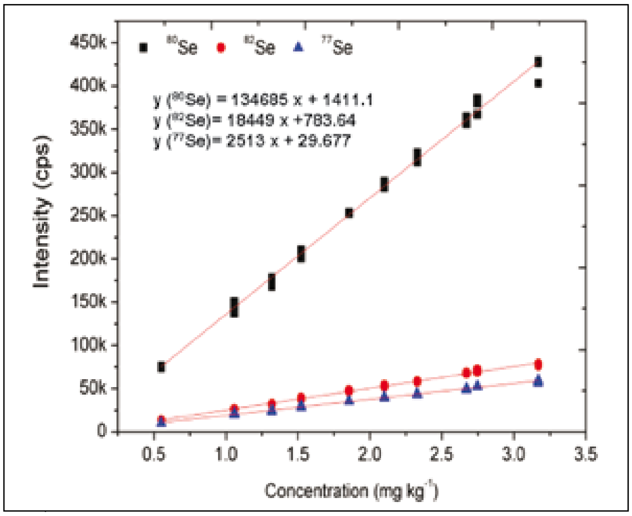

Figure 3. Calibration curve of SeMet; isotopes 82, 80 and 77.

The detection limit (calculated as the concentration for the net signal equivalent to three times the background noise in the chromatogram) was 36 μg kg^{-1} for ^{80}SeMet.

The method accuracy employed for Se speciation by HPLC-DRC-ICP-MS was evaluated by analysis of CRM SELM-1 and the results obtained for SeMet determination (3420 ± 295 mg kg^{-1}) were in good agreement with the certified value (3448 ± 146 mg kg^{-1}), indicating that the sample preparation allowed extracting SeMet and that neither degradation nor interconversion of the analyte was observed.

3.6 Selenium determination in extracted samples

3.6.1 Determination of total Se by ICP OES

The method employed for total Se determination by ICP OES was optimized (item 3.1) and validated (item 3.2). The results obtained (2048 ± 69 mg kg^{-1}) were in good agreement with the certified value (2059 ± 64 mg kg^{-1}).

Total Se in the enzymatic and non-enzimatic extracts was also determined by ICP OES using external calibration in the range of 0.2 – 1.0 mg kg^{-1}. All the sample extracts were adequately diluted in 2 % HNO_3 and analyzed under the experimental conditions given in Table I.

The values obtained for extraction employing non-proteolytic procedures were in the concentration range from 274 ± 18 mg kg^{-1} to 502 ± 32 mg kg^{-1} and for proteolytic procedures were in the range from 1888 ± 123 mg kg^{-1} to 2042 ± 120 mg kg^{-1}, corresponding to 12 - 22 % and 82 - 92 % of the total Se content in the yeast samples, respectively (values corrected for moisture).These values were obtained by the division of total Se concentration in the extracts by the total Se concentration in the digested yeast sample [28] (value 2170 mg kg^{-1} used as reference). Figure 4 shows the recovery of the selenium for different extraction procedures.

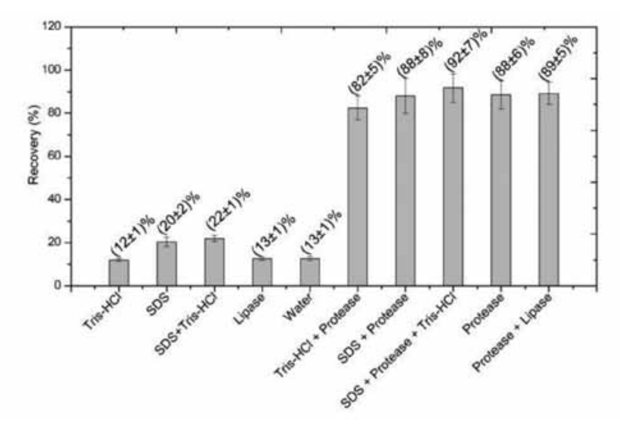

Figure 4. Recovery of selenium with different extration procedures.

