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ERRATUM

SCHEFFLER, G.L..; MOMBACH, C.; BENTLIN, F.R.S.; DEPOI, F.S.; POZEBON, D., Determination of elements constituints of button cells. Brazilian Journal of Analytical Chemistry, v.1, p. 251-257, 2011.

Title

Determination of elements constituints of button cells

Change for

Determination of elements constituents of button cells

In Memoriam

On behalf of all the staff of the BrJAC, I want to pay a sincere homage to Professor and friend Reinaldo Calixto de Campos, who passed away last February 7th. In these lines, I would like to emphasize some personal aspects of Reinaldo, since there is no doubt that he was one of the keystones of Analytical Chemistry in Brazil.

With a generous heart, good humor, passionate for all his ideas and naturally conciliatory, Reinaldo was one of the greatest human beings that I have known. Most probably these characteristics explain how it was easy to have Reinaldo as a good friend. At his funeral, Reinaldo´s wife told me: "I did not know that I shared my husband with so many people". Paraphrasing Emerson (1803-1882): "A friend may well be reckoned the masterpiece of Nature".

I have known Reinaldo for a long time and we made a diverse trips together, discussing science, education, politics…and other topics. I remember a trip from Santa Maria to Porto Alegre…at that time Reinaldo was already the Dean of the Scientific and Technical Center of PUC-Rio, but his simplicity as human being was always fascinating, even while occupying a distinguished position in the University. "Reinaldo, you are a true guy", I said. And yes, Reinaldo was a true man, as true and intense as also was his life, his scientific carrier and his actions as a human being.

On various ocasions I had the chance of working together with Reinaldo, and all of them were really pleasant because his enthusiasm, competence, and amazing ideas, as demonstrated in the organization of some events as the Brazilian Meetings on Chemical Speciation (EspeQBrasil2008 and 2010), and, recently, on the Colloquium Spectroscopicum Internationale – CSI XXXVII, of which he was the chair. Reinaldo also contributed effectivelly to the National Institute of Science and Technology for Bioanalytics, where, besides being a researcher, he was a member of the advisory committee.

Even after his cancer was diagnosed a little bit more than one year ago, and being submitted to chemo- and radioterapies, Reinaldo did not give-up his duties, within and outside of PUC. I have no doubt that he left us prematurely…chemistry is now less reactive due to his absence…however, the legacies of a great man, like Reinaldo, are good examples, and he leaves to us his ideas to sort out.

Good friend, our scientific community and your friends are greatfull to you. We see you again in the eternity.

Marco Aurélio Zezzi Arruda
Editor

In Memory of Reinaldo Calixto de Campos.

The editor of BrJAC kindly invited me to write some words about my Colleague Reinaldo de Campos who passed away prematurely on February 7, 2012. I accepted this difficult task hoping to be as objective as possible, but knowing it is inevitable to be subjective and emotionally involved when paying a tribute to a recently lost friend.

I first met Reinaldo in 1977 as a young graduate student of Analytical Chemistry in PUC-Rio where I was responsible for the Advanced Analytical Chemistry discipline. He immediately stood out among the other students for his keen intelligence and inquisitive nature, not to mention the charming personality. As a PhD student he was under the guidance of Adilson Curtius and Harald Berndt who were leading scientists in Brazil and Germany, respectively, in the field of atomic spectrometry. Because of his outstanding performance as a graduate student and his proved teaching skills Reinaldo became an Assistant Professor at PUC-Rio in 1989, almost immediately after his thesis approval. Since then he supervised almost 40 dissertations and thesis, published ca 100 papers in scientific journals and participated or coordinated several projects financed by CNPq, CAPES, FINEP, FAPERJ and Petrobras, among others. In his laboratory a solid group was established to develop research in the field of atomic spectrometry. More recently, he had developed growing interests in proteomics and in association with other scientists established lab facilities in PUC-Rio to progress in this challenging research field. The importance of Reinaldo in the Analytical Chemistry community is proved in several ways; he was a research fellow of the CNPq and Cientista do Nosso Estado in FAPERJ. He also organized several scientific meetings; the last one was the Colloquium Spectroscopicum Internationale XXXVII in 2011 attended by prominent scientists from all over the world.

Reinaldo had especial impact as an educator and in several occasions was honored by students as patron of class. As a Dean of the Centro Técnico Científico in PUC-Rio over the last six years he made efforts to raise awareness about the importance of the teaching activities and development of new learning approaches. He was working in the direction to strengthen partnership between the university and secondary educational institutions as a means of better prepare students for the tertiary educational level.

All the information give above can be extracted from Reinaldo's CV and his impact in the Analytical Chemistry in Brazil can be measured either by the numerous citations of his papers and by the number of students that were under his guidance or attended his classes. However, the example of courage, the optimism and joy of living will only persist in our memories. During the difficult year of 2011 when submitted to debilitating chemo- and radiotherapies Reinaldo continued to work with his students, conducting his projects and fulfilling his duties as a Dean. His engagement in the dream of making the best for his country as a scientist and a citizen was a way of living that here I testify.

Angela Wagener
Director of Chemistry Department
PUC-Rio

Reinaldo Calixto Campos, age 57, died of pulmonary embolism on February 7 in the city of Rio de Janeiro. He was Dean of the Scientific Technical Center of PUC-Rio since 1989 and associate professor in the Department of Chemistry at the same University. A native of Rio de Janeiro's Botafogo neighborhood, this team was, in fact, his passion. Reinaldo defended his doctoral thesis in analytical chemistry in 1988 with the title "Study of a New Technique for Introduction of Solid Fuel Samples for the Determination of Volatile Elements by Flame Atomic Absorption Spectrometry" under the orientation of Adilson José Curtius Harald Berndt. In the graduate program of the Department of Chemistry at PUC-Rio Prof. Reinaldo was responsible for the formation of a large number of teachers and researchers for the scientific community, with emphases on Rio de Janeiro as well as other regions of Brazil, as well as providing many young people from the technical schools with the possibility training in his laboratories. Prof. Reinaldo and his group published 120 scientific papers in the area of atomic spectrometry in national and international journals of analytical chemistry, many of them also presented at symposia, conferences and congresses. Prof. Reinaldo directed over 85 masters' and doctoral dissertations. Prof. Reinaldo also always showed great concern for the issues facing teaching and learning and participated strongly in the projects "PIUES" and "Condigital" dedicated to producing materials for improving the teaching of chemistry. Prof. Reinaldo has always been very communicative, easy to form relationships and a voracious reader. Perhaps this was the differential factor I his career's success among his students and other teachers of chemistry and the other different areas of knowledge. Thus, on February 7, 2012 Brazilian society lost a great chemist, but he remains in memory with a great legacy for future generations.

Prof. Pércio Augusto Mardini Farias
Associate Professor at PUC-Rio






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According to the Brazilian Ministry of Education, the number of professionals with post-graduation increased by around 100% in Brazil in the last ten years. In 2002, the country had 23,400 researchers with a master degree and 6,800 with PhD degrees. In 2012, 41,300 researchers will obtain a master's degree and 13,300 a PhD degree.

CAPES has just recognized the quality of BrJAC ranking it as B4 in the Qualis classification. For a new Brazilian Analytical Chemistry journal, published in English in order to consolidate the integration of academic, business and research institutes, the news could not be better.

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Analytical Chemistry in Innovation Time

During the 20th century and the first decade of the 21rst, the chemical world together to other sciences such as information technology, instrumentation and automation, chemometrics, optics, image technology, magnetic fields, nanotechnology, physics and other scientific areas, underwent huge developments, working together to cover a wide field of applications like energy, petrochemicals, medicine, nutrition, the environment, green chemistry and many other areas to promote the development and improvement of science and industry, for the benefit of the community.

Looking forward, BrJAC has been created with the main objective of promoting integration and partnerships between academy and industry, of improving communication and propagating knowledge and development in chemistry. Certainly, after five issues of great publications we can affirm that the intended success has been achieved and we may sustain the expectation of great improvement in the next editions.

However under industry's optics, the great benefits of all this work is the capability to transform the results obtained into practical and wide applications in industrial activities to improve their productivity, competitiveness and profits, as well economic and social development. In this case we must have in mind the importance of some complementary, but not less important, tools, such as quality assurance, metrology and also the standardization process as an important subject because its final objective is know-how consolidation in such a way that it can be easily and widely used.

In the research and development field of knowledge this seems to be a little distant and difficult to achieve, but in fact the production of a technical standard should be seen as the last step of the development process, where new discoveries are transformed into an easy and simple procedures, processes, test methods or even new instruments. This is one practical way that can be used to effectively improve industry quality.

Keeping in mind that quality assurance, metrology and standardization are interesting possibilities to promote the continuity of our work, we wish you a nice reading of this issue of BrJAC.

Maura Moreira
Editor

Summary

Expedient

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TRENDS FOR ANALYTICAL CHEMISTRY DEVELOPMENT IN BRAZIL

In last decades, there was a strong increase in industrial production specially related to automation, microelectronics and informatics. This new wave of innovation, the so-called third industrial revolution, started with a reduced number of countries specially USA, Japan, Germany and more recently Korea and China. The technical progress changed the organization standards resulting in a strong increase of productivity and reduction of unitary production costs. As a consequence, it caused a higher technological distance of some countries from emerging economies as Brazil. Even considering that Brazil just keeps the increase of investment rate of last years, about more 20 years will be necessary to achieve the current level of European countries. Therefore, taking into account the status of organization of global economy and quick technological changes, Brazil must perform an enormous effort to advance in some specific strategic fields.

According to Brazilian Government (plan for *Brazilian Strategy of Science, Technology and Innovation for 2012 to 2015*), among the priority programs in our country some of them can be cited as the investments to develop Brazilian pharmaceutical industry, crude oil and gas field, advanced materials and nano- and bio-technologies.

As an example, in mining industry the chain of production and especially new developments related to rare earth elements (REE) and compounds, nowadays practically a monopoly of China, is of great concern for Brazilian Government. The whole process must be controlled but unfortunately there is a lack of reliable analytical methods concerning REE determination with the required accuracy at low levels. On the other hand, pre-salt is currently the most important productive chain that is able to increase the process of innovation and technological development in Brazil. There is a clear concern also for clean methods related to extraction and refining and new developments are necessary for determination of trace elements among other compounds of concern in crude oil and related products. Existing analytical methods must be updated in order to attend the new analytical challenges. In case of nanotechnology, according to *National Nanotechnology Initiative* (NNI) and *Nanobusiness Alliance*, about US$ 2,500 billion will be spent in 2015 (about 60% only for materials industry and electronics). However, the current status of analytical methods are not well suitable for analysis of advanced and nanomaterials and their products as well as for electronic devices and petrochemical products and related compounds as polymers.

Therefore, analytical chemistry probably will follow these tendencies providing suitable methods for the new challenges and requirements that will be necessary (obviously there are other important areas as sensors development, biotechnology and health sciences). Nowadays, Brazilian research groups present a relatively well established knowledge for many of required analysis in several matrices as water, food, biological and environmental matrices among others. However, despite the high relevance of these matrices, effort must be directed to the development of methods related to strategic fields as electronics, advanced and nanomaterials, ores, crude oil and its derivatives, polymers, pharmaceutical products, etc.

Fortunately, there is a strong and increasing development of analytical chemistry research in Brazil and recent works have been published in close cooperation with Brazilian companies in addition to basic research. Research applied to new materials or applications will require new methods development or updating the existing ones. For any case, analytical chemistry in Brazil will be asked for new responses and BrJAC - Brazilian Journal of Analytical Chemistry will be an strategic tool to help these new tasks providing an important space to high level analytical research.

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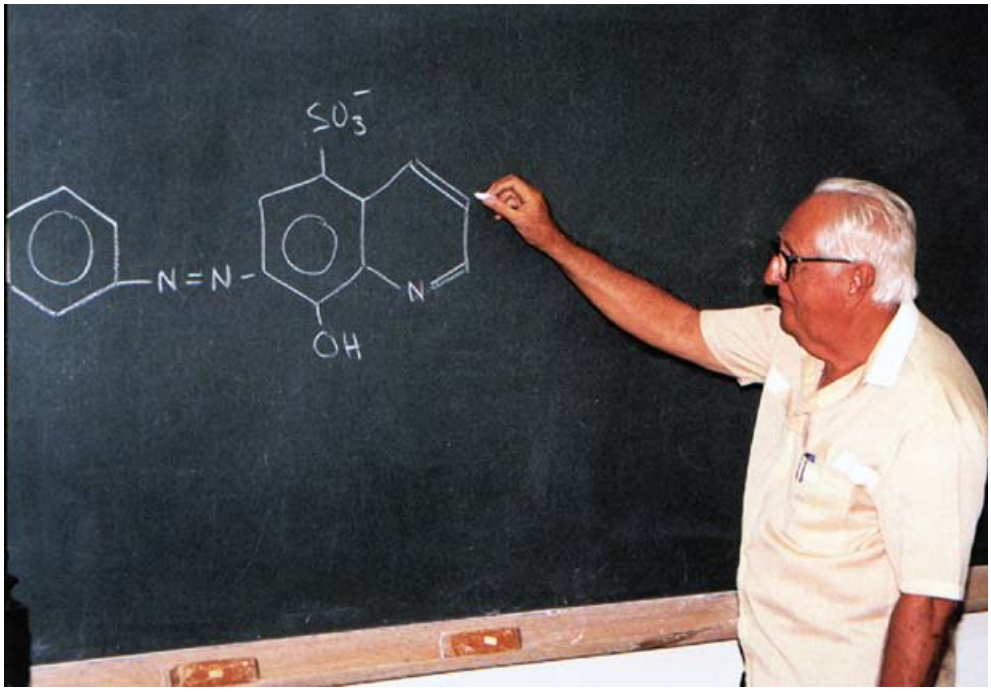
The analysis of the career of a professor shows a lot about his/her accomplishments, the challenges that were faced and technical problems that were solved during the teaching and researching years. However, this kind of metric, very proper for science production (which is huge for this chemist), does not show completely the importance of a professor in the academic lives of the graduation and specialization students around him. This is the case of professor Antonio Celso Spínola Costa, from Federal University of Bahia (UFBA), Brazil.

An example of a teacher



In a recent publication prepared by his co-workers and colleagues as a tribute to professor Spínola, all testimonials (and they were more than 20) are unanimous in referring to him as an extremely dedicated teacher and mentor. He is the "guru" for most chemists from Bahia, and for many in the whole country. The title of the publication is indeed "Example of a professor and scientist for his and for future generations", and it shows that Spínola was always concerned about the education in Chemistry in the country, in the school and high school years to begin with.

Such recognition, added to the many prizes and medals he won in his career, did not come by chance. It comes from hard work, intelligence, from

leadership and from a deep love for teaching. This last characteristic from professor Spínola is evidenced by the early age in which he reached his Habilitation (the post of free professor, which could be obtained in Brazil only after PhD title and after a very rigorous test). This allowed him to become the head of the Chemistry Chair in 1964 and full professor in 1968: he was only 38, and reached the top of the academic career 15 years after he graduated in Chemistry.

Looking back at his history, it is easy to see his fast movements. Professor Spínola, born in Salvador, Bahia, in 1930, entered to Polytechnic School, Federal University of Bahia (UFBA) in 1949, and during graduation, in 1951, he had his first contact with the teaching experience, playing the role of a volunteer monitor in a Geology laboratory, where he was responsible for most of the practical course. He graduated in Industrial Chemistry Engineering in 1953 and began his professional life immediately, as the responsible for the Analytical Laboratory of the Bahia Institute of Inorganic Technology. He also went to Rio de Janeiro for a fellowship in a Mineral Production Laboratory.

In June, 1956, the young chemist was hired as a professor to the chair of Analytical Chemistry in the Polytechnic School of UFBA, where in very poor facilities, he began to develop his first study, "Erichrome Black T as Qualitative Test for Magnesium", published in the School journal in the next year. This was the beginning of a very large production in the two main lines of studies of professor Spínola: the use of organic reagents in inorganic analyses (although he has taught in many disciplines, Spínola is recognized as a Brazilian pioneer in this area of analytical chemistry) and techniques of decomposition and solution for sample preparation. In the year of 1958, professor Costa published his first

international essay, in the Chemist-Analyst journal (currently Analyst). Two years later, he published in Analytica Chimica Acta and, in 1961, in Mikrochimica Acta. These three publications are considered milestones of his research line, which resulted in more than 70 papers. Fritz Feigl cited his techniques in his famous book Spot Tests. His international connections indeed improved after his fellowship with professor T. S. West in the Imperial College, in London.

While dedicating his time to teach and to researching, Spínola also got more and more involved in the university's affairs. In the year of 1969, a University Reformation was issued by the Ministry of Education in Brazil, and Spínola then worked in the creation of the Chemistry Institute of UFBA, which he founded that year and became the first director (he would director the Institute again between 1975 and 1979). In 1971, the Institute was already building its own premises in the university campus, allowing the installation of the laboratories necessary for teaching and researching. The UFBA Chemistry Institute is known as one of the best Brazilian research institutes in chemistry.

While conducting the Chemistry Institute, he did not forget his commitment with the university as a whole. From 1968 to 1971, he was part of the group dedicated to the university reformation, which lead to the creation of the first Master's Degree program in the university. By that time, he also coordinated the United Nations Development Program and to the United Nations Educational Scientific and Cultural Organization (UNDP-UNESCO) in order to reinforce the teaching of basic sciences in the country. From 1976 to 1980, he participated in the Research and Post-Graduation Rectory.

His reputation spread nationally. From 1981 to 1990, his was made counselor of the National and also de Regional Chemistry Councils, organs that regulate the professional activities of chemists in Brazil. He is known as a very rigidly ethical "ad hoc" consultant for these institutions as well as national financial research agencies that often seek for his expertise. He got many funding grants, due to the quality of his research, and he was named an Excellence Fellowship Scholar by CAPES (the organ from the Ministry of Education that coordinates the training of graduation and post-graduation teachers in Brazil). In 1991, he succeeded in founding the first Doctoral program in Chemistry in UFBA, and started advising students, in this and in other universities. During his academic path, professor Spínola supervised more than 47 Master's Degree and 8 PhD Degree applicants.

His students say about him:
"Patient with those who want to learn, impatient with all others"
"Inspiringly good humored"
"Acutely intelligent"

These graduate students were never left alone in their research activities. In an intense schedule of meetings and production targets, professor Spínola never let their enthusiasm to fade. Every week they had to meet with the supervisor to present the new accomplishments and to receive guidance. And it was not possible to skip these meetings, because even when he was abroad, in his conferences, professor Costa called them by phone regularly to be informed about the laboratory activities -- in the era before the internet, such international phone calls were expensive, but important.

The undergraduate students were also intensely stimulated. In classroom tests, they were allowed to consult any book they wanted. The evaluation of the chemistry knowledge was not based on individual information, but in the student's ability to think and solve problems. The undergraduate students used to fill large

supermarket paper bags with the books and bring them to help with the evaluation, but felt they were useless during the written tests. The teacher would say then: "those who really know the subject don't need books anymore".

In 2000, professor Spínola was 70 years old and, according to Brazilian law for federal university, he had to retire, leaving the space for younger teachers to work. "Educators do not retire", some would say. Professor Spínola had to, but he never stopped teaching, offering orientation to students and researchers and participating in workshops, conferences and other meetings with his colleagues. A resolution by the university some years before that had already created a special program for retired professors, aimed at avoiding the interruption of important researches and taking advantage of the skills and experience of its most productive professors. This had made it possible to allow those who were retired to resume or to continue their academic activities, including grant applications, doctoral degree supervising, participation in the quality judgment of thesis and coordination of research groups. Every two years, these professors are evaluated and can again present the application for the "job". The Emeritus Professor Spínola Costa has been doing that for the last 10 years.

You became senior professor only 10 years after you had become a teacher. What do you think has lead to a so consistent (and fast) progression as yours in UFBA? What do you think of the academic profession in Brazil?

I progressed quickly because I was devoted entirely to the academic career. About academic careers in Brazil, in my opinion, teachers are poorly paid and the state bureaucracy hinders the further development of science.

You have been an intern fellow with Professor T.S. West at Imperial College of Science and Technology in 1972. What have you brought from the experience?