The procedure employing Tris-HCl, close to the physiological pH 7.5, showed similar recovery (12 %) to the water only procedure (13 %). It can be explained by extraction of inorganic species and selenocompounds soluble in water. The use of SDS increased the selenium extraction (20 % for SDS and 22 % for SDS+Tris-HCl) corresponding to the free fraction (also extracted with the water procedure) and the fraction weakly bound to protein [29].

Lipase is able to release selenocompounds bound in the lipidic fraction. The results obtained by this extraction procedure were similar to those obtained with water and Tris-HCl extractions.

Enzymatic hydrolysis using protease has been one of the most common techniques for selenium compound extraction in yeast samples due to the high extraction yields, in the range 80-90 % [28-30]. As can be seen in Figure 4, the recovery for proteolytic procedures was higher than non-proteolytic ones. This fact can be explained by the action of protease on proteins with high selenium levels present in the sample yeast. This enzyme acts to release the selenium aminoacids in no specific way. Thus the enzymatic extraction with protease was selected for further analysis due to be the most efficient procedure for extracting selenium without degradation of selenoamino acids.

3.6.2 Determination of SeMet by HPLC-DRC-ICP-MS

The main problem concerning the development of analytical procedures for Se speciation is the lack of CRMs [31], qualified standard solutions of some selenium species and poor knowledge about the stability of this species in different matrices. Furthermore, sample preparation is the critical point to assure method accuracy, since it is necessary to obtain appropriately efficiency extractions without degradation or interconversion of the analytes.

Selenium speciation studies in yeast samples confirmed the presence of a variety of chemical forms in these organisms. In this sense, the use of different extraction protocols to distinguish between different fractions of selenium present in the sample was investigated.

The extraction protocols were described in the item 2.5. The non-proteolytic extracts were diluted 1:3 with Type I water before injection in the HPLC system. The chromatographic profiles obtained by Tris-HCl, SDS, SDS + Tris-HCl and water procedures were similar according to Figure 5. The selenium compounds obtained were not quantified due to absence of standards and low recovery, the signal intensities of some peaks on the chromatograms were close to quantification limits.

The chromatograms obtained using non-proteolytic extractions showed up to 6 Se species. Trying to assign the identity of the observed chromatographic peaks, their retention times were compared with those of the selenium standards available in our lab (selenite, selenate, selenomethionine and selenocystine) by analysis of a sample fortified with these standards.

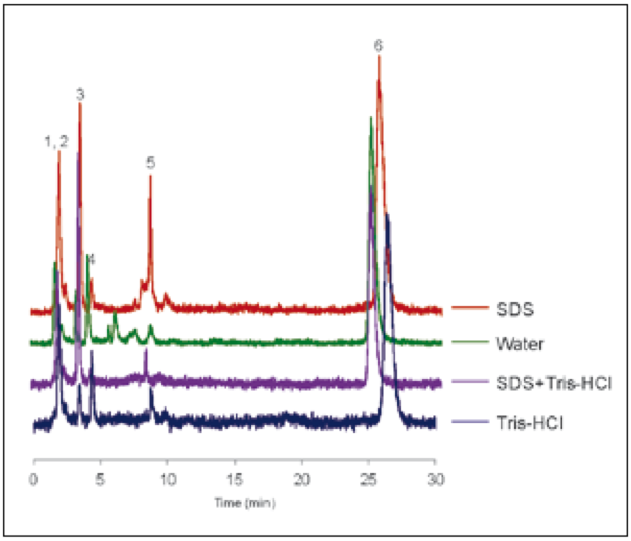

Figure 5. Non-enzymatic extraction, ^{80}Se.

According to Casiot [29], the compounds extracted include weakly bound and water-soluble selenite, selenoamino acids and possibly trimethylselenonium and selenoglutathione.

Looking at the extraction procedures in general, some topics were investigated. First, non-proteolytic extraction conditions showed low recoveries (< 25 %, see Figure 4), the SDS procedure tended to show a slight increase of selenium intensity when compared with other non-proteolytic extracts. SDS is widely used to denature proteins and to render them water soluble by forming ion pairs [20].

The chromatograms (Figure 5) showed Se species peaks near dead volume, probably Se(Cys)$_2$ and SeOMet, peaks 1 and 2. SeMet (peak 3) and Se(IV) (peak 5) were confirmed with retention times of 3.39 and 8.96 min, respectively, using spiked solutions. A major peak (peak 6) eluted at 28 min could not be identified due to the absence of selenium standards.

In the enzymatic hydrolysis with protease, the enzyme breaks the peptide bonds of unspecified forms, releasing the selenoaminoacids present in proteins without their degradation; on the other hand, the information of the proteins in the original sample is lost. According to Szpunar and collaborators [30] the predominant form of Se in yeast is selenomethionine (SeMet) (≈ 80 %), whereas other compounds are in smaller quantities.

The profiles observed for enzymatic hydrolysis with protease (Figure 6) showed selenomethionine as a major species, minor chromatographic peaks and others in the dead volume, the last ones could not be quantified due to their low intensity and lack of resolution. Gosetti *et al.* [32] found similar unknown peaks when extracting selenium from yeast using a similar extraction procedure and chromatographic conditions.

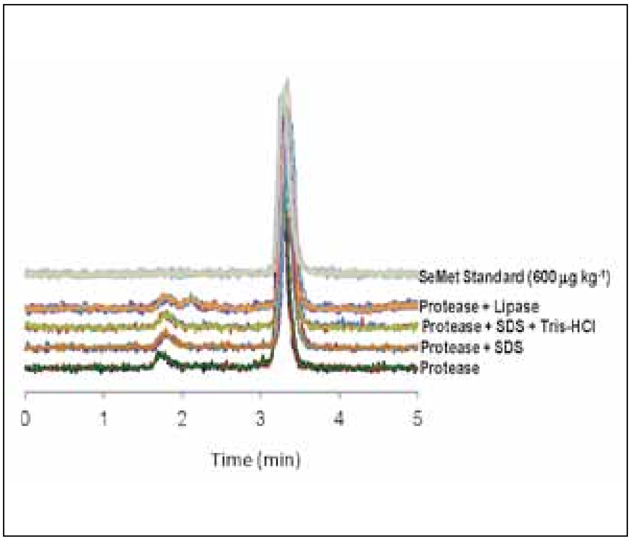

Figure 6. Different extraction procedures of SeMet from a yeast sample using protease, n=3.

The concentration of ^{80}SeMet was 3011 ± 288 mg kg^{-1} in the sample analyzed in triplicate using only protease and external calibration (0.10 – 0.85 mg kg^{-1}; $y = 168431x - 340$, $R^2 = 0.99594$). The high contents of SeMet, which appear in small quantities in the non-proteolytic extract, implies that most selenoamino-acid is incorporated mainly into selenoproteins in the solid sample [20].

The extraction solution using only protease was able to extract the selenium compounds from the yeast sample with high extraction yields, assuring reliable results with acceptable accuracy. For being simpler, faster and less susceptible to cross contamination, after validation with CRM SELM-1, this extraction is the authors' choice to be applied in the next steps of this work.

3.7 Measurement uncertainty

The estimative of the measurement uncertainty in speciation analysis is complex because of several reasons: (i) chemical measurements are more difficult to model; (ii) there are more uncertainty sources that are often very difficult to quantify; (iii) inhomogeneity of the samples and (iv) the separation of the chemical species to be determined [33].

According to Figure 1 (Ishikawa diagram), in this work the uncertainty sources investigated were: uncertainty of the SeMet concentration in the analytical solution (u_{C0}), the uncertainty of the sample dilution (u_{df}), the uncertainty of the sample extraction (u_{ext}), the uncertainty of the instrumental repeatability (u_{rep}) and the uncertainty associated with moisture (u_{moist}). Figure 7 shows the relative uncertainty of each source.