The Imperial College at the time was the largest center of Analytical Chemistry in the world. This enabled my access to frontier topics only available in England, such as use of organic reagents in inorganic analysis.

You have just turned 80 years old: what are your day to day activities? What do you like to do? Do you maintain contact with Chemistry? How was the ceremony that was held on your birthday at UFBA?

I was very moved by the ceremony organized at UFBA. At that time I could see my friends and colleagues who were present throughout my academic career. I love reading a book while listening to good music as well as enjoy my family, especially my grandchildren. I still go to the Yacht Club of Bahia each week to keep in touch with friends. I still keep contact with chemistry by reading journals and attending conferences.

What does delight you most in analytical chemistry?

What made you choose that line of research (organic reagents for inorganic analysis)?

What most amazes me in Analytical Chemistry is the obsession with accuracy in the experiments, besides the fact that it is extremely important for science as a whole. I first picked this area of research because of the shortage of equipment for research in Bahia at the time. It was still an underdeveloped field, and I felt it would be possible to explore it even with all the limitations of that time. After this start, marked by technical difficulties, I continued to believe that this area was lacking of researchers in Brazil. It was also an area that allowed me to exercise my creativity.

"What most amazes me in Analytical Chemistry is the obsession with accuracy in the experiments, besides the fact that it is extremely important for science as a whole."

Do you keep yourself informed about the progress of research in chemistry? What is your opinion about the current state of research of ultra-traces in Brazil? And what do you think about obtaining analytical information in real time? What are the latest advances and the challenges in analytical chemistry, a global projection for the future?

Yes, I keep myself informed by reading the international journals. I believe that the area of ultra-traces should be more explored in Brazil.

The BRJAC thanks the students of Chemical Engineering, Federal University of Rio de Janeiro (UFRJ) and Federal University of Sergipe (UFSE), Celso Eduardo Costa and Costa, grandchildren of professor Antônio Celso Spinola Costa, for their assistance in this interview, which was held on February 18, 2011

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Availability of Al, Cu, Fe, Mn, and Zn in Brazilian herbal teas

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Abstract

The availability of Al, Cu, Fe, Mn and, Zn were determined in Brazilian teas by flame atomic absorption spectrometry after different sample pretreatment procedures. The total element contents were assessed after microwave-assisted digestion using concentrated nitric acid and hydrogen peroxide. Extraction procedures using water and a simulated gastric juice solution were investigated. The tea infusions were prepared in the traditional way or using a domestic microwave oven. The results showed that in arnica (*Solidago microglossa*), stone breaker (P*hyllantus niruri*), lemon grass (C*ymbopogon citratus*) and ginger (Z*ingiber officinalis*) Fe presented the highest total content after cavity-microwave-assisted digestion. Results from infusions obtained using a domestic microwave oven were always higher than those from traditional infusion. Aluminum was not present in the infusion after applying either the domestic microwave oven or traditional infusion. After the infusion procedure Mn was the most abundant element in all of the herbal teas, while Cu was found at the highest values in arnica and ginger. Copper and zinc showed similar efficiency of extraction for all investigated procedures. Precision, expressed as repeatability, was found to have a relative standard deviation consistently below 6%. Limits of detection and quantification and linearity were calculated for all sample media. Accuracy was checked using NIST SRM 1547, and all values were in agreement with certified values at the 95% confidence level after applying the Student t-test.

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Keywords: Brazilian teas, availability, extraction procedures, FAAS

1. Introduction

Tea and herbal tea are two of the oldest, most popular, and widely consumed drinks after water, due to their refreshing and mild stimulant effects [1]. The history of tea dates back more than 5000 years, when it was first used by the Chinese as a medicinal drink and later as a beverage [2]. Traditional teas are classified into three types depending on the fermentation process: green tea (non-fermented), produced by drying and roasting the leaves; oolong (semi-fermented), obtained by partial fermentation; and black tea (fermented), which is fully fermented [3].

Herbal teas can be made with fresh or dried flowers, leaves, seeds or roots, typically by adding boiling water over the plant parts. They are consumed for their physical or medicinal effects, especially their stimulant, relaxant or sedative properties [4].

In recent years, herbal tea has been increasingly used by the general public to replace or complement conventional medicines [5]. According to the World Health Organization, 70–80% of the world population, especially in developing countries, relies on non-conventional medicines derived mainly from herbal sources in their primary healthcare [6].

Some trace elements have curative or preventive roles in combating diseases, so it is important to know the levels of macro- and micro-elements in medicinal plants and herbal medicaments to estimate their roles as sources of these components in the human diet. These same components at elevated levels, however, can be dangerous and toxic. Therefore, these precautions are indispensable when larger amounts of these products are consumed, i.e., when long-term therapy is need [5]. So, sample pretreatment procedures are useful to promote analyte quantification as a solution.

Sample pretreatment procedures should be performed in order to reconcile efficiency and reagent economy, increasing the simplicity of the method and the reliability of results through the optimization of loss, and reducing contamination and environmental impact [7].

Lésnieswicz et al. [5] determined the bioavailability and the total content of macro and micro-nutrients in Polish herbs by inductively coupled plasma optical emission spectrometry (ICP OES). The bioavailability was assessed by infusion, 0.1 mol L^{-1} HCl solution extraction and simulated

gastric juice solution. The authors also presented the daily intake recommended for a number of elements. For example, for Al, the maximum tolerable amount is 70000 µg/day, the recommended concentration of Cu is between 900-1000 µg/day, Fe has a range of 8000-18000 µg/day, Mn a range of 1800-2300 µg/day and Zn a range of 8000-11000 µg /day.

The infusion of elements by traditional or domestic-microwave simulates the way of preparation by a large portion of the general population [8]. Basgel and Erdemoglu [9] determined fourteen different elements in herbs consumed in Turkey for medical purposes. In these medicinal plants, there was a higher total content of Fe and a lower content of Cu. Aluminum and Fe presented the lowest values in the infusions.

Malik et al. [1] determined micro and macro-elements in 31 samples from infusions of stimulants (coffee and tea). Aluminum, Cu, Fe, Mn and Zn were quantified by ICP OES. After infusion, Fe was the element least extractable and Mn showed the highest concentration.

Flaten [10] dedicated a review to Al in tea leaves (*Camellia sinensis*) in which the bioavailability of this element from the normal infusion procedure and also simulated gastric and intestinal solutions was presented. The results showed that the use of plant infusion increases metal absorption. However, bioavailability of Al present in tea was shown to be not very different from other dietary sources.

This work evaluated the availability of Al, Cu, Fe, Mn, and Zn in teas popularly used in Brazil after different sample pretreatment procedures. The procedures used included infusion by either traditional or domestic microwave oven methods, and simulated gastric juice solution extraction. Cavity-microwave-assisted acid digestion was employed to estimate total element content. The element quantifications were realized by flame atomic absorption spectrometry (FAAS).

2. Experimental

Instrumentation

A Perkin Elmer Model AAnalyst 200 flame atomic absorption spectrometer (Norwalk, CT, USA) equipped with a deuterium lamp for background correction system was used. A multielement hollow cathode lamp composed of Cu, Fe, Mn, and Zn was used to quantify the analytes using an air-acetylene flame. For Al, quantification was performed using a nitrous oxide-acetylene flame. The wavelengths for Al, Cu, Fe, Mn, and Zn were 309.30, 324.75, 248.33, 279.48, and 213.86 nm, respectively.

The cavity-microwave oven Ethos 1 (Milestone, Sorisole, Italy) was used for closed vessel acid digestion of the samples. A 120 mL TFM® (modified polytetrafluoroethylene) closed vessel with a calibrated resealing pressure relief mechanism was inserted onto a rotating turntable inside the microwave oven cavity. The heating program was performed as following: in the first step, 10 minutes were spent to reach 200 °C. The heating was kept at 200 °C for 20 minutes. Ventilation step was applied as final step.

A digital shaker incubator bath (model CT-712, Cientec, São Paulo, Brazil) without refrigeration was used in the simulated gastric juice solution procedure.

Samples, reagents and solutions

The tea samples investigated included arnica (*Solidago microglossa*), stone breaker (*Phyllantus niruri*), lemon grass (*Cymbopogon citratus*), and ginger (*Zingiber officinalis*). These samples were chosen according to popular consumption and two samples of each tea (A and B) were purchased at a local market in Belo Horizonte, Brazil.

All solutions were prepared using analytical-grade reagents, and deionized Milli-Q water (18.2 MΩ cm^{-1}, Direct-Q 318, Milli-Q, Millipore) was used throughout, including for the infusions. Hydrogen peroxide (30% m m^{-1}) and concentrated nitric acid (Merck, Darmstadt, Germany) were used for sample digestions. Pepsin from the gastric mucosa of pigs (Sigma-Aldrich, P7000, St.Loius, MO, USA), sodium chloride (Synth, São Paulo, Brazil) and concentrated hydrochloric acid (Merck, Darmstadt, Germany) were used for preparing the simulated gastric juice solution.

The reference solutions of Al, Cu, Fe, Mn, and Zn for calibration curves were prepared daily by suitable dilution from 1000 mg L^{-1} stock solutions (Merck, Darmstadt, Germany) in acid, water and simulated gastric juice solution media.

Sample preparation

Herbal teas were dried at 60 °C until constant weight. After drying, the samples were submitted to cryogenic grinding to ensure particle sizes smaller than 63 µm, increasing homogeneity.

Cavity microwave-assisted acid digestion

About 200 mg of each tea sample was accurately weighed directly into a TFM® microwave reaction vessel. Then, 7 mL of concentrated HNO_3 was added and, after 30 minutes of contact, 1 mL of 30% m m^{-1} H_2O_2 was added. The vessels were closed and the heating program was executed. The digests were transferred to 50 mL polypropylene bottles and filled with deionized water. These samples were all prepared in triplicate.

Infusion preparation

Traditional infusions were made by transferring 500 mg of the sample into polypropylene bottles and adding 50 mL of boiling deionized water. This mixture was allowed to rest for 2 minutes, and then submitted to centrifugation at 3500 rpm for 20 minutes. The supernatant was removed for element quantification by FAAS.

To prepare the tea sample in a domestic microwave oven, 50 mL of deionized water was added to a beaker containing 500 mg of sample. The mixture was inserted

into the domestic microwave oven for 2 minutes and transferred to a polypropylene bottle for centrifugation at 3500 rpm for 20 minutes. The supernatant was removed for element quantification by FAAS.

All samples were prepared in triplicate, and the matrix effect was evaluated by comparing analytical curves prepared in water and supernatant infusion medium.

Procedure employing simulated gastric juice solution

For the procedure using simulated gastric juice solution, 500 mg of each sample were added into 30 mL of simulated gastric juice solution containing sodium chloride, hydrochloric acid and pepsin (1.6 g pepsin, 0.3 g of NaCl and 3.5 mL of 37% HCl, completed to 500 mL with deionized water). The closed Erlenmeyer was shaken for 1 h at 37 ºC at 200 rpm. A centrifugation step at 3500 rpm for 20 minutes was also realized. All investigated extracts were stored in clean polyethylene bottles at 4 ºC before analysis.

3. Results and discussion

3.1. Validation of the proposed procedures

In the present work, Al, Cu, Fe, Mn, and Zn contents were determined in each herbal tea by applying different sample pretreatment procedures. Table I summarizes the medicinal use associated with each of the tea samples investigated.

Table I. Teas consumed for medicinal purpose in Brazil

Tea (scientific name)	Indications
Stone breaker (Phyllanthus niruri)	Hepatitis, antitumor activity, antioxidant, antiviral and anticarcinogenic [11,12]
Lemon grass (Cymbopogon citratus stapf)	Digestive disorders, hypertension, tranquillize action, anti-inflammatory and antioxidant [13,14]
Arnica (Solidago microglossa)	Infections in the skin, against muscular pain, sedative, antiinflammatory [15]
Ginger (Zingiber officinalis)	Arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting and hypertension [16,17]

The quantitative determinations were carried out by an analytical calibration curve for the samples, blanks and NIST SRM 1547. The parameters of the calibration curves for the analytes in different extraction solutions were obtained by the least squares method, and linearity was checked through a curvature correlation coefficient (R). The values were always higher than 0.999 for all correlation coefficients in all evaluated media, except for Mn and Zn quantification in 2.0 mol L^{-1} HNO_3 (R=0.998).

Precision, expressed as relative standard deviation (RSD), was evaluated by repeatability in three different concentrations (low, medium and high level) using seven replicates. Values showed low standard deviation, with RSD <6% for all investigated concentrations in all levels.

Accuracy was checked using peach leaves NIST SRM 1547 after cavity-microwave-assisted acid digestion. It can be seen in Table II that results obtained for Al, Cu, Fe, Mn, and Zn are in agreement with the certified values at 95% confidence level, applying Student t-test.

The limits of detection (LOD) and quantification (LOQ) were calculated by measuring 10 independent blank solutions for all extraction solutions, including blanks from the cavity-microwave oven.

Table II. Determination of Al, Cu, Fe, Mn, and Zn in peach leaves NIST SRM 1547 after cavity-microwave-assisted digestion (mean ± standard deviation; n = 3).

	Al (µg/g)	Cu (µg/g)	Fe (µg/g)	Mn (µg/g)	Zn (µg/g)
Certified values	249 ± 8	3.7 ± 0.4	218 ± 14	98 ± 3	17.9 ± 0.4
Cavity-microwave	241 ± 3	3.6 ± 0.1	211 ± 4	97 ± 2	17.5 ± 0.2

3.2. Al, Cu, Fe, Mn, and Zn content in tea samples after sample pretreatment procedures

The total contents of Al, Cu, Fe, Mn, and Zn in each sample were determined using a cavity-microwave-assisted acid digestion procedure. These values were adopted as reference values to estimate the efficiency of extraction under each of the experimental conditions. Table 3 presents the Al, Cu, Fe, Mn, and Zn contents obtained for all sample pretreatment procedures. The values obtained in the extraction procedures can be used to estimate the dairy ingestion of the total metal amount in a cup of tea.

In Table III, it can be seen that, except for ginger and stone breaker A, Fe was present in the highest total contents in all samples. The contents of each of the elements for samples A and B for each tea were different except for Cu, whose values were very similar. Aluminum was not detected in ginger. Total content distribution of the analytes in the tea samples followed the order: Fe > Al ~ Mn > Zn > Cu. Kara [4] also noted the same distribution in the total contents of elements after quantification of different medicinal herbs.

The concentrations obtained by the cavity-microwave oven digestion procedure do not represent the availability of these elements for the general population that consumes tea as medicine, so extraction procedures are required. Mild procedures can be used depending on the purpose of the analysis and the matrix, and extractions using water or dilute acids present satisfactory results in the determination of compounds in medicinal plants [10].

The traditional and domestic microwave infusions were prepared to simulate the method of tea preparation at home.

Table III. Aluminum, Cu, Fe, Mn, and Zn contents in tea samples after different sample pretreatment procedures (n=3).

		Al (μg/g)	Cu (μg/g)	Fe (μg/g)	Mn(μg/g)	Zn (μg/g)
Cavity-microwave	Arnica A	259 ± 2	11.8 ± 1.9	1370 ± 24	963 ± 18	35.1 ± 0.8
	Arnica B	208 ± 12	11.7 ± 1.0	2058 ± 72	766 ± 31	33.7 ± 1.8
	B-stone A	1777 ± 14	6.36 ± 0.02	1641 ± 44	106 ± 1	17.5 ± 0.1
	B-stone B	640 ± 5	5.85 ± 0.33	935 ± 19.6	175 ± 4	32.7 ± 0.6
	Lemon grass A	193 ± 7	4.45 ± 0.30	410 ± 9	96.1 ± 2.9	20.7 ± 0.8
	Lemon grass B	161 ± 4	5.7 ± 0.3	391 ± 19	118 ± 12	22.5 ± 1.7
	Ginger	< LOD	7.64 ± 0.74	65.4 ± 1.1	67.3 ± 1.6	8.99 ± 0.15
Traditional infusion	Arnica A	< LOD	6.40 ± 0.53	2.12 ± 0.08	232 ± 9	17.1 ± 0.5
	Arnica B	< LOD	6.40 ± 0.27	2.52 ± 0.14	173 ± 8	16.2 ± 0.20
	B-stone A	< LOD	4.02 ± 0.24	5.49 ± 0.30	50.2 ± 1.5	7.26 ± 0.89
	B-stone B	< LOD	2.87 ± 0.11	4.46 ± 0.17	83.2 ± 6.9	16.7 ± 1.0
	Lemon grass A	< LOD	2.27 ± 0.11	5.88 ± 0.38	63.8 ± 2.5	5.25 ± 0.40
	Lemon grass B	< LOD	1.77 ± 0.13	5.62 ± 1.40	90.9 ± 8.3	5.58 ± 0.53
	Ginger	< LOD	5.40 ± 0.34	15.2 ± 0.4	22.5 ± 2.0	5.51 ± 0.68
Home-microwave infusion	Arnica A	< LOD	7.17 ± 0.57	7.38 ± 0.02	328 ± 8	22.5 ± 1.0
	Arnica B	< LOD	6.96 ± 0.33	8.35 ± 0.51	276 ± 4	22.5 ± 1.8
	B-stone A	< LOD	4.48 ± 0.24	9.73 ± 0.12	100 ± 5	14.0 ± 0.6
	B-stone B	< LOD	3.71 ± 0.23	14.20± 1.2	166 ± 1	25.3 ± 0.3
	Lemon grass A	< LOD	1.77 ± 0.22	3.09 ± 0.54	48.2 ± 0.7	6.26 ± 0.31
	Lemon grass B	< LOD	1.18 ± 0.18	4.39 ± 0.39	74.6 ± 2.8	6.38 ± 0.12
	Ginger	< LOD	7.07 ± 0.27	18.3 ± 0.60	31.6 ± 0.9	6.75 ± 0.14
Gastric juice solution	Arnica A	< LOD	7.97 ± 0.06	43.8 ± 1.0	374 ± 4	25.8 ± 1.1
	Arnica B	< LOD	7.46 ± 0.05	49.4 ± 1.4	312 ± 5	25.2 ± 0.5
	B-stone A	17.5 ± 0.9	4.97 ± 0.06	143 ± 2	98.8 ± 1.6	13.5 ± 0.4
	B-stone B	< LOD	3.09 ± 0.06	83.1 ± 4.0	153 ± 4	23.6 ± 0.5
	Lemon grass A	< LOD	2.41 ± 0.09	32.2 ± 0.7	73.9 ± 3.0	9.23 ± 0.31
	Lemon grass B	< LOD	3.04 ± 0.11	28.4 ± 0.9	95.4 ± 1.2	8.81 ± 0.24
	Ginger	< LOD	4.40 ± 0.11	31.2 ± 1.0	48.0 ± 0.6	5.96 ± 0.10

The results obtained after these procedures showed the metal concentrations ingested considering consumption of a cup of tea. For all elements, metal concentrations were superior using a domestic microwave, indicating that tea preparation in the domestic-microwave offers more essential elements for the body than a traditional preparation. This result confirms the superior efficiency of microwave radiation to transfer energy over conductive heating. The microwave radiation reached the sample directly, without energy loss to the reaction vessel, and consequently, the efficiency of energy transfer is high [18]. When conductive heating is used part of the energy is lost in the reaction vessel walls. Microwave radiation power, temperature and time are critical variables in extraction procedures and the efficiency of these procedures is strongly associated with them [19]. Aluminum concentrations, independent of the tea, were below to the LOD, after traditional and domestic microwave infusions. Manganese presented the highest content after the infusion procedure for all investigated samples, ranging from 22.5 to 328 μg g^{-1}. According Kumar et al. [20] tea leaves contain 300–900 μg/g of Mn, an essential element for plants, microorganisms and higher animals, including humans. Considering the recommended daily intake of 2–5 mg of Mn per day, the amount needed may be provided solely through the consumption of a few cups of tea per day, considering that each cup of tea contains around 200 mL.