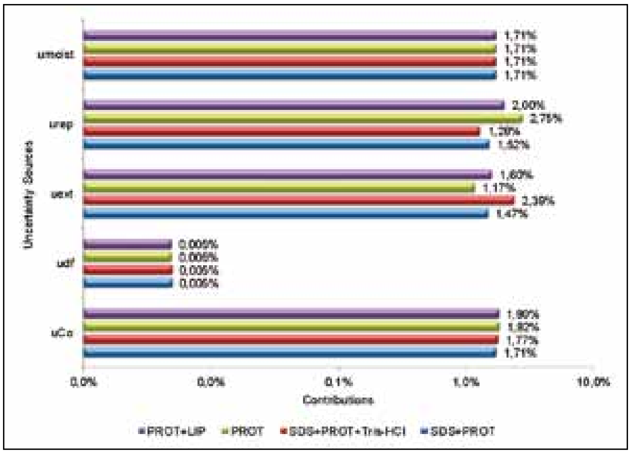

Figure 7. Relative uncertainty contributions.

The major relative uncertainty contribution for SeMet determination was instrumental repeatability (u_{rep}). This uncertainty source depends on several factors: (i) the interaction between mobile phase, column and analyte, (ii) efficiency of transport of the analyte in the interface HPLC-DRC-ICP-MS, (iii) repeatability of the volume injection, (iv) peak integration, (v) column temperature, (vi) imperfect functioning of mechanical parts of the chromatographic pump or electronic parts of the system, (vii) pulses from the perisistaltic pump (make-up flow rate), (viii) fluctuation of nebulizer gas, (ix) inefficient mixing between column effluent and make-up flow in the T piece, etc. [34]. These variables can explain the elevated contribution of this parameter to measurement uncertainty.

The relative uncertainty sources of u_{ext}, u_{C0} and u_{moist} have similar contributions (1.6 % - 1.8 % in relation to SeMet concentration in the sample).

The extraction procedure (u_{ext}) depends on enzyme activity, in other words, the ability of protease to fragment selenoproteins from yeast into its respective selenoaminoacids. The enzymatic activity depends on small variations in pH, temperature, sample to enzyme mass ratio and the time of reaction [24]. The specificity of the reaction and the spatial arrangement between the protease and the proteins also contribute to the vulnerability of the extraction procedure, resulting in greater dispersion of values.

Calibration curve regression is the major factor responsible for the variation of the SeMet concentration in the analytical solution (u_{C0}). The uncertainty of the estimated coefficients, the residual behavior of the calibration curve, assuming linearity in the case of slight nonlinearity of the calibration curve and instrumental repeatability are considered as critical points that increase the contribution of this uncertainty source [33].

The procedure used to determine sample moisture was made at 105°C in an air oven until constant weight. The water amount in yeast samples can be problematic for accurate analysis. Generally, high values of uncertainty (u_{moist}) can be explained due to volatilization problems of some component of the samples and the difficulty of removing all of the water molecules originally present without changing the mass of matrix and the chemical species [35], important in speciation analysis.

4 Conclusion

The HPLC-DRC-ICP-MS method developed in this work was optimized and validated. Good agreement of the certified value of SeMet was obtained for CRM SELM-1 (value obtained: 3420 ± 295 mg kg^{-1} and certified value: 3448 ± 146 mg kg^{-1}) and the figures of merit were considered adequate for the intended use.

The DRC effectively reduced the argon dimer interferences enabling the use of the most abundant isotope ^{80}Se and consequently to obtain a higher signal/noise ratio.

Different extraction protocols were evaluated to determine the presence of species of selenium in selenized yeast. The results obtained showed that the main Se species present in yeast is SeMet. Higher extraction efficiencies were obtained using the proteolytic method. SeMet appears in lower amounts when non-proteolytic procedures were used, indicating that this aminoacid is not free, but incorporated into selenoproteins.

This work provides metrological information on selenomethionine determination in yeast samples since it is part of a broader project aimed to production of a national certified reference material. The main uncertainty sources which affect the measurand (C_{SeMet}) were the instrumental repeatability (u_{rep}) and SeMet concentration in the analytical solution (u_{C0}), that contributed 35 % and 23 %, respectively, for ^{80}SeMet and protease extraction.

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References

1. Heras, I.L.; Palomo, M.; Madrid, Y.; *Anal. Bioanal. Chem.*, 2011, 400, 1717.
2. Clark, L.C.; Combs, G.F.; Turnbull, B.W.; Slate, E.H.; Chalker, D.K.; Chow, J.; Davis, L.S.; Glover, R.A.; Graham, G.F.; Gross, E.G.; Krongrad, A.; Lesher, J.L.; Park, H.K.; Sanders, B.B.; Smith, C.L.; Taylor, J.R.; *J. Am. Med. Assoc.*, 1996, 276,1957.
3. MacFarquhar, J.K.; Broussard, D.L.; Melstrom, P.; Hutchinson, R.; Wolkin, A.; Martin, C.; Burk, R.F.; Dunn, J.R.; Green, A.L.; Hammond, R.; Schaffner, W.; Jones, T.F.; *Arch. Intern. Med.*, 2010, 170, 256.
4. Yang, L.; Sturgeon, R.E.; McSheehy, S.; Mester, Z.; *J. Chromatogr. A*, 2004, 1055, 177.
5. Dumont, E.; Vanhaecke, F.; Cornelis, R.; *Anal. Bioanal Chem.*, 2006, 385, 1304.