Simulated gastric juice solution extraction can indicate the bioavailability of analytes present in tea to the digestive tract (Table III). The concentrations of Cu and Fe found for this method were around 2 to 8 μg g^{-1} and 28 to 143 μg g^{-1}, respectively. Copper was more efficiently extractable in B-stone breaker than A, reaching 78%, while Fe was more extractable in ginger, with 48%, when compared to the cavity-microwave digestion procedure. For Mn and Zn, the values were more variable in the range of 48 to 374 and 6 to 26 μg g^{-1}, respectively. Manganese extraction values were 39, 87, 81, and 71% for arnica, B-stone, Lemon grass, and ginger, respectively. Extractable values around 70% were found for Zn in arnica and B-stone. Again, Mn and Zn extraction values were compared to the cavity-microwave digestion procedure. For Al, values between LOD and LOQ were obtained, and the concentrations found were in a range of 12 to 20 μg g^{-1}, with a RSD less than 10%. The results of Mn obtained are in agreement with the literature, which reports that Mn is the most bioavailable element to the human body. These authors pointed out that under simulated intestinal conditions, a single serving of tea contributed about 40% of the average daily dietary intake of manganese [20].

The availability of the elements found in tea leaves in a biologically useful form is still unknown. For this reason, extraction results can be seen as preliminary data for the estimation of ingested concentrations. For example, determination of Al concentrations could be critical for patients with renal failure. However, for most samples, Al was poorly extracted in most media, reaching values as low as 7%, as was previously reported [3].

Extraction efficiency was calculated assuming cavity-microwave digestates values as 100% efficiency. The levels obtained by the sample pretreatment procedures decreased according to the following order: simulated gastric juice extraction > domestic microwave infusion > traditional infusion. One exception was noted for Zn, for which domestic microwave infusion was superior to simulated gastric juice solution extraction.

For nutritional information, infusions and simulated gastric juice solution represent the metal concentration that is ingested and, consequently, available to the human body. Using these solutions, extraction values were always below to values considering toxic to humans (Al 70000 μg/day, Cu 10000 μg/day, Fe 45000 μg/day, Mn 11000 μg/day and Zn 40000 μg/day) indicating that some cups of herbal tea can be consumed daily [5].

Despite the high total Fe content present in the tea samples studied, this element is poorly available for human body absorption, showing that ingestion from herbs leaves a deficiency of this element when compared to the daily

recommended requirement [5]. On the other hand, the high availability of manganese suggests that tea is a rich source of this dietary element for humans. These results were confirmed by Powell et al. [21], who demonstrated that tea is not a rich dietary source of essential metals for humans, except for manganese.

4. Conclusion

The determination of Al, Cu, Fe, Mn, and Zn in herbal tea was done using digestion and extraction procedures. The extraction of the elements depends on its characteristics, heating procedure, and on the extraction solution employed. Microwave procedures were found to be more efficient than conductive heating, likely because it provides better control of the energy transferred. The simulated gastric juice procedure was found to be a simple and effective procedure to estimate the bioavailability of the investigated elements in tea samples.

The evaluated procedures showed that Al and Fe were not efficiently extractable from tea, while Mn was the most easily extractable element.

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Development and validation of a gradient RP-HPLC method for quantitative determination of potential manufacturing impurities in deferasirox (API) and pharmaceutical formulations

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Abstract

Three impurities in deferasirox drug substances were detected and quantified using a simple gradient reversed phase HPLC method. The chromatographic separation was achieved on a Phenomenex Luna C-18(2) 100 Å 3 µm, 150X4.6 mm column using a mobile phase consisting of phosphate buffer (pH-8.00) - acetonitrile with UV detection at 252 nm and flow rate of 0.8 ml/min. The column temperature was maintained at 40 ^{0}C throughout the analysis. This method is simple for the separation of deferasirox and potential manufacturing impurities from each other in 45 minutes of run time. The proposed method has been validated as per international guidelines on method validation and can be used for the routine quality control analysis of deferasirox as active pharmaceutical ingredient (API) and in pharmaceutical formulations. Also the method was determined to be robust with regards to the following parameters: mobile phase apparent pH; mobile phase organic content; detection wavelength and time dependence of sample and standard stability.

Keywords: Deferasirox; Potential manufacturing impurities; HPLC; Pharmaceutical formulation; API

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1. Introduction

Deferasirox (4-[3,5-bis (2-hydroxyphenyl) -1,2,4-triazol-1-yl]benzoic acid) is the first in a new class of tridentate, oral, iron-chelating agents used in the treatment of chronic iron overload secondary to multiple RBC transfusions, i.e., transfusional iron overload [1,2]. Iron overload is a serious complication caused by frequent blood transfusions in patients with blood transfusion-dependent anemias, such as β-thalassemia sickle cell disease, other rare anemias, and myeloproliferative disorders [3]. Deferasirox has been developed as an alternative to deferoxamine, the standard treatment of chronic iron overload, which is administered parentrally in patients with chronic iron toxicity. Chelation treatments are effective for rapid iron removal, maintenance of low iron stores, prevention of heart and other organ damage caused by iron overload and, ultimately, improved survival [4-7]. Deferasirox is an orally active iron chelator that binds selectively to Fe^{3+} [8]. It is a tridentate ligand that requires two molecules to form a stable complex with each iron atom. The efficiency of chelation of this compound is defined as the ratio of the amount of iron actually excreted to the amount of iron that could have been theoretically bound.

Deferasirox is highly selective for iron, while it does not appear to promote dietary absorption of iron. Although deferasirox has very low affinity for zinc and copper, there are variable decreases in the serum concentration of these trace metals after the administration of deferasirox. These transient decreases may, however, be clinically irrelevant. The iron complex of deferasirox appears to be inert and is excreted to a large extent in the feces, rather than being redistributed. Deferasirox is the first FDA approved oral drug for chronic iron overload [9].

The presence of impurities in an API can have a significant impact on the quality, efficacy, stability and safety of drug products and, as per regulatory requirements, an impurity profile study has to be carried out for final drug [10,11]. ICH guideline for API indicate that new impurities at levels of > 0.15% for a ≤ 2 g/day daily dose or >0.05% for a >2 g/day daily dose should be qualified or reduced by purification of the batch prior to use in clinical studies [12]. The possible organic impurities present in the drug may be starting materials, intermediates, by-products, reagents, ligands and process degradation products [13]. A literature survey revealed that numerous techni-

ques have been reported for estimation of deferasirox in plasma and pharmaceutical formulations, including UV and visible spectrophotometery [14], electro-catalytic oxidation on a nickel oxyhydroxide-modified electrode [15], magnetic resonance imaging [16], HPLC coupled With MS/MS [17], visible spectrophotometery methods [18], and fluoroscence [19]. Some of reported methods however suffer from such disadvantages as poor selectivity, sensitivity, accuracy and precision. Separation of deferasirox from its potential manufacturing impurities is required for accurate and precise quantification of related substances present in formulated pharmaceuticals and API. This set of potential related substances was originally derived from synthetic considerations. To the best of our knowledge this would be the only method reported which is capable of resolving and quantify these manufacturing impurities. The chemical structure of deferasirox is shown in Fig. 1.

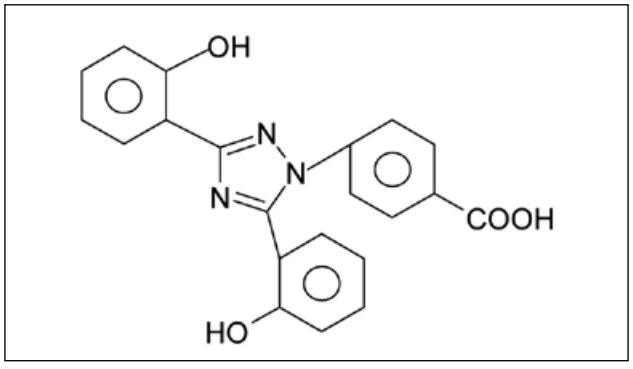

Figure 1. Chemical structure of deferasirox

2. Experimental

2.1 Reagents

Ammonium dihydrogen phosphate and ammonia solution were obtained from Rankem. HPLC-grade methanol, acetonitrile and water were obtained from Merck. All reagents used were of HPLC gradient grade.

2.2 Standards

Standard pharmaceutical grade deferasirox (Clearsynth Limited) and its formulation tablet from a commercial source were used. Salicylic acid and salicylamide was from Aldrich. 2-(2-Hydroxyphenyl)-4H-1,3-benzoxazin-4-one (DEF-I), Certified 99.9% pure was obtained from Medilux Laboratories India, Ltd.

2.3 Solution and sample preparation

Mobile phase A was 50-mmol/L, pH 8.0, ammonium dihydrogen phosphate solution, while mobile phase B was acetonitrile (HPLC gradient grade, Merck). The mobile phase flow rate was 0.8 mL/min. The column temperature was kept at 40 ^{0}C. The gradient profile and run time were the same with each column, initially a 70:30 v/v ratio of buffer and acetonitrile was run isocraticaly and then the linear gradient from 30 to 80% acetonitrile was applied from 5 to 25 min. From 25 to 35 min, the mobile phase composition was constant with 80% acetonitrile and 20% 50 mmol/L ammonium dihydrogen phosphate buffer. From 35 min to 37 min the mobile phase composition was changed to the initial composition, and remained the same until the end of the run. Each separation was stopped after 45 min.

A sample solution containing deferasirox at a concentration of 500 µg/ml was used for accurate and precise quantification. This sample solution was prepared from the composite powder of 30 130 mg deferasirox tablets. Methanol was added to the flask and shaken vigorously until the product clumps were dissolved. The flask was then sonicated at 25 ^{0}C for 5 min. The content of the flask was diluted to volume with methanol. Approximately 15 ml content of flask was transferred to a centrifuge tube and centrifuged at 3000 rpm for 10 min. A portion of the supernatant liquid was transferred to a HPLC vial. Deferasirox and related substances working standards and test solutions were prepared directly without centrifuging and were utilized for quantification.

2.4 Apparatus

Sample analyses were performed on an chromatographic system of the Waters Alliance series equipped with a built-in solvent degasser, quaternary gradient pump and Waters-2696 Photodiode array detector with variable injector and auto sampler with cooler. The chromatographic column utilized in these studies was a Luna C18 (2), 100Å, 150 x 4.6 mm x 3 µm. The detection wavelength was 250 nm. Mobile phase flow rate was 0.8 ml/min. Twenty microliters of sample was injected into the HPLC for every analysis. A Waters column heater module was used to maintain a constant column temperature of 40 ^{0}C. Photodiode array spectra were obtained from the Waters separation module equipped with a model 2696 photo diode array detector. Peak purity analysis was carried out over a wavelength range 200-300 nm through the use of the Empower$_{Tm}$-2 Build-2154 software. The stability chamber utilized during forced degradation studies was controlled by a temperature controller. All measurements were carried out at a temperature of (40±2 ^{0}C). The pHmetric studies were carried out on Decibel, Db-1011 digital pH meter fitted with a glass electrode as indicator and saturated calomel as reference electrodes.

2.5 Synthetic route of deferasirox

Salicylic acid and thionyl chloride is reacted with salicylamide in solvent to obtain 2-(2-hydroxyphenyl)-4H-1,3-benzoxazin-4-one. This intermediate is reacted with 4-hydrozinobenzoic acid in a suitable solvent to get deferasirox.

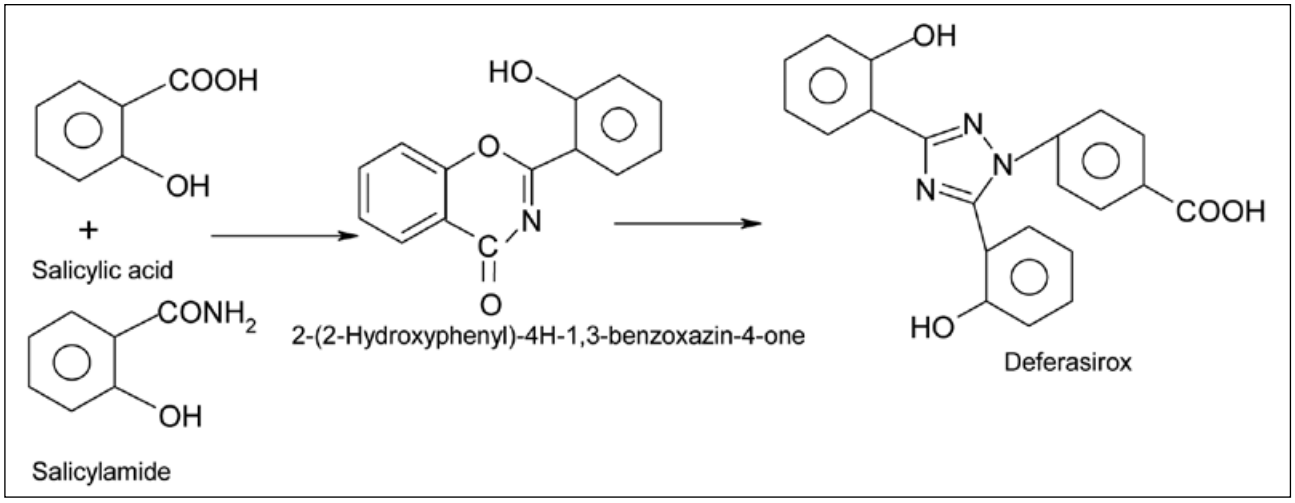

Figure 2. Synthesis scheme of deferasirox

3. Results and Discussion

3.1 Method development

Optimal separation of related substances from each other and from deferasirox was achieved with ammonium dihydrogen phosphate buffer and acetonitrile usin a gradient HPLC method. A mobile phase temperature of 40 ^{0} C was employed for the separation. No significant degradation of deferasirox was observed at 40 ^{0}C during its elution time. A typical chromatogram with retention time and elution order observed for deferasirox and potential manufacturing impurities are depicted in Fig. 3 and Table I, showing retentions and relative retention times of deferasirox and related impurities. Quantitation of all related substances was conducted on an area/area basis for the deferasirox peak area after each injection. The external standard method was utilized for quantitation.

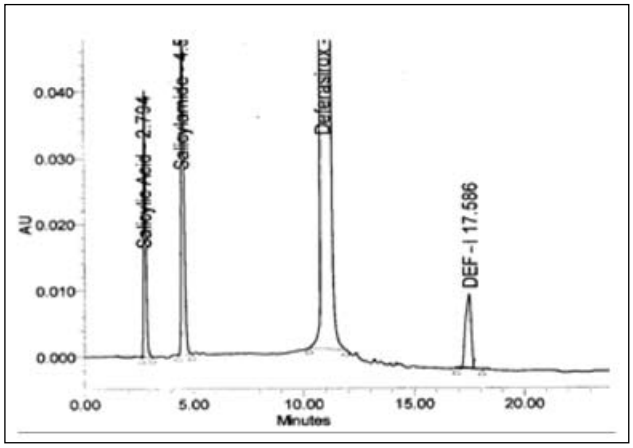

Figure.3 Analytical HPLC chromatogram of laboratory batch showing the elution order of deferasirox with the impurities salicylic acid, salicylamide and DEF- I. The flow rate was 0.8 mL/min and the detection wavelength was 252 nm.

Table I. Typical retention times observed for deferasirox and its related substances using the conditions of the proposed method.

Component	Retention time range (min)	Relative retention time
1. Deferasirox	10.6 -11.2	1.0
2. Salicylic Acid	2.3 - 3.1	0.21 – 0.27
3. Salicylamide	4.1 - 4.9	0.38 – 0.44
4. DEF- I	17.2 – 17.8	1.56 – 1.64

3.3 Column selection

Numerous HPLC columns are commercially available now. Each individual stationary phase provides different levels of selectivity for different impurities due to various modifications, such as end capping, mixed-modes, amide bond linkages and phenyl and cyano bonding. The goal of a RP-HPLC impurity profile method is to separate different impurities from the main API peak and from each other as well. Thus, it is recommended that different stationary phases that possess large differences in polarity, wet stability and retention ability should be screened. This increases selectivity since the properties of the APIs and each impurity will differ greatly. Such properties include ionic status, molecular size and whether or not the chemical is hydrophobic or hydrophilic. Some impurities may possess structural features and properties very similar to the API. Therefore, they easily co-elute with the API peak. In those cases the screening of different column becomes even more important. The accurate number of columns for a screening is case dependent. Eight columns, including Zorbax SB phenyl, Luna C18 (2), Symmetry C-18, RP-8, Thermo Hypersil ODS, Thermo Hypersil Gold CN, YMC Pro-C18 and YMC AQ ODS were screened with a fixed mobile phase. The Luna C18 (2)100A, 150 x 4.6mm x 3

µm column gave an adequate separation between deferasirox and its related manufacturing impurities.

3.4 Effect of ionic strength in mobile phase

When acetonitrile was fixed at 30%, we observed the effect of the buffer concentration in the mobile phase on the separation by adding different quantities of ammonium dihydrogen ortho phosphate (0.01 mol/L) solution. It was found that the buffer concentration in the range of 70% - 90% has little influence on the retention times of all compounds involved, but their retention times increased significantly when the proportion was more than 70%. Accordingly, we choose 70% as the buffer proportion.

3.5 Effect of organic content in the mobile phase

In this experiment, we fixed the proportion of buffer (70% of 0.01 mol/L) and adjusted the contents of acetonitrile ranging from 15-40% in the mobile phase. By comparing with separation phenomenon under various acetonitrile contents, it indicated that all compounds could be separated perfectly when acetonitrile proportion was 30%.

4. Method validation

The method was validated according to ICH guidelines [21,22]

4.1 Leaching study

An investigation was conducted to determine the sonication time required for complete leaching of the deferasirox from the sample matrix. Eight samples of 0.50 g portions of the composite deferasirox tablets were prepared. For each strength studies, two samples were also prepared using a control procedure known to fully leach the deferasirox. The remaining samples were tested for various sonication times of 10, 30, 60 min. Table II represents the assay results for sample solutions for 125 mg deferasirox tablets prepared using different sonication times during sample leaching. Based on the results obtained, a 10 min sonication time at a temperature of 25 ^{0}C was specified in the proposed method.

Table II. Effect of sample sonication time on leaching of deferasirox for composite sample of tablets (125 mg).

Sample Nº	Sonication time (min)	Deferasirox Label Claim (%)
1[a]	10	99.64
2[a]	10	100.54
3	10	101.24
4	10	101.48
5	30	100.16
6	30	101.09
7	60	104.38
8	60	103.48

a) control procedure known to fully leach deferasirox from tablet

4.2. Selectivity

A separate selectivity test was performed by applying the proposed method to the determination of deferasirox in synthetic mixtures consisting of deferasirox, starch, lactose, calcium gluconate and magnesium stearate. Deferasirox was extracted with three 25 ml diluents and filtered. The filter was washed with diluents and diluted up to the final volume with diluents. The response of the analyte in this aliquot was compared with the response of pure deferasirox. It was found that assay results were not changed.

4.3 Specificity

Specificity is the ability of the method to measure the analytical response in the presence of all potential impurities [23]. For the specificity test, a chromatogram of the standard solution of deferasirox was recorded under selected conditions. The response of the analyte in this mixture was compared with the response of pure deferasirox. It was found that assay results were not changed.

4.4. Accuracy and Precision

The accuracy of the method for quantitation of salicylic acid, salicylamide and DEF-I was evaluated by analysis of solutions of actual samples of standard deferasirox and tablets[24]. The sample solutions were prepared with deferasirox at target concentrations of 500 µg/ml and were spiked with salicylic acid, salicylamide and DEF-I at the following percent (w/w) levels. i.e. 10.0, 8.0, 5.0, 2.0, 0.1 and 0.05. For each strength study, four sets of accuracy analysis were conducted by each of two analysts. Accuracy results are presented in table III, IV and V. A review of the data indicated that the mean recovery value at the 0.05% spiking level differs significantly from the mean recovery value at higher levels. It is important to note that the 0.05% level represent the LOQ for related substances in this method.

4.5 System repeatability

System repeatability or instrumental precision was determined by six replicate injections of standard solution during the studies conducted for determination of the accuracy of the method. The first six replicate injections were performed by both analysts on two different days and the relative standard deviations found were 0.29% for deferasirox.

4.6 Linearity

The linearity relationship of detector response was measured as peak area versus concentration. Solutions were prepared so that the concentrations analyzed for all related impurities were evaluated over the range of 0.25-6.00 µg/ml, equivalent to 0.04-1.0% with respect to test sample concentration. Six replicate sets of each concentration level were prepared and checked for linearity. A calibration curve was established [25] between the average response and concentration of the analyte. The product correlation coefficient, R^2, was 0.99996 was achieved. The data is presented in table VI.

Table III. Results for spiked salicylic acid recovered from samples of deferasirox applying the proposed method.

	Sample Nº	Percentage (w/w) added	Recovery (%)				Mean	SD	% RSD
			Analyst–1		Analyst–2				
			Day–1	Day–2	Day–1	Day–2			
	1	10	99.8	99.7	99.6	99.8	99.73	0.10	0.10
	2	8	99.9	99.6	99.8	99.7	99.75	0.13	0.13
	3	5	99.8	99.8	100.6	100.2	100.10	0.38	0.38
	4	2	100.6	100.1	100.1	100.8	100.40	0.36	0.35
	5	0.1	102.5	101.2	103.2	102.9	102.45	0.88	0.86
	6	0.05	75.8	90.6	119.6	124.6	102.65	23.35	22.74
Mean				96.40	98.50	103.82	104.67		
SD				10.14	3.91	7.84	9.84		
% RSD				10.52	3.97	7.55	9.40		

Table IV. Results for spiked salicylamide recovered from samples of deferasirox applying the proposed method.

	Sample Nº	Percentage (w/w) added	Recovery (%)				Mean	SD	% RSD
			Analyst–1		Analyst–2				
			Day–1	Day–2	Day–1	Day–2			
	1	10	99.7	99.6	99.8	99.9	99.75	0.13	0.13
	2	8	99.8	99.8	99.9	99.8	99.83	0.05	0.05
	3	5	99.8	99.8	99.6	99.7	99.73	0.10	0.10
	4	2	100.2	100.2	99.8	99.6	99.95	0.30	0.30
	5	0.1	100.8	99.8	99.7	100.1	100.10	0.50	0.50
	6	0.05	80.1	95.5	112.3	118.1	101.50	17.19	16.93
Mean				96.73	99.12	101.85	102.87		
SD				8.16	1.78	5.12	7.46		
% RSD				8.43	1.80	5.03	7.26		

Table V. Results for spiked DEF-I recovered from samples of deferasirox applying the proposed method.

	Sample Nº	Percentage (w/w) added	Recovery (%)				Mean	SD	% RSD
			Analyst–1		Analyst–2				
			Day–1	Day–2	Day–1	Day–2			
	1	10	99.9	99.7	99.9	100	99.88	0.13	0.13
	2	8	99.7	99.6	99.9	99.8	99.75	0.13	0.13
	3	5	99.8	99.8	99.8	99.7	99.78	0.05	0.05
	4	2	99.9	100.2	99.9	99.8	99.95	0.17	0.17
	5	0.1	100.6	100.2	99.5	100.1	100.10	0.45	0.45
	6	0.05	82.1	96.5	113.4	115.2	101.80	15.60	15.33
Mean				97.00	99.33	102.07	102.43		
SD				7.31	1.41	5.55	6.26		
% RSD				7.53	1.42	5.44	6.11		

Table VI. Average linearty of impurities and the diluted reference solution.

Concentrations µg/ml	Average area of salicylic acid	Average area of salicylamide	Average area of DEFI	Average area of deferasirox reference solution
0.25	20786.75	15205.00	93011.00	11313.50
0.50	39573.56	29412.75	181722.25	22627.58
0.60	48688.25	35895.25	212354.56	27052.40
1.20	99775.45	72785.65	446552.80	53304.85
2.40	199452.80	136072.65	892905.60	107609.60
3.60	289329.20	218952.45	1339358.40	161914.40
6.00	498862.00	364920.25	2332264.45	251524.40
Intercept	-1090.00	-1626.00	-20303.00	2894.00
Slope	82756.00	60799.00	38764.00	42274.00
Correlation value	0.999	0.999	0.999	0.998

4.7 Quantification limit:

The accuracy results are presented in Tables III, IV and V. The means of recovery of salicylic acid, salicylamide and DEF-I were 101, 100 and 100 %, respectively. In a review of the data, it was noted that the 0.05% spiking level represents the quantification limit for the method and that this spiking level is 0.05% of 500 µg/ml deferasirox, so the LOQ was found to be 2.5 µg/ml.

4.8 Ruggedness

This parameter evaluates the consistency of the results when external factors such as analyst, instruments, laboratories, reagents and days are varied deliberately. Ruggedness of the proposed method was estimated by changing days and analyst with each analyst running two sets, each set on a different HPLC system using a different column. Two different HPLC systems (Waters and Merck) were utilized to conduct the analyses. Finally, the results are within acceptable limits, so that the method was considered rugged.

4.9 Robustness

This parameter evaluates the consistency of the results when internal factors such as column, flow rate, mobile phase composition, temperature, injection volume or any other variable inherent to the method of analysis are varied deliberately. Robustness of the proposed method was estimated by changing the mobile phase buffer concentration ±0.01 mol/L, mobile phase organic content ±3.0%, flow rate from 0.8 ml to 1.2 ml / min, mobile phase temperature ±5.0 ^{0}C , detection wavelength setting ±2.0 nm, changing column brand and mobile phase composition. System suitability parameters were found to be within acceptable limits.

4.10. Stability

HPLC studies of samples on stability testing of deferasirox under different condition suggested the following behaviors [26].

4.10.1 Processed sample stability

Two sets of samples with a low and a high concentration of deferasirox were analyzed and left in the auto-sampler at ambient temperature. The samples were analyzed using a freshly prepared calibration samples 5 days later.

4.10.2 .Long term stability

Two sets of samples with a low and a high concentration of deferasirox were stored in the freezer at -18 ^{0}C for 30 days. The samples were analyzed using a freshly prepared calibration samples. The results are within the acceptable ± 15% limit of the nominal concentration but after that the peak response decreased significantly. The samples were found to be stable for 20 days at -18 ^{0}C.

5. Conclusion

The above-mentioned data demonstrates that the proposed method is accurate, precise, linear, specific and robust for the determination of related substances in deferasirox API and pharmaceutical formulations. Also the method was determined to be robust with regards to the following parameters: mobile phase buffer composition, mobile phase organic content; detection wavelength and time dependence of sample and standard stability.

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Solid surface room-temperature phosphorimetry: a metrological study to compare the performance of cellulose and nylon as measurement substrates

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Abstract

Solid surface room-temperature phosphorimetry is an attractive ultra-trace analysis technique that relies on the immobilization of the analyte of interest on the surface of a substrate. The choice of substrate is crucial in terms of the quality of the analytical measurement. For the determination of camptothecin and norfloxacin, significant improvements in detectabilty and precision (evaluated as the combined uncertainty of the measurement) was achieved using nylon in contrast with these same figures obtained using cellulose previously treated to reduce background. For chrysene, there was no significant difference in detection performance as a function of the type of substrate because the characteristic excitation/emission pair is placed in a low noisy region of the cellulose substrate. The use of nylon also enabled practical advantages such as short drying time and no need for pre-treatment to reduce background.

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1. Introduction

Phosphorimetry is a sensitive and selective analytical technique for the ultra-trace determination (trace quantities of analytes in microsize samples) of many classes of substances of biologycal and environmental interest. Room temperature phosphorescence is obtained under specific conditions to immobilize and protect the luminescent species. When analytes are absorbed on solid substrates, the non-radiative processes of deactivation of the excited state such as molecular vibration and collisional quenching are minimized. In addition, the solid substrate is an excelent matrix to place phosphorescence enhancers (salts of elements of high atomic mass) in close proximity to the analytes in order to get efficient external heavy atom effects. The external heavy atom effect induces or significantly increases phosphorescence from organic molecules by promoting the change of multiplicity of their excited state population (from singlet state to triplet state) and, therefore, improving both phosphorescence quantum yield and phosphorescence velocity rate. When a solid substrate is used to measure phosphorescence, the analytical technique is called solid surface room temperature phosphorimetry (SSRTP). SSRTP can be a very attractive analytical method because of its operational simplicity, the high selectivity achieved by the proper use of a selective heavy atom phosphorescence enhancer, low limits of detection and low operational costs [1].

Different materials have been used as solid substrates including cellulose (chromatographic or filter paper), sucrose, sodium acetate, silica gel, cyclodextrin, starch, polyacrylic acid among others. However, the cellulose substrate is the most employed because it is easy to deal with, economic, easily available and effective for the luminescence induction of a wide variety of compounds. However, this substrate presents two critical problems: a high background signal and a high moisture affinity, which are unfavorable characteristics to measure phosphorescence [2,3]. In order to reduce background, paper substrates are often treated. Such treatment consists in washing paper strips with boiling water, drying and then exposing them to UV radiation to degrade natural phosphorescent components of the paper. However, these operations are time-consuming and it is not totally efficient, leaving a measurable background that limits the detection capability of the method.

Nylon has been pointed out as a promising new material to be used as a substrate in SSRTP [4]. The primary chemical structure of nylon consists of amide groups separated by methylene sequences. Nylon-6.6 is a polyamide synthesized from 1,6-hexamethylene diamine and adipic acid, and contains a mixture of polymer chains with ends terminating in amine, carboxylic acid or a combination of the two groups. Reports in the literature show that differences in end group configuration can lead to significant differences in the polymer morphology [5]. The amide group is essentially planar due to the partial double-bond character of the C-N bond and the chains are oriented in such a way as to maximize hy-

drogen bonding between the amino and carbonyl groups. The hydrophilic amide groups enhance the affinity of the substrate for water. Nylon-6.6 is a typical semicrystalline polymer that can be crosslinked, dramatically changing polymer properties [6]. The nylon membrane can have different pore sizes that enable different backgroud levels and may significantly influence its ability to retain the analytes, which affects their luminescence properties. Nylon substrates are ready to use not requiring any further treatment to improve performance and it is readily available on the market at a reasonable cost. Only a few analytical SSRTP based methods are described in the literature using nylon as substrates [4,5,7-9], probably because the advantages of nylon have not been properly evaluated. Such an evaluation can be made by a careful performance study to compare the signal-to-ratio values obtained from different analytes on nylon and on cellulose substrates and to properly access the precision of such measurements through uncertainty calculations [10].

Uncertainty is a metrological term which characterizes the range of measured values within which the true value is expected to lie with a specified level of confidence. For measurements to be of practical value it is necessary to have some knowledge of their reliability which can be accessed by the calculated uncertainty [11]. In analytical chemistry, each measurement has an uncertainty associated to it, resulting from errors arising in the various stages of sampling, sample preparation, analysis and from imperfect knowledge of factors affecting the result.

The goal of this article is to perform a metrological study to compare the performance of phosphorescence measurements using both cellulose and nylon substrates. The evaluation was made by (i) comparing the analyte net signal observed, (ii) assessing the magnitude of the uncertainties associated to these measurements (using an incomplete model) and (iii) evaluating practical aspects that affect the analytical procedure. Three different analytes (from different classes of substances) were selected: The alkaloid camptothecin (Figure 1A), chrysene, a polycyclic aromatic hydrocarbon, (Figure 1B) and the fluoroquinolone norfloxacin (Figure 1C).

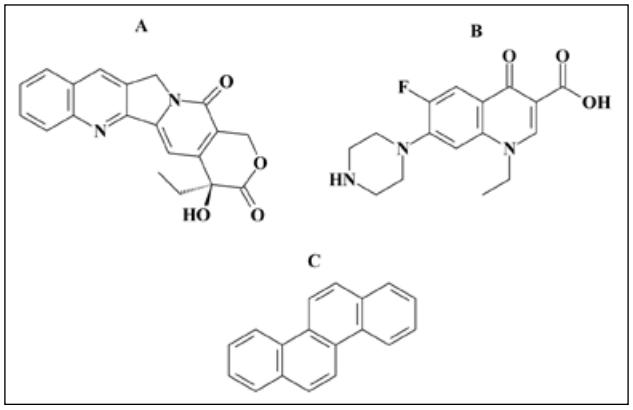

Figure 1. Chemical structures of (A) campthotecin (CPT); (B) norfloxacin (NOR) and (C) chrysene (CHRY).

2. Experimental

2.1. Instruments

Phosphorescence measurements were performed on a luminescence spectrometer from Perkin-Elmer, LS-55 (Perkin-Elmer, CT, USA), coupled to a solid surface analysis apparatus (Perkin-Elmer) modified to allow a flow of purging gas (nitrogen dried by passing it through a silica gel bed) on the sample holder. This instrument was equipped with a pulsed xenon arc lamp as the excitation source and a R928 response type photomultiplier detector. The instrument was operated with 1500 nm min^{-1} scan velocity. A delay time of 3 ms, gate time 3 ms and a spectral bandwidth of 10 nm were used. The maximum excitation/emission pairs chosen for each of the substrates are indicated in Table I. A laboratory-made photochemical reactor was employed to treat the paper substrates in order to reduce their background. The photochemical reactor was constructed using a circular oven unit cabinet that was adapted by covering its internal parts with aluminum foil and placing six mercury sterilization lamps (6 W each) around the internal upper semicircle part of the cabinet. During operation, the internal temperature of the reactor was constant at 30 ± 2 ^{0}C.

Statistical calculations were made using the Statistica 7.0 software package.

2.2. Reagents

All experiments were made using analytical grade chemicals and ultrapure water from an ultrapurifier (Milli-Q system, gradient A10, Millipore, USA). Campthotecin (CPT) and norfloxacin (NOR) were obtained from Sigma-Aldrich (Saint Louis, USA) and chrysene (CHRY) was from Acros Organics (Fairlawn, NJ, USA). Thallium nitrate (Acros Organics), thorium nitrate (RP ACS, Milan, Italy) and silver nitrate (Vetec, Rio de Janeiro, Brazil) were used as phosphorescence inducers/enhancers. Methanol, ethanol, acetone, acetic acid, boric acid, phosphoric acid and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Filter paper (Whatman N°42) was used as cellulose substrate. The nylon substrate (Nylon-6.6, 0.2 µm pore size) used was obtained from Sigma-Aldrich. Nylon substrates of different pore sizes (0.45 and 0.6 µm, Sigma-Aldrich) were also tested. Nitrogen (99.996 %) was from Linde, Rio de Janeiro, Brazil and it was further purified by passing it through an ammonium metavanadate solution and dried over a silica gel bed.

2.3. Solutions and materials preparations

CPT stock solutions (4 x 10^{-4} mol L^{-1}) were prepared in methanol. CPT standard solutions with lower concentrations were made by further dilution of the stock solutions with water/Britton-Robinson buffer (pH 10.5) 50/50% v/v. NOR stock solutions (1 x 10^{-3} mol L^{-1}) were prepared in acetone/water 50/50% v/v. More diluted solutions of NOR were prepared in acetone/water/Britton-Robinson buffer (pH 12) 20/50/30% v/v/v. Chrysene stock solutions (5 x 10^{-4} mol L^{-1}) were dissolved in ethanol and standard solutions with lower concen-

trations were made by further dilution of the stock solutions with ethanol/water 50/50% v/v. The Britton-Robinson buffer solution (0.04 mol L^{-1}) was prepared by mixing acetic acid, boric acid and phosphoric acid aqueous solutions. The pH of buffer solution was adjusted by addition concentrated sodium hydroxide solution. Ultrapure water was used to prepare all heavy atom salt solutions whose concentration were: $TlNO_3$ (0.25 mol L^{-1}), $AgNO_3$ (0.03 mol L^{-1}) and $Th(NO_3)_4.4H_2O$ (0.25 mol L^{-1}).

Cellulose substrates (filter paper) background reduction consisted of washing the paper strips with boiling water in a Soxhlet apparatus for 2 h. After drying under an infrared lamp, the paper was exposed to UV light for another 2 h. These solid substrates were cut in circles (18 mm in diameter) to be used during the analysis. The nylon substrates (cut in 18 mm diameter circles) were used as purchased, not requiring any further treatment to reduce background.

2.4. General procedure

Five µL of analyte standard solutions were spotted on the center of the solid substrates using a calibrated adjustable (1–10 µL) automatic pipette. A volume of 5 µL of the heavy atom solution was applied to the center of the substrates prior to the application of the samples or blanks. Solid substrates spotted with samples or blanks were dried at room temperature in a vacuum desiccator (120 min for cellulose and 40 min for nylon). The desiccator was covered with aluminum foil to shield substrates from ambient light. Since the heavy atom salts and the analytes are in their solid form on the surface of the substrates after drying, they could be stored, under vacuum and shielded from light, for long times without significant changes in the measured phosphorescence. In order to perform analytical measurements, the circles were placed on a clean sample holder and inserted into the surface instrument accessory. The sample compartment was continuously purged with dry nitrogen gas for 2 min prior to each measurement in order to minimize quenching effects from oxygen and air moisture. Excitation radiation was focused in the center of the substrate, where sample was spotted, and luminescence was collected at 90^0 to minimize stray and scattered light.

3. Results and discussion

3.1. Preliminary studies in the nylon membrane

Experimental conditions to measure phosphorescence from CPT, NOR and CHRY are indicated in Table I. These conditions were selected based on previous papers using cellulose as substrate for SSRTP [7,12,13]. It is important to point out that the heavy atom salts chosen for each of the analytes were the ones that induced or better enhanced their phosphorescence. Such mechanisms are fairly selective for classes of analytes. For instance, Th(IV), which is an excellent phosphorescence inducer for NOR, does not work for either CHRY nor CPT. Further information on the effect of these heavy atom salts on the phosphorescence characteristics of the analytes can be found elsewhere [7,12,13]. The analyte carrier solutions may also play an important role in the measured phosphorescence after the analyte is dried on the surface of the substrate. This is the case for analytes that may act as donors or acceptors of protons due to the hydrogenionic concentration of the carrier solution (which is the case of fluoroquinolones and alkaloids). Analyte charge or conformational modifications induced in solution affect how these analytes will interact with the sites of the substrate, which reflect, for instance, on how efficient is the immobilization process or how deep they can penetrate inside the layers of the substrate.

Table I. Experimental conditions to induce room-temperature phosphorescence from camptothecin (CPT), norfloxacin (NOR) and chrysene (CHRY) using Cellulose[a] and nylon[b] substrates.

Substance	CPT	NOR	CHRY
Heavy atom salt (amount)[c]	$TlNO_3$ (332 µg)	$Th(NO_3)_4$ (784 µg)	$AgNO_3$ (25 µg)
Carrier solution[d] (proportion)	water/BR buffer[e] pH 10.5 (50/50 v/v)	acetone/water/BR buffer[e] pH 12 (20/50/30 v/v/v)	ethanol/water (50/50 v/v)
Excitation/emission wavelength pair	370/570 nm	283/460 nm	274/510 nm
Reference	[7]	[12]	[13]

a) Whatman filter paper treated to reduce background.
b) Nylon-6.6.
c) Amount of salt deposited on the center of the substrate using 5 µL of heavy atom salt solution: ($TlNO_3$, 0.25 mol L^{-1}), ($Th(NO_3)_4$, 0.25 mol L^{-1}) and ($AgNO_3$, 0.03 mol L^{-1}).
d) Solution used to dissolve the analyte and place it in the center of the substrate using 5 µL carrier solution.
e) Britton-Robinson buffer (0.04 mol L^{-1}).

In a preliminary study concerning the choice of the nylon substrate, phosphorescence from the three analytes was measured (under the specific experimental conditions indicated in Table I) from nylon membranes with different pore sizes (0.2 µm, 0.45 µm and 0.6 µm). No phosphorescence was measured from the analytes placed on the 0.6 µm pore size nylon membrane. Larger pore sizes might not retain the analytes on the surface of the membrane and the analyte molecules that penetrated into the internal layers of the membrane do not interact with the excitation radiation [3]. Although phosphorescence from all the analytes could be measured from both 0.2 and 0.45 µm pore size nylon membranes, significant differences were observed both in substrate background emission and in phosphorescence intensities of the analytes. The 0.2 µm pore size membrane presented lower background and good properties in retaining the analytes, which, in turn, represented significantly superior signal-to-noise ratio (from two to four times higher, depending on the analyte) than the ones observed from the 0.45 µm pore size nylon. Therefore, the 0.2 µm pore size nylon membrane was selected for this study.