6. Larsen, E.H.; Sloth, J.; Hansen, M.; Moesgaard, S.; *J. Anal. At. Spectrom*., 2003, 18, 310.
7. Vacchina, V.; Moutet, M.; Yadan, J.; Baene, F.; Kudla, B.; Lobinski, R.; *J. Chromatogr. B*.,2010, 878, 1178.
8. Dernovics, M.; Lobinski, R.; *J. Anal. At. Spectrom*., 2008, 23, 744.
9. Kitaguchi, T.; Ogra, Y.; Iwashita, Y.; Suzuki, K.T.; *Eur. Food Res. Technol.,* 2008, 227, 1455.
10. Wróbel, K.; Wróbel, K.; Kannamkumarath, S.S.; Caruso, J.A.; Wysocka, I.A.; Bulska, E.; Światek, J.; Wierzbicka, M.; *Food Chem.,* 2004, 86, 617.
11. Wrobel, K.; Wrobel, K.; Caruso, J.A.; *J. Anal. At. Spectrom.,* 2002, 17, 1048.
12. Grant, T.D.; Montes-Bayón, M.; LeDuc, D.; Fricke, M.W.; Terry, N.; Caruso, J.A.; *J. Chromatogr.* A., 2004, 1026, 159.
13. McSheehy, S.; Pohl, P.; Szpunar, J.; Potin-Gautier, M.; Lobinski, R.; *J. Anal. At. Spectrom*., 2001, 16, 68.
14. Far, J.; Preud'homme, H.; Lobinski, R.; *Anal. Chim. Acta.,* 2010, 657, 175.
15. Fang, Y.; Zhang, Y.; Catron, B.; Chan, Q.; Hu, Q.; Caruso, J.A.; *J. Anal. At. Spectrom.,* 2009, 24, 1657.
16. Roman, M.; Sánchez, M.L.F.; Sanz-Medel, A.; Iglesias, H.G.; Cescon, P.; Barbante, C.; *J. Anal. At. Spectrom*., 2011, 26, 100.
17. Montes-Bayón, M.; Molet, M.J.D.; Blanco-Gonzalez, E.; Sanz-Medel, A.; *Talanta*, 2006, 68, 1287.
18. Cañas, B.; Pineiro, C.; Calvo, E.; López-Ferrer, D.; Gallardo, J.M.; *J. Chromatogr. A*, 2007, 1153, 235.
19. Mesko, M.F.; Hartwig, C.A.; Bizzi, C.A.; Pereira, J.S.F.; Mello, P.A.; Flores, E.M.M.; *Int. J. Mass Spectrom*., 2011, 307, 123.
20. Huerta, V.D.; Sánchez, M.L.F.; Sanz-Medel, A.; *J. Anal. At. Spectrom*., 2004, 19, 644.
21. Reyes, L.H.; Sanz, F.M.; Espílez, P.H.; Marchante-Gayón, J.M.; Alonso, J.I.G.; Sanz-Medel, A.; *J. Anal. At. Spectrom*., 2004, 19, 1230.
22. Stefánka, Z.; Ipolyi, I.; Dernovics, M.; Fodor, P.; *Talanta*, 2001, 55, 437.
23. Infante, H.G.; O'Connor, G.; Rayman, M.; Wahlen, R.; Entwisle, J.; Norris, P.; Hearna, R.; Cattericka, T.; *J. Anal. At. Spectrom*., 2004, 19, 1529.
24. Peachey, E.; McCarthy, N.; Goenaga-Infante, H.; *J. Anal. At. Spectrom*., 2008, 23, 487.
25. Capelo, J.L.; Ximénez-Embún, P.; Madrid-Albarrán, Y.; Cámara, C.; *Anal. Chem.,* 2004, 76, 233.
26. *Guide to the Expression of Uncertainty in Measurement*. International Organization for Standardization (ISO), JCGM 100:2008.
27. Tanner, S.D.; Baranov, V.I.; Bandura, D.R.; *Spectrochim. Acta Part B*, 2002, 57, 1361.
28. Huerta, V.D.; Reyes, L.H.; Marchante-Gayón, J.M.; Sánchez, M.L.F.; Sanz-Medel, A.; *J. Anal. At. Spectrom*., 2003, 18, 1243.
29. Casiot, C.; Szpunar, J.; Łobiński, R.; Potin-Gautier, M.; *J. Anal. At. Spectrom.*, 1999, 14, 645.
30. Połatajko, A.; Banaś, B.; Encinar, J.R.; Szpunar, J.; *Anal. Bioanal. Chem*., 2005, 381, 844.
31. Rodríguez-Gonzalez, P.; Alonso, J.I.G.; *J. Anal. At. Spectrom*., 2010, 25, 239.
32. Gosetti, F.; Frascarolo, P.; Polati, S.; Medana, C.; Gianotti, V.; Palma, P.; Aigotti, R.; Baiocchi, C.; Gennaro, M.C.; *Food Chem*., 2007, 105, 1738.
33. Leito, S.; Mölder, K.; Künnapas, A.; Herodes, K.; Leito, I.; *J. Chromatogr. A*, 2006, 1121, 55.
34. Barwick, V.J.; *J. Chromatogr. A*, 1999, 849, 13.
35. ElSayed, N.I.; Mekawy, M.M.; Megahed, F.M.; *Aust. J. Basic Appl. Sci.*, 2011, 5, 582.

POINT OF VIEW

ANALYTICAL CHEMISTRY - CHALLENGES FOR A HIGH LEVEL RESEARCH

In last years, Brazilian science has grown in a fast way. It has been caused, among other reasons, by the increase of public investments in research and in infrastructure of new and well-established universities. Support for research as the National Institutes of Science and Technology program has been important to increase the scientific development and improve the spread of research information. In this sense, Analytical Chemistry has given an important contribution for the increase of publications in international journals. Fortunately and contrarily to other countries where the concern of young students in Analytical Chemistry is decreasing, Brazil is one of the few examples following a different direction.

If we consider the recently law of National Education Plan (PNE) that was approved in Brazil, we should have very good news in next ten years as the investments in education should achieve about US$ 22 billions up to 2023. It means around twice the current investments and should result in more than 60,000 masters and 25,000 PhD students per year. This new is important to motivate young students to follow Analytical Chemistry field as research support for equipments and structure should be guaranteed.