3.2. Comparison between cellulose and nylon: detectability performance

The detection capability of an analytical method is defined by its ability to detect the substance of interest and it is evaluated, in this work, using the limit of detection (LOD), which is defined by minimum quantity of analyte that can be detected using a specific method, which should correspond to the quantity (mass, concentration, etc) that yields an analytical signal that can be distinguished, at a established confidence level, from the signals obtained from the blank measurements [14]. The criteria adopted to calculate LOD (in mol L^{-1}) was the concentration capable of generating a signal equal to $x_b + 3s_b$, where x_b is the average blank signal and s_b is the standard deviation of consecutive blank measurements, which are phosphorescence measurements from substrates where 5 µL of the heavy atom salt solution and 5 µL of the carrier solvent system used to dissolve the analyte were deposited. Detectability was also reported in terms of mass values in the substrate (absolute limits of detection, ALOD) that is calculated using the equations: ALOD = LOD × V × M, where LOD is the limit of detection (calculated in mol L^{-1}), V is the volume of the analyte solution deposited on the substrate (5 x 10^{-6} L) and M is the molar mass of the analyte (g mol^{-1}). The LOD and the ALOD depend upon three parameters: (i) the magnitude of the analyte phosphorescence, which depends on the characteristics of the analyte and upon the analyte-substrate interaction (how efficient is the immobilization of the analyte in the substrate), (ii) the magnitude of the blank signal (x_b) and (iii) the standard deviation of the blank signal (s_b), in other words, the quality of successive measurements.

Another useful figure of merit for comparison is the sensitivity of the analytical curves, defined by the inclination (angular coefficient) of the linear range of the analytical curve. Analytical curves for the three substances were constructed under specific experimental conditions for each of the analytes in order to compare sensitivities, therefore, to evaluate how efficiently each type of substrate promotes a better environment to protect the analyte from radiationless processes of the triplet excited state.

Since substrate blank signal (x_b) and the quality of the repetition of successive measurements (repeatability indicated by s_b) are critical to achieve better detection limits, it is necessary to evaluate the total phosphorescence of the substrate material. The total excitation and emission phosphorescence spectra from both nylon and low background filter paper are displayed in Figure 2, where it can be observed that, despite the similarities in wavelength profiles, critical differences in background intensities are found, showing, in principle, a clear advantage of the nylon substrate over the cellulose one, which, in turn will reduce x_b. However, substrate background varies depending on the wavelength pair used (characteristic of the analyte being measured) and to the heavy atom enhancer employed, since the phosphorescence of the substrate is also affected by them. This can be observed in the typical phosphorescence spectra from the analytes and from the substrates taken under specific experimental conditions (Figure 3). From these spectra, a lower background for nylon substrates (down to 8 times less intense) can be clearly seen, despite the excitation/emission wavelength pair and the heavy atom enhancer used. In addition, analyte signal improvement is observed for NOR (2.7 times) and CPT (1.8 times), which contributed to the improved LOD values calculated for these analytes (Table II). The ALOD values obtained using nylon were up to 8 times better than the ones achieved in cellulose substrates. For NOR and CPT, the improvements in the detection capability are caused by both the improvement in analyte signal and the significant decrease in background. For CHRY, the analyte signals generated in both substrates are equivalent. The sensitivity values calculated from the calibration curves, constructed under the optimized experimental conditions, confirmed the signal improvements for NOR and CPT when using nylon substrates.

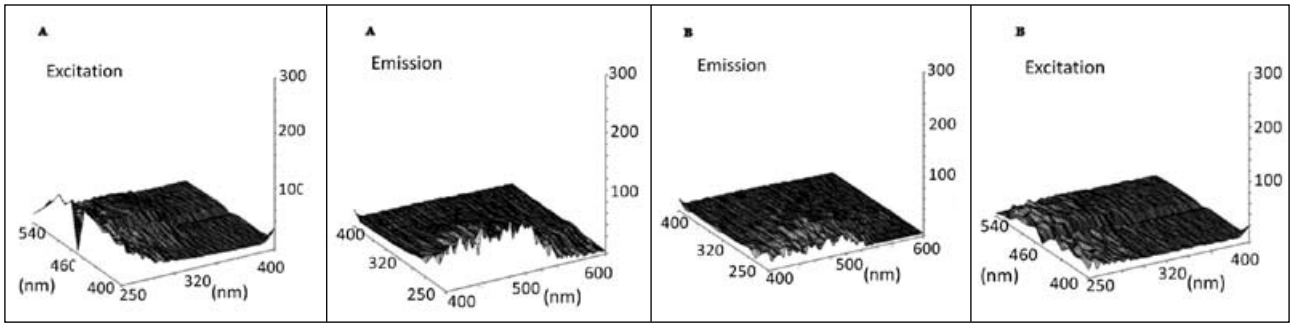

Figure 2. Total excitation and emission phosphorescence spectra from both (A) low background cellulose paper (B) nylon.

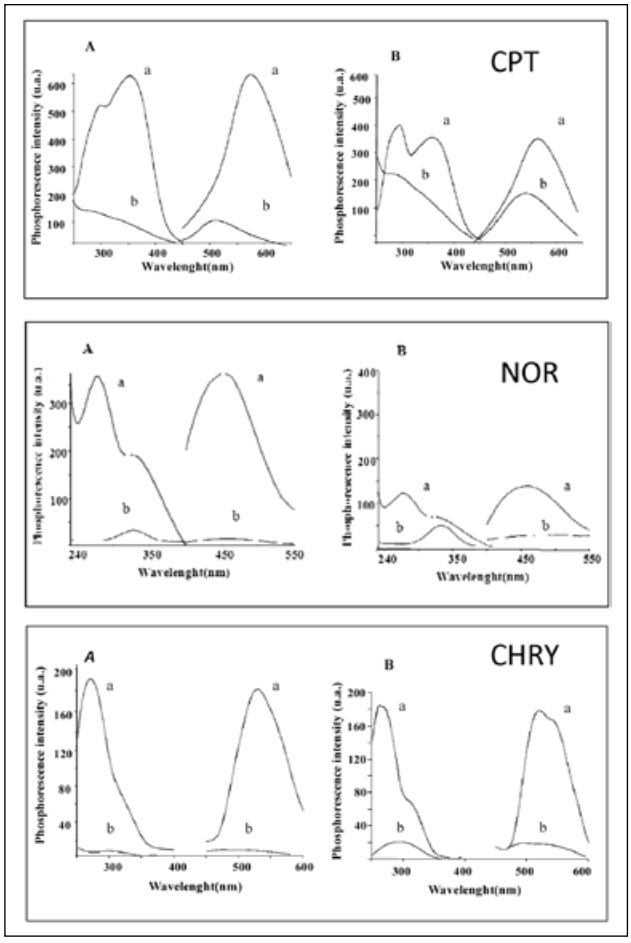

Figure 3. Phosphorescence excitation and emission spectra from (A) nylon and (B) low background cellulose in the presence (a) and in the absence (b) of analyte: CPT (5 µL of 4.8 x 10^{-5} mol L^{-1} and 332 µg of $TlNO_3$); NOR (5 µL of 6 x 10^{-5} mol L^{-1} and 748 µg of $Th(NO_3)_4$); CHRY ((5 mL of 2 x 10^{-5} mol L^{-1} and 25 mg of $AgNO_3$.

Table II. Analytical figures of merit achieved for camptothecin (CPT), norfloxacin (NOR) and chrysene (CHRY) using cellulose and nylon substrates obtained under the experimental conditions described in Table I.

Substance	CPT		NOR		CHRY	
Substrate	Cellulose	Nylon	Cellulose	Nylon	Cellulose	Nylon
LOD (mol L^{-1})	2.8 x 10^{-5}	1.2 x 10^{-5}	1.9 x 10^{-5}	3 x 10^{-6}	5.5 x 10^{-6}	5 x 10^{-6}
ALOD[a] (ng)	48	21	39	5	6.3	5.8
Sensitivity[b] (L mol^{-1})	3.9 x 10^{6}	1.2 x 10^{7}	1.2 x 10^{6}	6.6 x 10^{6}	5.1 x 10^{6}	4.7 x 10^{6}

a) Effective mass of analyte detected in the substrate.
b) Higher concentrations of the linear ranges of the curves and their determination coefficients in cellulose and in nylon: CPT (8 x 10^{-5} mol L^{-1}; 0.985 and 0.9984), NOR (2 x 10^{-4} mol L^{-1}; 0. 9990 and 0. 9801), CHRY (6 x 10^{-5} mol L^{-1}; 0.9860 and 0.9831).

3.3. Comparison between cellulose and nylon: uncertainty

A reliable evaluation of the quality of the phosphorescence measurement can be made by taking into consideration four groups of factors that contribute to the overall uncertainty of the SSRTP measurement: repeatability (u_r); intermediate precision (u_{ip}); preparation of solutions (u_s) and calibration curve (u_{curve}). Since SSRTP is not an absolute analytical method, calibration curves are needed to perform quantification. Therefore, the method relies on comparison of signals from samples with the ones measured from standards using a mathematical function (a linear equation of the type $y = mx + b$, where y is the sample phosphorescence measured from the sample, x is the concentration of analyte responsible for the phosphorescence, m is the angular coefficient of the calibration curve and b is the linear coefficient of the calibration curve). It is important to mention that the experimental variables such as heavy atom quantity deposited on the substrate and pH of the carrier solution were chosen to be in the robust range of the analytical response so that any variation due to them is included in u_r. A complete uncertainty calculation was made for two different analyte concentrations (or masses on the substrate), except for NOR for which only one concentration was used. This was done in order to evaluate the performance in two different regions of the calibration curves in their linear ranges as indicated in Table III (CPT), Table IV (NOR) and Table V (CHRY). These masses were delivered on the substrate using 5 µL of analyte solutions of specific concentrations. Each of these working solutions was prepared by diluting a specific volume of their analyte stock solutions, which were prepared by weighing the analyte mass into the 10 mL volumetric flask. The working analyte solutions had their volumes adjusted with the specified carrier solutions. The combined uncertainty (u_c) was calculated by the square root of the quadratic summation of the four uncertainty values (Equation 1) considering that the contributing groups are independent from each other in the overall variability of the measurement.

$$u_c = [(u_r^2 + u_p^2 + u_s^2 + u_{curve}^2)]^{1/2} \quad (1)$$

The u_r values were obtained from the evaluation of repeatability (measured as s_b). For CPT, the u_r values obtained from the cellulose substrate were 13% (at the 2.4 x 10^{-5} mol L^{-1} level) and 6.5% (at the 4.8 x 10^{-5} mol L^{-1} level) of the average signal generated by the working solutions added to the substrates. A decrease in the u_r of about three times was achieved by using nylon as substrate, reflecting the improvement of the quality of measurement. For NOR, when nylon was employed, a significant improvement was observed with the u_r value decreasing from 14% (value achieved using cellulose) to 6% of the average analyte signal. For CHRY, such an improvement was not remarkable regardless of the concentration level of the working solution used to spot

the analyte on the substrate (from 13 and 8% in cellulose to 10 and 7% in nylon) probably because the detection occurred at 510 nm, a region of similar background variation in both substrates. As repeatability studies were made with each replicate with different paper or nylon circles, results reflected the lesser variation of signal between substrates. Background from cellulose substrates tend to vary, even if the measured substrates are taken from the same lot and sheet of filter paper (inhomogeneous presence of quantities of phosphorescent components). Nylon is more homogeneous in composition, resulting in better repeatability.

The u_{ip} values were obtained from the analysis of variance that compared measurements made by two different analysts, each one preparing ten different substrates with the same analyte solution. The impact on the u_{ip} was also detectable for CPT at the two concentration levels tested (from 3 and 1.5% in cellulose to 0.5 and 0.8% in nylon), and for NOR (from 4% in cellulose to 2% in nylon), since less noisy substrates improved the quality of the measurement made by both the analysts and, therefore, bring the independent results into closer agreement. Such an impact was not observed for CHRY, which provided similar intermediate precisions.

The u_s value was calculated from the expanded uncertainties from the balance, U_{bal}, and from the volumetric apparatuses: in this case, the expanded uncertainty from the microliter pipettes, U_{mp}, and the volumetric flask, U_{vf}, used in the preparation of the analyte solution. The uncertainty was obtained from $u_{bal} = U_{bal}/k$, from $u_{mp} = U_{mp}/k$ and from $u_{vf} = U_{vf}/k$, where U values were obtained from calibration certificates and k = 2 (the chosen coverage factor, 95.4%). The u_s value was calculated by the square root of the quadratic summation of the uncertainty values of the balance (u_{bal}) and of the volumetric apparatuses (u_{vf} and u_{mp}) multiplied by the uncertainty of a dilution factor. Since this component of the overall uncertainty is attributed to procedures that do not depend upon the substrates (preparation of solutions), the values employed in the expression of combined uncertainties are comparable (small differences are due to the variation in the analyte concentration of solutions used to spot each type of substrate).

The u_{curve} was calculated using parameters of the analytical curves that were constructed using five different analyte concentrations. The standard deviations for both the sensitivity (*m*) and the linear coefficient (*b*) of the analyte addition curve were calculated in order to get their respective uncertainties u_m and u_b. A detailed description on how calculate u_{curve} can be found in Cunha *et al*. [13]. Because of the better quality of measurements in nylon, the components u_m and u_b were smaller for this substrate, generating smaller u_{curve} values for CPT. Similar contributions were found for the uncertainty component for CHRY, as expected. An apparent contradiction has been found for NOR, for which a better result was achieved in cellulose substrates. This can be explained by the degradation of the linear coefficient value of the calibration curve prepared using nylon (where more intense signals were measured) which caused the signal measured from the higher concentrations to be at the beginning of the saturation region of the analytical response where signal response starts to deviate from linear behavior.

Finally, through the combination of these uncertainties, the u_c values were generated, which indicated nylon as the substrate that enabled better results for CPT and NOR with the measurement uncertainty decreased by 2 times. For CHRY, the uncertainties were almost equivalent (results were slightly better using nylon). Table III, Table IV and Table V also shows the expanded uncertainty (*U*), providing an interval within the measured value is believed to lie with a higher level of confidence. *U* is obtained by multiplying u_c by the coverage factor k whose value is chosen based on the desired confidence level (k = 2; 95.4%).

Table III: Uncertainty results (reported in mol L^{-1} as the concentration of analyte that generates a signal equivalent to the calculated uncertainty value) for the room-temperature phosphorescence measurement of CPT in substrates at two different concentration levels: cellulose: (A) 2.4 x 10^{-5} and (B) 4.8 x 10^{-5} mol L^{-1}; nylon: (A) 3.2 x 10^{-5} and (B) 5.5 x 10^{-5} mol L^{-1}. Values for nylon are in parenthesis.

Uncertainty sources	Uncertainty values in equivalent analyte concentration - mol L^{-1}	
	Concentration level	
	A	B
u_r	3.2 x 10^{-6} (1.7 x 10^{-6})	3.1 x 10^{-6} (1.3 x 10^{-6})
u_{ip}	7 x 10^{-7} (7.7 x 10^{-7})	7.1 x 10^{-7} (4.8 x 10^{-7})
Volumetric flask (u_{vf})	1.4 x 10^{-6}	
Microliter pipette 5µL (u_{mp})	2.2 x 10^{-8}	
Microliter pipette 100 – 1000 µL (u_{mp})	3.5 x 10^{-7}	
Balance (u_{bal})	5 x 10^{-6}	
Dilution factor (uf)	1.4 x 10^{-1} (1 x 10^{-1})	7 x 10^{-2} (6 x 10^{-2})
u_s	5.3 x 10^{-7} (1.5 x 10^{-7})	3.8 x 10^{-7} (8.4 x 10^{-8})
Linear coefficient (b)	5.1 x 10^{2} (2.1 x 10^{2})	
Sensitivity(m)	1.5 x 10^{11} (1.4 x 10^{11})	
u_{curve}	1.5 x 10^{-6} (1.1 x 10^{-6})	
u_c	3.7 x 10^{-6} or 6.4 ng (2.2 x 10^{-6} or 3.9 ng)	3.5 x 10^{-6} or 6.1ng (1.8 x 10^{-6} or 3.1 ng)
$U_{(95\%, k=2)}$	7.3 x 10^{-6} or 12.7 ng (4.4 x 10^{-6} or 7.7 ng)	7 x 10^{-6} or 12.2 ng (3.6 x 10^{-6} or 6.2 ng)

Table IV: Uncertainty results (reported in mol L^{-1} as the concentration of analyte that generates a signal equivalent to the calculated uncertainty value) for the room-temperature phosphorescence measurements of NOR in substrates at the concentration levels: cellulose: 2 x 10^{-5}; nylon: 3.5 x 10^{-5}. Values for nylon are in parenthesis.

Uncertainty sources	Uncertainty values (in equivalent analyte concentration - mol L^{-1})
u_r	2.7 x 10^{-6} (2.1 x10^{-6})
u_{ip}	7.8 x 10^{-6} (2 x10^{-7})
Volumetric flask (u_{vf})	1.4 x10^{-6}
Microliter pipette 5µL (u_{mp})	2.2 x10^{-8}
Microliter pipette 100 – 1000 µL (u_{mp})	3.5 x 10^{-7}
Balance (u_{bal})	5 x 10^{-6}
Dilution factor (uf)	4.2 x 10^{-1} (2.8 x 10^{-1})
u_s	9.1 x 10^{-7} (7.5 x 10^{-7})
Linear coefficient (b)	1.1 x 10^{1} (2.9 x 10^{1})
Sensitivity(m)	1.1 x 10^{9} (2.6 x 10^{10})
u_{curve}	3.8 x 10^{-6} (5.1 x 10^{-7})
u_c	4.9 x 10^{-6} or 7.7 ng (2.3 x 10^{-6} or 3.6 ng)
$U_{(95\%, k=2)}$	9.7 x 10^{-6} or 15.5 ng (4.5 x 10^{-6} or 7.2 ng)

Table V: Uncertainty results (reported in mol L^{-1} as the concentration of analyte that generates a signal equivalent to the calculated uncertainty value) for the room-temperature phosphorescence measurement of CHRY in substrates at two different concentration levels: cellulose: (A) 6 x 10^{-6} and (B) 6 x 10^{-5} mol L^{-1}; nylon: (A) 6 x 10^{-6} and (B) 5 x 10^{-5} mol L^{-1}. Values for nylon are in parenthesis.

Uncertainty sources	Uncertainty values (in equivalent analyte concentration - mol L^{-1})	
	Concentration level	
	A	B
u_r	7.7 x 10^{-7} (5.8 x10^{-7})	5 x 10^{-6} (3.5 x10^{-6})
u_{ip}	2.9 x 10^{-7} (1.9 x10^{-7})	1.9 x 10^{-6} (1.3 x10^{-6})
Volumetric flask (u_{vf})	1.4 x10^{-6}	
Microliter pipette 5µL (u_{mp})	2.2 x10^{-8}	
Microliter pipette 100 – 1000 µL (u_{mp})	3.5 x 10^{-7}	
Balance (u_{bal})	5 x 10^{-6}	
Dilution factor (uf)	8.3 x 10^{-1} (6.9 x 10^{-1})	7 x 10^{-2} (8 x 10^{-2})
u_s	1.3 x 10^{-6} (1.2 x 10^{-6})	3.7 x 10^{-7} (4.1 x 10^{-7})
Linear coefficient (b)	1.6 x 10^{2} (1.8 x 10^{1})	
Sensitivity(m)	1.7 x 10^{11} (1.9 x 10^{10})	
u_{curve}	1.2 x 10^{-6} (1.1 x 10^{-6})	
u_c	2 x 10^{-6} or 2.2 ng (1.7 x 10^{-6} or 2 ng)	5.4 x 10^{-6} or 6.2 ng (3.9 x 10^{-6} or 4.5 ng)
$U_{(95\%, k=2)}$	3.9 x 10^{-6} or 4.5 ng (3.5 x 10^{-6} or 4 ng)	1.1 x 10^{-5} or 12.4 ng (7.8 x 10^{-6} or 9 ng)

3.4. Practical aspects

In practical terms, some advantages of nylon over cellulose can be pointed out. First, the nylon membrane does not need pre-treatments to reduce background as required for cellulose substrates. Second, the required drying time before phosphorescence measurement can be shortened when nylon is employed. A specific study was made to observe the time required to dry the substrates after spotting them with the solutions. Dry substrates are required since traces of solvent decrease the phosphorescence measured in substrates due to the disruption of the interaction forces established between analyte and substrates and because solvents might carry dissolved oxygen that is a natural quencher of the triplet excited state. Proper drying in a vacuum desiccator took 40 min for nylon substrates while 120 min is usually required for cellulose substrates [15].

4. Conclusions

A metrological study has demonstrated the advantage of using nylon over cellulose as substrate for room-temperature phosphorescence. For the three analytes studied, a great improvement of performance (better detectability and lower uncertainty) was achieved for CPT and NOR. For these two analytes, nylon allowed more intense phosphorescence to be measured in a lower background environment. The quality of measurement, evaluated as a combined uncertainty, was also significantly improved in nylon. A significant decrease in repeatability (each replicate in a different substrate circle) was achieved because of variations in the phosphorescence background of nylon substrates are negligible even if they come from another lot of nylon sheets. The other relevant source of repeatability (due to small variations in the position where the analyte solution was spotted on the substrate which causes variations in the amount of analyte effectively interacting with the incident excitation light) is not affected by the use of nylon instead of cellulose. With nylon, the quality of the intermediate precision, evaluated by comparing the performance of two analysts, is also improved bringing the independent results into closer agreement. For CHRY the advantages, in terms of detectability and uncertainty of

measurement, of using nylon instead of cellulose were not so obvious. Nylon also grants operational advantages such as the shorter drying time required before phosphorescence measurements and no need for treatment to reduce background as is necessary for cellulose.

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An analytical method for quantifying dimethicone in a 30% simethicone emulsion using gas chromatography.

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Abstract

Gas chromatography-flame ionization detection (GC-FID) was evaluated as a potential technique for the quantification of dimethicone in a 30% simethicone emulsion. Fourier transform infrared spectroscopy (FT-IR) was employed for validation of the proposed chromatographic method. The following validation parameters were studied: specificity/selectivity, linearity, accuracy, precision (repeatability and intermediate precision) and robustness. The acceptance criteria were established in agreement with the legislation from the Brazilian National Health Surveillance Agency (ANVISA). Data were analyzed by a series of statistical tests, namely, analysis of variance (ANOVA), Student's *t*- and *F*-tests, Bartlett and Cochran methods, and Grubb's test. The use of GC-FID was shown to provide a viable, reliable and suitable method for the quantification of dimethicone in a 30% simethicone emulsion. The comparison between the validated methods with those of the American pharmacopeia for the equality of variances test showed that the precision between the methods is different. In the comparison between the methods, Student´s *t*-test indicates that the values, when repeated, where found to be equal. The gas chromatographic method developed afforded an alternative approach, with better precision, when compared to the established spectroscopic method.

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Keywords: dimethicone; simethicone; validation; GC-FID; FT-IR.

1. Introduction

Dimethicones are poly(dimethylsiloxanes) of different grades. Simethicone is a mixture of liquid dimethicones containing finally divided silica to enhance the defoaming properties of silicon. Dimethicone and simethicone are used to relieve flatulence and abdominal discomfort due to excess gastrointestinal gas. These compounds can lower surface tension and, when administered orally, can cause bubbles of gas in the gastrointestinal tract to coalesce, thus aiding their dispersion. [1]

As raw material, simethicone can be used either directly in solid formulations, or as an emulsion (30% simethicone emulsion, USP) for inclusion in liquid suspension formulations. The lack of chemical properties, easily analyzed by routine techniques, is reflected in the small number of methods devoted to the analysis of dimethicones. The British Pharmacopeia does not contain a monograph on simethicone and its dimethicone monograph does not contain a quantification assay. The United States Pharmacopeia (USP) does not contain a dimethicone monograph, but its simethicone monograph presents an assay that can be used to quantify dimethicone, which is based on the measurement of the infrared absorption band at 1265 cm^{-1}.[2] This method was shown to suffer from interference from matrix components (such as antacid components). [3] Nevertheless, FT-IR has been shown to be adequate for quantification of dimethicone in commercial tablets and capsules, complying with the USP requirements. The use of gel permeation chromatography (GPC) coupled to refractive index detection [4] and high performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD) [5] for the analysis of simethicone have already been reported in the literature.

The instruments required for techniques such as FT-IR, GPC or HPLC-ELSD are not common and are not usually found in the average pharmaceutical laboratory in Brazil. Nor are these techniques part of the routine assays employed in quality control. Therefore, in the present study, we investigated the viability of developing an analytical method employing gas chromatography-flame ionization detection to quantify dimethicone in a 30% simethicone emulsion. After development, analytical assay methods have to be validated to ensure their quality and suitability. [1,6-10] For pharmaceuticals, detailed guidelines exist, which describe the requirements to validate analytical methods. The linearity, precision, accuracy, specificity, detection and quantification limits and/or the range of the

method are then evaluated, depending on the type of analytical procedure. [11-16]. It is also advisable to consider performing a robustness test. In the present investigation, a series of validation parameters, as well as their respective acceptance criteria were established in agreement with the legislation and regulations from the Brazilian National Health Surveillance Agency (ANVISA). Thus, this paper compares the results of quantification of dimethicone in a 30% simethicone emulsion by GC-FID and FT-IR. Both methods were validated according to pre-established criteria which satisfy the Brazilian legislation.

2. Methodology

2.1 Materials

Simethicone (100%, USP) and simethicone (30% emulsion, USP) were supplied by Dow Corning (Brazil). Toluene (HPLC grade, ≥99.9%, Carlo Erba, Italy or ≥99.9%, Merck, Germany) was used without additional treatment. Hydrochloric acid and sodium sulfate (anhydrous, 101.2%) were purchased from Nuclear (Brazil).

2.2 Instrumentation

An Agilent gas chromatography system (model 6889N, USA) equipped with a flame ionization detector and coupled to an automatic sampler (model 7683, Agilent, USA) was used for the chromatographic analyses. Table I describes the experimental conditions. Infrared analyses were carried on a Nicolet FT-IR spectrometer (model Avatar 370 DGTS, Thermo, USA). Table II describes the experimental parameters.

Table I. Chromatographic parameters.

Parameter	Experimental conditions
Oven program	0.0 °C/min 80°C 0.5 min 15.0°C/min 180°C 0.0 min 35.0°C/min 300°C 0.4 min
Injection temperature	260°C
Split ratio	15
Detector (FID) temperature	290°C
Gases for detector	H_2:N_2:synthetic air (30:35:350)
Carrier Gas (N_2)	1.8 mL min^{-1}
Injection volume	2 µL
Retention Time (min)	Peaks 2.95, 4.15 and 5.8 (dimethicone group)
Column	HP-5 J&W Scientific (30 m × 0.32 mm × 0.25 µm)

Table II. Spectroscopic parameters.

Parameter	Experimental conditions
Wavenumber	1260 cm^{-1}
Absorption range	1321 – 1209 cm^{-1}
Sample window	KBr
Spacer	0.2 mm
Scans (number)	32

2.3 Sample assay

Ca. 800 mg of the 30% simethicone emulsion was weighed in a 100 mL Erlenmeyer flask, followed by the addition of 20.0 mL of toluene. The flask was magnetically stirred for 10 min, after which 50 mL of hydrochloric acid/water solution (2:5) was added and the resulting solution was stirred for an additional 30 min. This solution was transferred into a decanting flask and allowed to rest for 30 min. After discarding the aqueous phase, a 10 mL aliquot from the organic phase was introduced to a centrifuge tube containing anhydrous sodium sulfate (1.0 g). After centrifugation, the supernatant was separated for chromatographic and spectrophotometric measurements. The final concentration was 12.0 mg mL^{-1}.

2.4 Validation Methodology

The validation methodology was based on resolution 899 from ANVISA, which provides a guide for validation of analytical and bioanalytical methods. [7] The following parameters and strategies were employed:

- *Specificity / selectiviy*. The chromatograms and spectra of the employed diluent solvent were compared to those of the analyte reference standard.

- *Linearity and linear range*. Linearity was evaluated by preparing seven standards of dimethicone at 50%, 80%, 90%, 100%, 110%, 120% and 150% of the sample solution concentration. For the quantification of dimethicone in a 30% simethicone emulsion the chosen concentration range was between 6 and 18 mg L^{-1} of analyte. The intermediate value of each range was considered as the target concentration corresponding to 100%. Standard samples were analyzed in triplicate and the linearity was evaluated by linear regression.

- *Accuracy*. Nine samples in each method were analyzed at the minimum, middle and maximum of the linear range: three at a low concentration level (50%), three at a middle concentration level (100%) and three at a high concentration (150%) level. Each sample was analyzed in triplicate.

  Accuracy was evaluated by the percent recovery of analyte (% Rec) (Equation 1), by the relative error (RE) (Equation 2) and by the relative standard deviation (RSD) among the triplicates.

$$\%\mathrm{Rec} = \frac{\mathrm{Cexp}}{\mathrm{Ctrue}}\, x100 \quad \text{(Equation 1)}$$

where:
$\%\mathrm{Rec}$ = percent recovery of analyte;
Cexp = experimentally found concentration;
Ctrue = true analyte concentration.

$$RE = \frac{Cexp - CTrue}{Ctrue} x100 \quad \text{(Equation 2)}$$

where:
RE = Relative error in percent (%);
Cexp = experimentally found concentration;
Ctrue = true analyte concentration.

- *Precision*. To evaluate method precision, estimations of the repeatability and the time-different intermediate precision were derived from the experimental set-up used. For the repeatability, six samples and the reference standard in 100% of the target concentration were analyzed in triplicate within the same day, by the same analyst, and using the same equipment. The intermediate precision was calculated from the samples employed for the accuracy determination, measured on three different days at three concentration levels at the target concentrations: 50%, 100% and 150%. Therefore, mean recovery values, relative standard deviation between triplicates and relative standard deviation among the three days of analyses were done for each examined range according to equation 3.

$$CV\% \text{ (or } RSD\%) = 2^{(1-0.5\log C)} \quad \text{(Equation 3)}$$

where:
CV = coefficient of variance
C = concentration of the analyte in the experiment expressed as mass fraction in exponential notation (e.g., 1 mg g^{-1} =10^{-3}).

- *Robustness*. For the chromatographic methods, the following experimental parameters were considered critical, according to a factorial experiment design: initial oven temperature, analyst, carrier gas flow, injection volume, split ratio, injector temperature. Experimental conditions employed for dimethicone quantification in a 30% simethicone emulsion are described in Table III. Analyses were performed in triplicate.

Table III. Robustness tests in the quantification of dimethicone in simethicone 30 % emulsion by GC-FID.

Parameter	Experiment							
	S	T	U	V	W	X	Y	Z
$T_{initial\text{-}oven}$ (°C)	80	80	80	80	85	85	85	85
Analyst	C	C	A	A	C	C	A	A
Flow rate (mL min^{-1})	1.8	1.7	1.8	1.7	1.8	1.7	1.8	1.7
$V_{injection}$ (µL)	2	2	1	1	1	1	2	2
Split ratio	15	20	15	20	20	15	20	15
$T_{injector}$ (°C)	260	265	265	260	260	265	265	260

T: Temperature; V: volume.

For FT-IR measurements, the robustness was evaluated in a random way by varying the number of scans, the solvent (different trademarks) and the ambient temperature.

2.5 Validation parameters and acceptance criteria

The acceptance criteria employed for the validation of the analytical methods based on gaseous chromatography and FT-IR spectroscopy are presented in Table IV. These criteria established a set of numerical limits that, when exceeded, signal a significant departure from operating conditions. Statistical methods were employed for setting process validation acceptance criteria.

Table IV. Validation parameters and acceptance criteria.

Validation parameters		Acceptance criteria
Specificity/Selectivity		The blank may not present any peak bearing the same GC retention time as dimethicone or any IR absorbance band in the same region as dimethicone absorbance.
Linearity		Linear correlation coefficient (R^2) higher or equal to 0.99. Relative standard deviation (% RSD) between triplicates lower than 5%.
Accuracy		Recovery between 93 and 107% of the target concentration. Relative standard deviation (% RSD) between triplicates lower than 5 %.
Precision	**Repeatability**	Recovery between 93 and 107% of the target concentration. Intra-day relative standard deviation (% RSD) lower than 5%.
		Relative standard deviation (% RSD) between triplicates lower than 5%.
	Intermediate	Recovery between 93 and 107% of the target concentration. Inter-day relative standard deviation (% RSD) lower than 5%.
		Relative standard deviation (% RSD) between triplicates lower than 5%.
Robustness		Recovery between 93 and 107% of the target concentration.
		Relative standard deviation (% RSD) between the tests lower than 5%.
		Relative standard deviation (% RSD) between triplicates lower than 5%.

Data was treated by the Assistat software

3. Results and discussion

Standardization

The use of reference materials is of extreme importance in a validation process. Such materials, prepared by trustworthy and known procedures, allow for tracking but are very expensive and are occasionally not available for routine analysis. In the present study, the primary standard was the simethicone (USP) standard, and a 30% simethicone emulsion were employed as the reference material, in order to render the validation process feasible.

For the standardization procedure, the USP validated method based of FT-IR spectroscopy, described on the monograph for simethicone, was employed [2]. Standard and sample preparation is described in the experimental

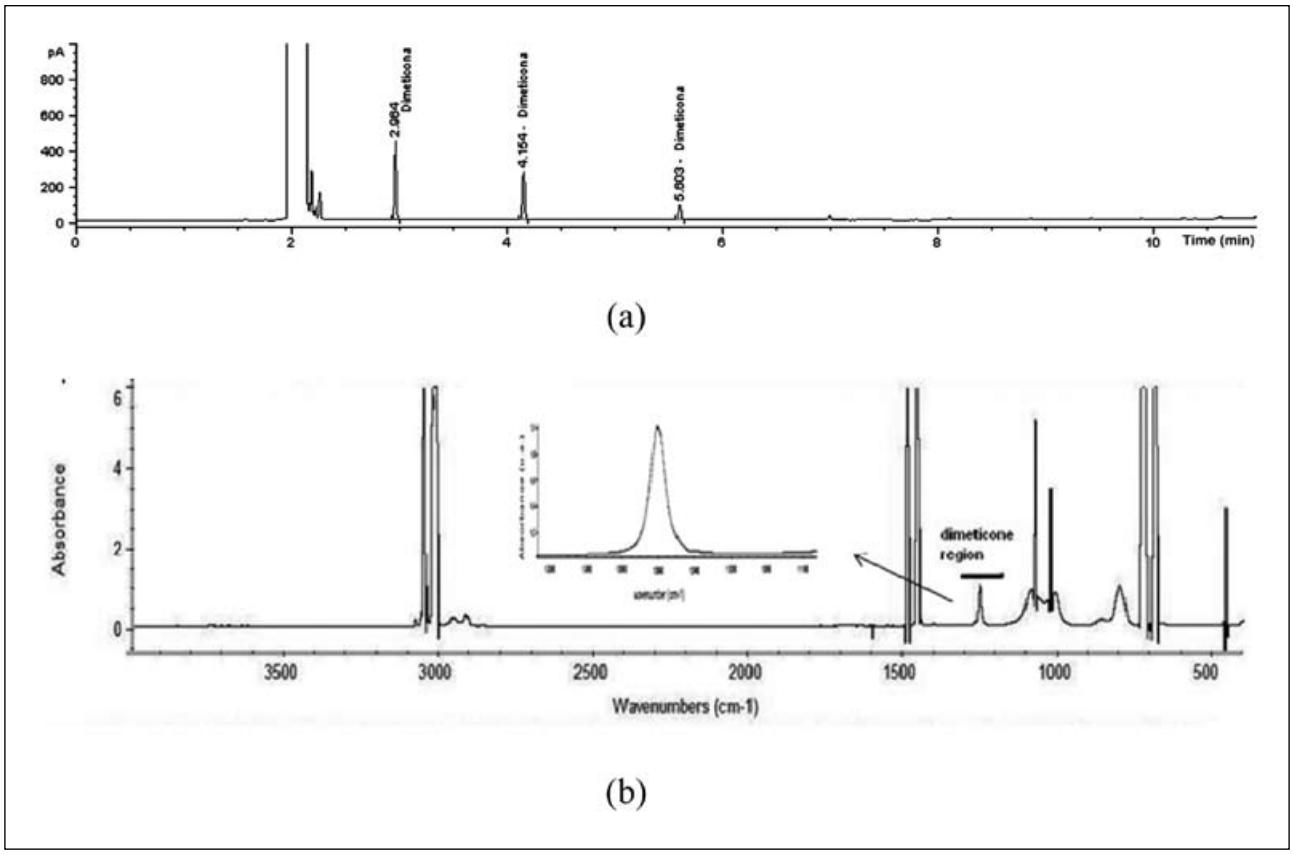

Figure 1. (a) Gas chromatography with flame ionization detection (GC-FID) chromatogram of the dimethicone reference standard; (b) FT-IR spectrum of the dimethicone reference standard. Insert: expanded region around dimethicone region.

section. In this case, 50.0 mg of material was used, which is in agreement with the American compendium. The analyses were performed in triplicate. The mean obtained for the 30% simethicone emulsion was 27.39%. These samples were employed as standard for the sample preparation for the validation procedure.

The results obtained by the chromatographic and spectroscopic methods will be discussed in sequence, according to the following parameters: specificity, linearity and linear range, accuracy and intermediate precision, repeatability and robustness.

Specificity

Figure 1 shows the dimethicone peaks in the GC-FID chromatogram and the absorption band in the FT-IR spectrum. Analysis of simethicone by GC-MS (quadrupole detection) allowed for such peaks to be identified as polysiloxanes. The infrared band centered at 1265 cm^{-1} is assigned to the symmetric stretching deformation of Si-CH_3 functional group.

In the chromatographic method, the blank chromatogram showed no peak at the same retention time of the analyte. Similarly, in the spectroscopic method, no absorption band was observed in the range of 1321-1209 cm^{-1} in the blank sample. Such results indicate that both chromatographic and spectroscopic methods are specific for the investigated analyte.

Linearity and linear range

The linearity curves were built with dimethicone standard, having concentrations corresponding to 50%, 80%, 90%, 100%, 110%, 120% and 150% of the target concentration of 12 mg mL^{-1} versus equipment signal. The resulting curves for the chromatographic and spectroscopic method were $y = 95.1392\times + 79.8702$ ($R^2 = 0.9995$) and $y = 1.1422\times + 0.2999$ ($R^2 = 0.9905$), respectively. The resulting correlation factors were higher than 0.99, and were therefore within the acceptance criteria. It is worth noting that the chromatographic method exhibited a better linear correlation between the measured concentration and the nominal one. In order to further evaluate curve linearity, the regression validity test based on the statistical method of analysis of variance (ANOVA) for a linear model was employed [17-19]. Table V describes the value obtained for the quantification of dimethicone in a 30% simethicone emulsion by both methods.

Table V. ANOVA parameters of the quantification methods of dimethicone in simethicone 30% emulsion.

Method	Variation source	Degrees of freedom (v)	Sum of squares (SS)	Mean Squares (MS)	F
GC-FID	Regression	1	2127980	2127980	33805.5
	Residual	19	1196.009	62.9478	
	Total	20	2129176		
FTIR	Regression	1	306.6905	306.690	1989.00
	Residual	19	2.9293	0.1542	
	Total	20	309.620		

GC-FID: Gas chromatography with flame ionization detection; FTIR: Fourier transform infrared spectroscopy F: ratio between the quadratic average to regression (MQ_{Reg}) and the quadratic average due to the residue (MQ_{Res}).

As shown in Table V, the analysis of variance with $F = MQ_{Reg}/MQ_{Res}$ reached an F value much higher than the critical F at 5% confidence level (1.19) = 4.38155, indicating that $a \neq 0$, i.e., at 95% confidence level, the slope of the regression curve is not null. Thus there is regression [20].