In Brazil, some research groups have achieved a consolidated status where they can compete in the border of knowledge with competitive groups in developed countries. However, we have observed that although there is a relatively high number of published papers, a big number of them is not bringing a vertical and real improvement but mainly little or even marginal scientific contribution. Even some of them have tried to discover the wheel again showing little knowledge of previous publications in literature where some "new discoveries" were already performed. No problems by revisiting previous works, it is extremely suitable and even recommended, but new publications must bring some advantage over existing methods or application for samples or analytes still not studied and not only repeating old works without new improvements. It has been partially highlighted in the excellent and critical point of view by Dr. L. T. Kubota (State University of Campinas) in April-June/2012 BrJAC edition.

With the fast expansion of universities in Brazil (increase of 100% of undergraduate students in the last ten years) many young PhD researchers have achieved a permanent position and started research activities in an independent way or in connection with established groups in older universities. However, this increase was not accompanied by a scientific quality improvement and it can be partially explained by a recent research showing that about 38% of undergraduate students in Brazilian universities can be classified as "functional unlettered" that are unable to understand even reasonably scientific texts and data in tables or charts. It is worrying and obviously can explain the difficulties of students motivation for good science. Moreover, the effect of the so called "social networks" is apparently not helping to decrease this problem and in many cases has even worsened the motivation of young students (and, unfortunately, also university teachers). It has been frequently observed that undergraduate and PhD students spend more time using Facebook, Tweeter or other distraction tools instead of using internet facilities to increase the scientific knowledge or make better the scientific quality of own research. Senior researchers must try to inform the young students (and even young researchers) the difference of real and serious research and that like "Facebook" research. Obviously, this situation is not particular of Analytical Chemistry but similar to all the other fields of science.

Among many other necessary tasks, one important way to show the correct direction is to have a more critical selection of oral presentations in scientific meetings and especially the evaluation of posters that must be evaluated (not only but mainly) by researchers with recognized quality of scientific work and not by a democratic (but never real) or popular selection of awarded works than rarely can distinguish the best works.

Therefore, considering the expressive increase of research support in next years, in my point of view it is imperative to have an urgent and critical discussion of the direction to be followed by Brazilian Analytical Chemistry, the new challenges in science and the quality level we expect for PhD students (and new generation of young researchers) in order to assure a healthy and continuous development in our country.

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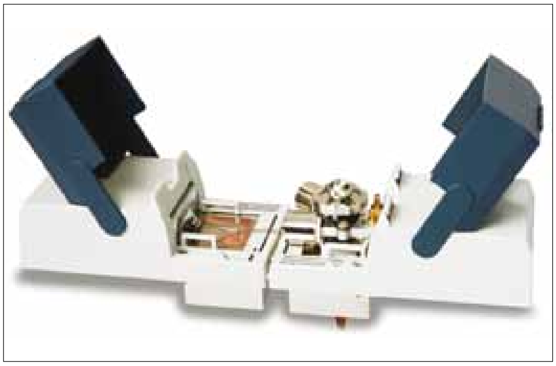

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- ***Software:***

9. Sheldrick, G. M.; SHELXL-93; *Program for Crystal Structure Refinement,* Gottingen University, Germany, 1993.

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

- ***Material presented in Congresses:***

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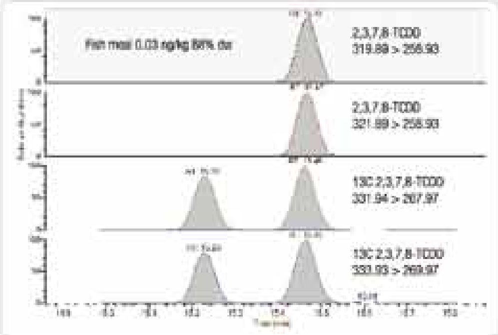
Fish meal 0.03 ng/kg 88% dw
2,3,7,8-TCDD
319.89 > 256.93
2,3,7,8-TCDD
321.89 > 258.93
13C 2,3,7,8-TCDD
331.94 > 267.97
13C 2,3,7,8-TCDD
333.93 > 269.97