In order to check the outliers (data far from the mean) on the linearity curve, Grubbs's test was applied [21, 22]. The obtained values are shown in Table VI.

Table VI. Results of Grubbs´s test of the quantification methods of dimethicone in simethicone 30 % emulsion.

Method	General Mean	Standard Deviation	G Min	G Max	G Critical
GC-FID	1164.2	326.3	1.704	1.663	2.557
FTIR	13.30	3.934	1.794	1.537	2.557

G Min: minimum value of Grubbs' test; G Max: maximum value of Grubbs' test; G critical value of Grubbs' test for 21 degrees of freedom. GC-FID: Gas chromatography with flame ionization detection; FT-IR: Fourier transform infrared spectroscopy

As shown in Table VI, both G minimum and G maximum are smaller than the critical G. Therefore, one can state that there are no discrepant data in this set for either of the methods.

The normality of the set of residues obtained after the linear regression model fitting of both chromatographic and spectroscopic methods was checked with the aid of Assistat software [23]. The evaluation was carried out applying the Anderson-Darling and Shapiro-Wilk methods. Data were considered in normal distribution.

For the homoscedasticity (constant variance) test, Bartlett and Cochran tests were employed [22]. Analyses of variance were performed at the different levels of concentration in order to check the homogeneity of the results obtained by chromatography and by spectroscopy.

In the Bartlett test of variance comparison, the calculated χ^2 was found to be smaller than the table χ^2 value, indicating that the calibration curve, at the 5% significance level, is accepted in the homogeneity evaluation (Table VII).

Table VII. Results of Bartlett's test for GC-FID and FT-IR methods for quantification of dimethicone in simethicone 30% emulsion

Method	$\chi^2_{calculated}$	$\chi^2_{critical}$ α=0,05	Homogeneity
GC-FID	11.289	12.592	Yes
FTIR	2.552	12.592	Yes

GC-FID: Gas chromatography - flame ionization detection; FTIR: Fourier transform infrared spectroscopy

The calculated Cochran test values were 0.5426 and 0.2316 for the chromatographic and spectroscopic methods, and were lower than the critical value of 0.5612 at 5% significance, thus indicating that the method is homoscedastic for the chosen linearity range both for the investigated methods for the quantification of dimethicone in the 30% simethicone emulsion.

Accuracy and intermediate precision

According to Table VIII, recoveries ranged between 98 and 102% for the chromatographic method and between 95 and 104% for the spectroscopic method. Each prepared sample was analyzed three times and the RSD among the triplicates was lower than 1%, being within the acceptance criteria of the validation parameter. Table VIII also shows the RE in the analyte recovery, indicating that all the found recovery values were shown to be within the acceptance criteria stipulated for methodology validation.

Table VIII. Accuracy and intermediate precision of the quantification methods of dimethicone in simethicone 30% emulsion.

	ACCURACY					INTERMEDIATE PRECISION			
	Day	Sample	Rec (%)	RSD_R (%)	RE (%)	$Mean_{ID}$ (%)	SD_{ID} (%)	RSD_{ID} (%)	Student's t-Test(α=0.01)
GC-FID	1	50	101.07	0.11	1.07				
	2	50	98.33	0.57	-1.67	99.23	1.59	1.60	-0.836
	3	50	98.30	0.14	-1.70				
	1	100	100.38	0.48	0.38				
	2	100	100.34	0.21	0.34	99.73	1.09	1.09	-0.428
	3	100	98.48	0.22	-1.52				
	1	150	99.40	0.35	-0.60				
	2	150	98.58	0.31	-1.42	99.23	0.59	0.59	-2.260
	3	150	99.72	0.33	-0.28				
FTIR	1	50	98.79	0.06	-1.21				
	2	50	95.73	0.05	-4.27	99.30	3.86	3.89	-0.312
	3	50	103.40	0.76	3.40				
	1	100	100.57	0.06	0.57				
	2	100	99.06	0.05	-0.94	100.13	0.93	0.93	-0.239
	3	100	100.76	0.07	0.76				
	1	150	99.85	0.06	-0.15				
	2	150	96.01	0.06	-3.99	98.93	2.59	2.61	-0.713
	3	150	100.94	0.06	0.94				

Rec = recovery (%) analyte; RE = relative error; ID: intraday; RSDR = relative standard deviation between replicates; SDID = standard deviation intraday; RSDID =relative standard deviation intraday; GC-FID: Gas chromatography with flame ionization detection; FT-IR: Fourier transform infrared spectroscopy.

For intermediate precision, the Horwitz equation (Equation 3) can be used to calculate the limit of the relative standard deviation according to the European Community [24]. The resulting value, using a concentration of 12 $mg.mL^{-1}$, was 3.89%. The results of the RSD in this parameter is within the limits stipulated by the Horwitz equation and by resolution 899 from the ANVISA. [7] Thus, the methods can be considered precise.

In order to check accuracy of the measurements of intermediate precision, the Student's *t*-test was employed at the 95% confidence level, with n = 3 and 2 degrees of freedom. The value for the calculated *t* (see Table 8) were found to be smaller than the critical *t* (4.30), indicating that the recovery values can be considered equal to 100%. Thus, the intermediate precision was within the acceptance criteria established in the present study.

Table IX. Repeatability results of the quantification methods of dimethicone in simethicone 30% emulsion.

Samples	GC-FID		FTIR	
	Rec (%)	RSD (%)	Rec (%)	RSD (%)
A	100.17	0.16	103.96	0.04
B	98.77	0.18	97.18	0.04
C	99.38	0.21	103.12	0.04
D	98.49	0.36	105.85	0.05
E	98.85	0.08	105.70	0.04
F	98.78	0.09	103.92	0.04
Mean (%)	99.07		103.29	
SD (%)	0.611		3.18	
RSD $(\%)_{BS}$	0.617		3.08	
Student´s *t*-test , calculated	-3.708		2.532	
Student´s *t*-test, critical (0.01,5) bi-caudal	4.032		4.032	
CV, Horwitz, calculated (%)	2.594		2.594	

GC-FID: Gas chromatography with flame ionization detection; FTIR: Fourier transform infrared spectroscopy. RSD: relative standard deviation; SD: standard deviation; BS: between samples; CV: coefficient of variance.

Repeatability

The repeatability was evaluated by the RSD % among the six samples analyzed within the same day, with the same equipment, with the same analyst and under the same environmental conditions. The acceptance criteria were also the RSD between triplicates and the recovery was between 93 and 107% (see Table IX). According to Table IX, this parameter was considered acceptable.

According to the European Community [24-26], the coefficient of variance (CV%) or the RSD%, calculated by the Horwitz equation (Equation 3), multiplied by 0.667 can be employed to evaluate the intra-laboratorial precision (repeatability). The value found in this calculation with mass fraction of 12×10^{-3} was 2.594. Thus, only the chromatographic method is capable to remain within this parameter. Nevertheless, the spectroscopic parameter can also be considered precise, and was in agreement with the acceptance criteria established in the present study, since the RSD has to be lower than 5% when taking into account the legislation of ANVISA.

The Student's *t*-test comparison of the mean obtained from the experimental recoveries of analyte with that of the expected value provides information with a statistical basis of the accuracy of the method from the recoveries in the repeatability tests.

The values of the calculated *t* for the investigated methods were: -3.708 for the 30% simethicone emulsion as determined by GC-FID, and 2.532 as determined by FT-IR, both being lower than the tabled *t* value at the 99% confidence level (degree of freedom = 5, and bi-caudal), which is 4.043. Therefore, the null hypothesis is accepted and the mean generated by the samples is equal to the expected value of 100%.

Robustness

The element of robustness for the chromatographic method was evaluated using the Youlden plan, [10] known as Plackett-Burman [27], employing a factorial plan. The results of the robustness test are shown in Table X.

Table X. Robustness of the quantification method by GC-FID of dimethicone in simethicone 30% emulsion.

Experiment	GC-FID	
	Rec (%)	RSD (%)
S	98.95	0.08
T	99.50	0.16
U	98.73	0.22
V	100.66	0.33
W	99.82	0.13
X	99.17	0.48
Y	99.40	0.10
Z	99.93	0.37
Mean (%)	99.52	
SD (%)	0.61	
RSD (%) between samples	0.62	

GC-FID: Gas chromatography with flame ionization detection; RSD: relative standard deviation; SD: standard deviation.

The values obtained for the chromatographic method were within those established for the acceptance criteria.

For the infrared method, the robustness was tested with three factors and two levels, in a random planning and with six analysis runs. [21] Table XI presents the employed factors and levels, and the recovery results. The RSD remained within the acceptance criteria for this parameter.

Table XI. Robustness of the quantification method by FT-IR of dimethicone in 30 % simethicone.

Random Planning			Analysis	Recovery FT-IR	
Scans	Solvent	Temperature		Content (%)	RSD(%)
16	X	20	1	103.10	0.04
16	X	25	2	103.16	0.05
32	Y	20	3	103.22	0.05
32	X	25	4	103.54	0.05
16	Y	25	5	103.05	0.04
32	X	20	6	103.13	0.04
Mean (%)				103.20	
SD (%)				0.175	
RSD (%) between analysis				0.17	

FT-IR: Fourier transform infrared spectroscopy; RSD: relative standard deviation; SD: standard deviation;

In order to verify if the changes in the robustness test were significant, the Minitab software package [28] was employed to calculate the effects and the critical value for each methodology. The calculated effects, both for the chromatographic and the spectroscopic methods, were not significant at the 5% confidence level.

Comparison between the chromatographic and spectroscopic methods

The comparison between the methods was done by the F-test, which compares the variances of the repeatability parameter, employing the null hypothesis that both methods are equally precise. Equation 4, employed for this test, uses the higher variance value in the numerator, and the lower one in the denominator. Considering 5 degrees of freedom, the critical values for F-test at the 95% confidence level is 5.050, the F value found for both methods was 27.115, indicating that they are not equally precise.

$$F = \frac{V1}{V2} \quad \text{(Equation 4)}$$

where:

$V1$ = variance of the FT-IR method;

$V2$ = variance of the GC method.

In order to check if the means of the results of tests of repeatability of both methods can be considered equal, the Student's *t*-test was employed for the comparison between the means of different variances. The calculated *t* value was 3.188, while the one from the table at a 98% confidence value was 3.35, thus accepting the null hypothesis that the results of the means of both methods are equal.

4. Conclusions

The quantification methods of dimethicone in a 30% simethicone emulsion by flame ionization detection and by infrared molecular absorption spectroscopy reached the acceptance criteria established in the present study and were validated. The use of statistical methods was an important tool in the evaluation of the validation parameters, both in terms of decision-making and supporting the reliability of the methods under development.

The comparison between the validated methods with those of the American pharmacopeia by the equality of variances test has shown that they are not equally precise. Nevertheless, this does not invalidate the utility of the chromatographic methods, but rather suggests that the precision between the two methods is different. In the comparison between the methods, the Student's *t*-test indicated that the values found in the repeatability test can be considered equal. In sum, the development of a chromatographic method for the quantification of dimethicone in a 30% simethicone emulsion can be considered as validated.

The developed chromatographic method afforded an alternative approach, with better precision, when compared to the consolidated spectroscopic method. In practical terms, both methods demand a well-trained analyst. From the economical point of view, the choice will reside in the availability of equipment. If an infrared spectrometer is operational in the laboratory, the already reported method is easily feasible. Nevertheless, if just a gas chromatography system is available, the method investigated here is completely validated and trustworthy.

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Qualitative headspace aroma profiling of wines from Syrah and hybrid grapes using solid phase microextraction-gas chromatography-mass spectrometry

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Abstract

Varieties of grapes from the *Vitis vinifera* group, including the Syrah grape, are widely used for winemaking. A hybrid grape (Maximum- IAC 138-22) obtained by crossing Syrah and Seibel 11342 grapes has shown great adaptability for São Paulo State, Brazil, apparently producing a high quality wine. This study has compared the headspace volatile aroma composition of wines made from the Maximum IAC 138-22 grape with wines made from Syrah varietals originated from different regions of the world. Using static solid-phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS) analysis, the principal volatile compounds were identified. Hierarchical clustering analysis (HCA) showed that the wine from the hybrid grape Maximum 138-22 has volatile aroma composition very similar to most of the high quality Syrah grape wines studied.

Keywords: wine, volatile compounds, syrah, HS-SPME-GC-MS

1. Introduction

The best and most famous wines in the world are produced from cultivars of *Vitis vinifera,* which includes the Syrah varietal.[1] The importance of American cultivars and their hybrids is undeniable for viticulture in the Americas. The American varieties are endowed with vegetative characteristics that best fit our environmental conditions, but they frequently fail to display the excellent qualities of flavor and aroma intrinsic to fine European varieties and their hybrids.

In Brazil, over 75% of wine production is obtained from American grape cultivars or hybrids. In the state of Sao Paulo, wine production (3% of national production) is based mainly on grape cultivars such as Isabel, Seibel 2, Niagara Branca, and Niagara Rosada. The Maximum IAC cultivar 138-22 is also being increasingly used. This hydrid grape, originally obtained in 1946 by Antonio Santos Neto working in a very large program for wine grapes adapted to the state of Sao Paulo, is the result of the crossing between Syrah and Seibel.[2] IAC 138-22 has short-cycle plants, is very resistant to pests, rot and cracking. The "Maximum" wine made from its grapes is red, neutral, well balanced with a pleasant aroma and taste superior to that of ordinary wines typically produced in Brazil.[3]

The occurrences of different groups of non-volatile precursors, which during the winemaking process are transformed into wine aromas, are mostly related to the grape varieties. These compounds are secondary metabolites arising under different genetic controls; some are related to specific cultivars and their phenotypic expression depends on various factors such as climate, soil and viticultural practices. Samples of the wines used in this study were produced from the Syrah varietal, which is known to be ubiquitously cultivated in Brazil. Hybridization with Seibel was thought to be beneficial so as to provide greater resistance to diseases. The Maximum hybrid IAC-138-22 also has early-maturing crops (before the intense summer rains), giving wines with low acidity, dyeing and tannic composition.

In wine, over 1000 volatile aroma compounds have been so far identified with concentrations varying between the mg L^{-1} to ng L^{-1}.[4] These aroma compounds, which originate from the grapes or are formed during fermentation and aging, play important roles in the organoleptic quality of wine.[5] Various classes of compounds are found in wine aroma such as hydrocarbons, alcohols, terpenes, esters, aldehydes, ketones, acids, ethers, lactones, bases, sulfur-

compounds, halogenated compounds and nitriles.[6] Various parameters, such as grape variety, climate, soil, fermentation conditions (pH, temperature, yeast flora), enological methods, treatment substances and bottle maturation, influence the final aroma composition.[7] Major aroma components can be analyzed by gas chromatography coupled to mass spectrometry (GC-MS),[8] but many are found at very low concentrations (ng L^{-1}) requiring special analytical protocols.[9]

Several isolation and pre-concentration methods have been developed for the analysis of volatile aroma components in wine, but it is generally admitted that they all fail to fulfill the ideal requirements, and various operational or technical difficulties have limited their use.[10,11] Some are too laborious, some have very low analyte recoveries and high detection limits, whereas others display low reproducibility, possibility due to solvent cross-contamination and insufficient selectivity.[12]

Headspace solid phase microextraction followed by GC-MS (HS-SPME-GC-MS) has been widely applied to wine analysis.[13] As a selective concentration technique, HS-SPME, particularly when coupled to mass spectrometry detection[14], offers many advantages such as speed, low cost, low sample requirements and ease of automatization.

The aim of this study was to evaluate the aroma profile of the wine made from the hybrid grape Maximum IAC 138-22 via comparison of a representative set of its most abundant aroma components, as detected in its volatile headspace composition, to that of several high quality Syrah wines produced in different regions of the world. This comparison was done via HS-SPME-GC-MS followed by chemometric hierarchical clustering analysis (HCA).[15, 16]

2. Materials and methods

2.1. Wine samples

A characteristic sample of red wine from the Maximum IAC 138-22 grape (A) was analyzed and compared to high quality Syrah wines produced in different countries: (B) Valle del Maipo /Chile, (C) Rivadavia, Mendoza/Argentina, (D) Alentejo Estremoz/Portugal, (E) Côtes du Rhône /France, (F) Tuscany /Italy, (G) Western Cape /South Africa, (H) Mendoza /Argentina and (I) Epanomi /Greece. All Authentic samples were purchased at local markets.

2.2. Reagents

All reagents were of analytical grade. The C7-C32 hydrocarbon mixture used for determination of Kovats' retention indices was obtained from Sigma Aldrich. Water was from a Milli-Q purification system (Millipore).

2.3. Solid Phase Microextraction coating

The DVB/CAR/PDMS fiber (coated with 50/30 µm of divinylbenzene / carboxen / polydimethylsiloxane) was purchased from Supelco (Bellefonte, PA, USA). The fiber was conditioned at 250 °C for 15 min in accordance with the manufacturer's instructions before its use.

2.4. Volatile compound analysis

HS-SPME of the wine samples were performed by the method described below. Both wine samples and the standard n-alcane solutions were analyzed in 40 mL glass vials. A 10 mL aliquot of wine was transferred to a 40 mL glass headspace sample vial containing 6.0 g of NaCl and 10 mL of Milli-Q water. Analyses were done in triplicate and the DVB/CAR/PDMS fiber was placed in into vials containing wine stirred for 15 min at 25 °C at 1200 rpm before extraction. Then extraction was continued for more 15 min until equilibration. The fiber was then inserted into the GC injector (held at 250 °C) for five min to desorb the volatile compounds and analyses were carried out using a HP-5 MS capillary column (30 m x 0.25 mm ID x 0.25 µm thickness film). GC-MS analysis and aroma identification were performed on a HP 5890 Series II Gas Chromatograph coupled with a HP 5970 Series Quadrupole Mass Selective Detector (MSD). The oven temperature was raised from 40 °C to 160 °C at 3 °C min^{-1}, and then to 260 °C at 10 °C min^{-1} (held for 10 min). The carrier gas was helium at a flow rate 1.0 mL min^{-1}. Injections were performed in split mode (split ratio 1:50). The temperatures of the injector and of the detector were 250 °C and 260 °C, respectively.

The compounds were identified by comparison of their mass spectra with those from the NIST MS Search 2.0 library database and by their retention indices. The Kovats Indices were calculated from retention times. The calibration mixture contained aliphatic hydrocarbons. Hierarchical Clustering Analysis (HCA) was performed using Pirouette 3.11.

3. Results and discussion

A sample of the wine from the hybrid Maximum IAC-138-22 grape (A) was compared via HS-SPME-GC-MS to high quality Syrah wines (B-I). A total of 31 compounds were detected and, among them, 14 were found as the most common and abundant. Figure 1 displays the chromatographic profiles of some selected wine samples. When the HS-SMPE-GC-MS data of the Brazil wine (A) is compared with other high quality wines samples, many similarities were indeed found. All chromatograms show very similar sets of the most intense peaks, and most of these peaks correspond to higher alcohols and esters. Therefore, the wine from the Maximum IAC-138-22 grape is found to display the same qualitative aroma features, as judged by the 14 sets of most common and abundant components, found in most of the high quality wines from Syrah varietals. Table 1 summarizes the compounds with the Retention Indices and percentages of area related to the itensity signal of each analyte found in each sample of the wine by HS-SPME-GC-MS. Note in Table I the predominance of higher alcohols and their ethyl esters, and the similar profiles.

Table I. Detected compounds with the Retention Indices and mean percentages of area found in each sample of the wine.

Nº	Compound	Gota Pura (A)	Chile (B)	Argentina[A] (C)	Portugal (D)	France (E)	Italy (F)	South Africa (G)	Argentina[B] (H)	Greece (I)	*RI lit	*RI calc
1	**3-methyl-1-Butanol**	14.3	17.99	20.47	41.9	16.83	15.77	40.73	44.02	18.8	734.0	721
2	**ethyl isobutyrate**	0	0	1.14	2.1	0	0	1.72	0	1.52	n.f.	-
4	**ethylbutanoate**	0.49	0	1.38	0	0.96	0	0	1.4	1.6	800.0	804
5	**ethyl lactate**	0	0	0	0	0.72	0	0	2.67	1.94	n.f.	-
6	**ethyl isovalerate**	0	0	0	0	0	0	0	0	1.24	856	850
7	**o- xilene**	0	0	0	0	0	0	0	1.08	0	n.f.	863
8	**isoamylacetate**	17.85	4.87	0	3.76	6.27	1.84	8.1	7.8	0	876	875
9	**hexyl acetate**	0	0	0	0	0.92	0	0	0	0	1008.00	1016
10	**ethyl hexanoate**	26.99	14.13	17.49	11.84	22.14	21.17	22.26	8.97	15.93	996	1002
11	**phenylethyl alcohol**	0	0	3.86	0	2.25	8.57	2.32	3.54	0	1110	1105
12	**ethyl octanoate**	17.67	39.21	30.16	13.22	41.48	38.17	7.5	13.28	0	1195	1151
13	**diethyl succinate**	1.33	4.88	8.86	0	1.64	8.22	2.15	1.92	0	1179	1143
14	**o-cresol**	0	5.36	0	0	0	8.57	0	3.54	0	n.f.	-
15	**Vitispirane**	0	0	0	0	0	2.14	0	0	0	1272	1271
16	**ethyl decanoate**	0.92	5.39	2.33	0	4.64	2.27	1.02	1.72	0	1394	1298
17	**4-methyl-1-pentanol**	3.37	0	0	0	0	0	0	0	0	n.f.	-
18	**hexyl acetate**	5.54	0	0	0	0.92	0	0	0	0	n.f.	-
19	**2-methyl-pentane**	0	3.74	0	0	0	0	0	0	0	n.f.	-
20	**toluene**	0	3.94	4.61	0	0	0	0	0	0	n.f.	-
21	**2-methyl-decane**	0	4.97	0	0	0	0	0	0	0	n.f	-
22	**1 hexanol**	3.58	7.69	2.07	0	0	0	3.91	0	0	867.00	868
23	**1 heptanol**	0	16.4	0	0	0	0	0	0	0	n.f	-
24	**3-hidroxy-2-butanone**	0	0	1.96	0	0	0	0	0	0	n.f.	-
25	**2-methyl-1-hidroxy-propane**	0	0	2.62	0	0	0	0	0	0	n.f.	-
26	**3,3-dimethyl -1-butanol**	0	0	0	0.74	0	0	0	0	0	n.f	-
27	**ethyl Pentanoate**	0	0	0	1.29	0	0	0	0	0	n.f.	-
28	**2,3-dimethyl -hexane**	0	0	0	2.53	0	0	0	0	0	n.f.	-
29	**4-methyl-1-pentanol**	0	0	0	0	0	0.98	0	3.18	1.78	n.f.	833
30	**Acetaldeído**	0	0	0	0	0	0	2.6	0	0.71	n.f.	-
31	**2,3-butanediol**	0	0	0	0	0	0	1.64	0	0	n.f.	-

*RI lit Retention Index from the literature (NIST, NIST2008 mass spectral library)
*RI calculated Retention Index calculated
n.f. data not found in the literature.

HCA was then used to statistically compare the "Maximum" wine (A) with the high quality Syrah wines. In the HCA dendrogram (Figure 2) two main groups are formed. The wines from other varieties of grapes, such as Cabernet Savignon, Merlot and Cammenere (data not shown), were also analyzed via GC-MS and HCA, and were found to be substantially dissimilar to the Syrah wines. The first group belongs to wines samples from Brazil(A), Mendoza/Argentina (C), Portugal (D), France (E), South Africa (G), and Greece(I), whose major similarity is the presence of 3-methyl-1-butanol, ethyl isobutyrate, ethyl lactate, ethyl butyrate, 1,2-dimethyl benzene, ethyl 2-methylbutyrate, 4-methyl 1-pentanol, 2,3-dimethylhexane, ethyl valerate, 2,3-butanediol, isoamyl acetate, ethyl isobutyrate, hexyl acetate and 3,3-dimethyl-1-butanol.

The second group is formed by the Syrah wines from Rivadavia/Argentina (C), Italy (F) and Chile (B), which are mainly characterized by the presence of hexyl acetate, 3-hydroxy-2-butanone, 2-phenylethanol, vitispirane and isobutyl methyl ether. The sample from Chile (B) was the most unique, displaying as characteristic volatile components in its aroma: ethyl octanoate, diethyl succinate, ethyl decanoate, 2-methyl pentane, toluene; 2-methyldecane and 1-heptanol.

From the HCA dendogram, it appears therefore that the phenotypic expression of the major volatiles in varietal Syrah wines from the different origins investigated is also present in the Maximum IAC 138-22 hybrid (A), thereby favoring the genetic constitution inherited from the parental Syrah used as male parent, in detriment to the Seibel 11342 varietal used as the female. The subtle differences found in each sample and the expression of different wines studied herein show that the hybrid Maximum IAC 138-22 (A) is best grouped with wines of the Côtes du Rhône/France (E), Western Cape/South Africa (G), Alentejo-Estremoz/Portugal (D), Mendoza/Argentina (C) and Epanomi/Greece (I).

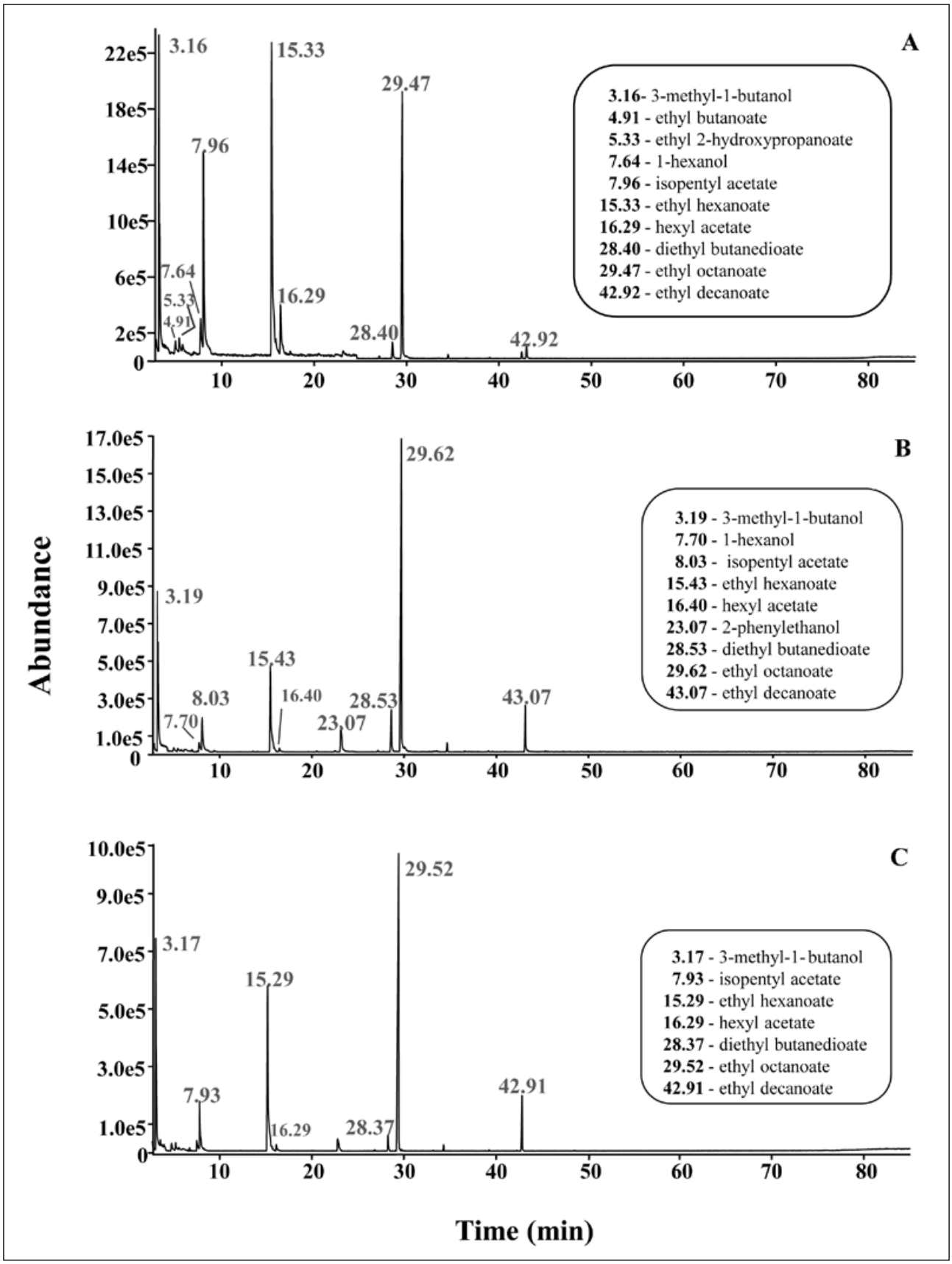

Figure 1. Total ion current (TIC) HS-SPME/GC-MS chromatograms of representative wine samples: São Paulo /Brazil (A), Valle de Maipo/Chile (B), and Côtes de Rhône/France (C).

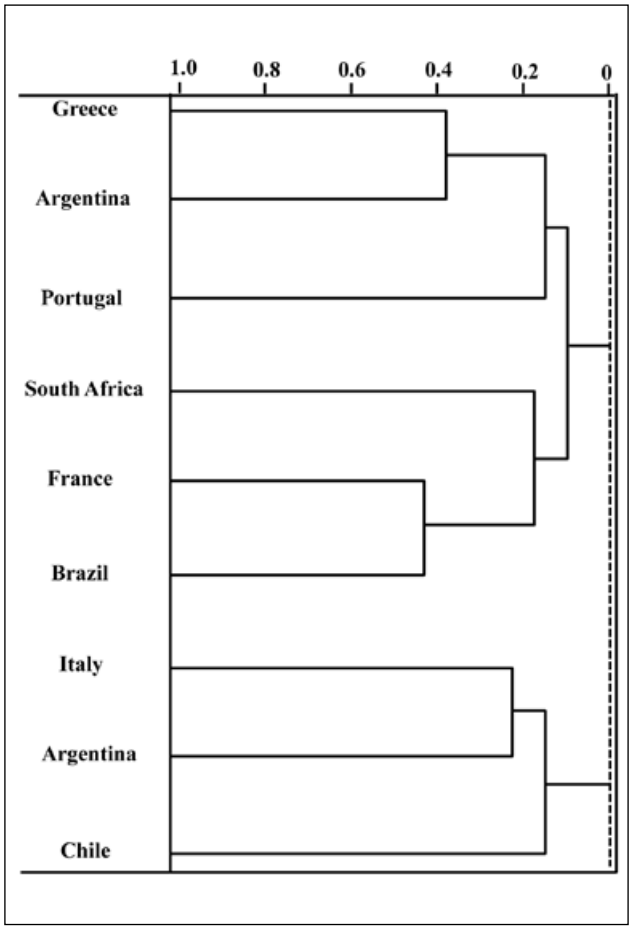

Figure 2. HCA dendrogram for the HS-SPME-GC-MS data (Table 1) of the 9 wine samples analyzed.

4. Conclusions

Using HS-SPME-GC-MS, the major aroma components in the headspace of different wine samples were detected and identified. When the chromatographic profiles of a set of 14 most common and abundant components were compared via HCA statistical analysis, the wine produced from the Maximum IAC-138-22 varietal was found to display a volatile aroma composition quite similar to several high quality Syrah wines originating from different regions of the world.

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A simple method based on HR-CS FAAS for multi-element determination of Cu, Fe, Mn and Zn in medicinal plants

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Abstract

A simple method to determine Cu, Fe, Mn and Zn in single aliquots of medicinal plants by HR-CS FAAS is proposed. The main lines for Cu, Mn and Zn, and the alternate line measured at the wing of the main line for Fe at 248.327 nm allowed calibration within the 0.025 – 2.0 mg L^{-1} Cu, 1.0 – 20.0 mg L^{-1} Fe, 0.05 – 2.0 mg L^{-1} Mn, 0.025 – 0.75 mg L^{-1} Zn ranges. Nineteen medicinal plants and two certified plant reference materials were analyzed. Results were in agreement at a 95% confidence level (paired *t-test*) with reference values. Limits of detection were 0.12 µg L^{-1} Cu, 330 µg L^{-1} Fe, 1.42 µg L^{-1} Mn and 8.12 µg L^{-1} Zn. Relative standard deviations (n=12) were ≤ 3% for all analytes. Recoveries in the 89 – 105% (Cu), 95 – 108% (Fe), 94 – 107% (Mn), and 93 – 110% (Zn) ranges were obtained.

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Introduction

Tropical rainforests contain a large diversity of plant species, some of which present medicinal values and have been used in a great number of modern therapeutic agents [1,2]. Besides low cost and abundance in tropical areas, medicinal plants may be sources of nutrients to humans such as the essential minerals Cu, Fe, Mn and Zn [2]. Although these elements are essential to biological systems, they may be toxic if present at concentrations higher than those required to accomplish their biological functions [3]. The concentration of micronutrients in plants depends on conditions influencing their translocation from soil to plant tissues [4]. Phytomedicine requires safety and efficacy, which may be acquired by accurate quality control of medicinal herbs [5]. In this context, the development of analytical technologies for accurate determination of Cu, Fe, Mn and Zn in medicinal plants is relevant [6].

The determination of Cu, Fe, Mn and Zn in plant materials by atomic absorption spectrometry is frequently carried out by line-source flame atomic absorption spectrometry (LS FAAS) [7]. Since the content of Fe in plant tissues is higher than those for Cu, Mn and Zn, the determination of Fe, Cu, Mn and Zn by LS FAAS is a demanding analytical job because each run requires optimized operating parameters and new calibrations and exchange and conditioning of radiation sources, which are time consuming and lead to increased analytical costs. For large-scale analyses of plants, the simultaneous determination of several elements, employing a single-sample injection is particularly helpful because time and analytical costs may be significantly reduced.

The combination of a high-resolution double-Echelle monochromator, a charge-coupled device detector and a xenon short-arc lamp continuum source allow fast-sequential multi-element determinations by high-resolution continuum source flame atomic absorption spectrometry (HR-CS FAAS) [8]. Besides multi-element determination, the integration of absorbance over the atomic absorption spectrum to enhance sensitivity [9], the measurement of absorbance at the sides of the maximum atomic peak to extend the linear working range [10] and background correction by least-squares [11] are among main benefits of the HR-CS FAAS.

The determinations of macro- and microelements in plant leaves by HR-CS FAAS are found in the literature [12], but a three-step sample preparation method and different aliquots of the sample are required. However, limited attention has been given to the multi-element analysis of medicinal plants for Cu, Fe, Mn and Zn determination in single aliquots of samples.

The objective of this work is the development of a simple, rugged and fast method for determination of Cu, Fe, Mn and Zn in single aliquots of medicinal plants by exploring potential features of the HR-CS FAAS technique.

Experimental

Instrumentation

All measurements were carried out using an Analytik Jena ContrAA 300 high-resolution continuum source flame atomic absorption spectrometer equipped with a xenon short-arc lamp XBO 301 (GLE, Berlin, Germany) with a nominal power of 300 W operating in a hot-spot mode as a continuum radiation source [8]. This new equipment presents a compact high-resolution double-Echelle grating monochromator corresponding to a spectral band width < 2 pm per pixel in the far ultraviolet range and a charge-coupled device (CCD) array detector.

An air-acetylene oxidizing flame for the atomization of Cu, Fe, Mn and Zn was used. Air Liquide acetylene, 99.7% purity (Sertãozinho, Brazil), was used as fuel gas. All measurements were carried out in triplicate using an injection module (SFS 6) enabling the computer-controlled aspiration of blanks, analytical solutions and samples. The aspiration rate was fixed at 5.0 mL min^{-1} and the equipment was adjusted under optimum conditions.

An Anton Paar Multiwave® 3000 microwave oven (Graz, Austria) equipped with a 48-position rotor and 25-mL Tefon reaction vessels was used for sample preparation.

Reagents and analytical solutions

High purity de-ionized water (resistivity 18.2 MΩ cm) obtained using a Millipore Rios 5® reverse osmosis and a Millipore Milli-Q Academic® deionizer system (Bedford, MA, USA), and Merck Suprapur® hydrochloric and nitric acids (Darmstadt, Germany) were used to prepare solutions and/or samples.

Reference analytical solutions (0.025 – 2.0 mg L^{-1} Cu, 1.0 – 20.0 mg L^{-1} Fe, 0.05 – 2.0 mg L^{-1} Mn, 0.025 – 0.75 mg L^{-1} Zn) were prepared daily by appropriate dilution of individual Carlo Erba Normex™ 1000 mg L^{-1} single stock standard solutions (Milan, Italy) and acidified to 1% (v/v) with HNO_3. All solutions were stored in Nalgene® high-density polypropylene bottles (Rochester, USA). Plastic bottles and glassware materials were cleaned by soaking in 10% (v/v) HNO_3 at least 24 h and rinsed abundantly in de-ionized water before use.

Procedure

Samples of *Peumus boldus*, *Matricaria chamomilla*, *Baccharis trimera*, *Rhamnus purshiana*, *Equisetum arvense*, *Centella asiatica Urban*, *Echinodorus grandiflorus*, *Lippia alba*, *Pimpinella anisum*, *Maytenus ilicifolia*, *Ginkgo biloba*, *Panax ginseng*, *Annona muricata*, *Casearia sylvestris*, *Mentha spp*, *Melissa officinalis*, *Bauhinia forficata*, *Mentha pulegium* and *Cassia angustifólia* were purchased at a local market in Araraquara city, SP, Brazil. Dried and powdered samples were mineralized in triplicate in a closed-vessel microwave-assisted acid-digestion system. A mass of 0.25 g of sample was accurately weighed and transferred to a microwave flask followed by 2 mL of 30% (m/m) H_2O_2 + 3.5 mL of concentrated nitric acid + 0.5 mL of concentrated hydrochloric acid. The following optimized program involving power/ramp time/hold time was: step 1, 600 W/10 min/5 min; step 2, 1400 W/15 min/5 min; step 3, 0 W/0 min/15 min (ventilation). After digesting and cooling, the resulting digests were transferred to 25 mL volumetric flasks and the volume completed with de-ionized water. Two certified reference materials (1573a Tomato Leaves; 1547 Peach Leaves) from the National Institute of Standards and Technology (Gaithersburg, MD, USA) were also analyzed to furnish additional information for method validation.

Measurements were carried out at the principal lines for Cu (324.754 nm), Mn (279.482 nm) and Zn (213.857 nm). The influence of wavelength-integrated absorbance on sensitivity [9] for Cu, Mn and Zn was studied by varying the number of pixels used for detection from 1 to 9 pixels, i.e., from only the central pixel (CP) to central pixel ± 4, respectively.

The side pixel registration approach [10] was investigated to extend the linear working range calibration for Fe because most plants contain Fe at concentrations higher than the upper limit of linear response (1 mg L^{-1}) in calibration plots using the main line at 248.327 nm. This approach was evaluated by measuring at wings of the principal line for Fe at the wavelengths 248.333, 248.332, 248.331, 248.329, 248.325, 248.324, 248.323, and 248.321 nm. For comparison purposes, the secondary line for Fe at 252.744 nm was also evaluated. For all lines, the wavelength-integrated absorbance equivalent to 3 pixels was used. Recovery tests for spiked samples were carried out in two levels by adding appropriate aliquots of 1000 mg L^{-1} single stock standard solution to plant digests in order to obtain extracts containing 0.10 mg L^{-1} Cu, Mn, Zn plus 1.0 mg L^{-1} Fe, and 0.25 mg L^{-1} Cu, Mn, Zn plus 2.0 mg L^{-1} Fe.

The limits of detection (LOD) and limits of quantification (LOQ) for Cu, Fe, Mn and Zn were calculated according to IUPAC recommendations [13].

Results and Discussion

The concentration of Fe usually found in plant tissues is significantly higher (a hundred times or more) than those for Cu, Mn and Zn. This impairs the determination of Cu, Fe, Mn and Zn in a single sample by LS FAAS due to the different diluting factors necessary to adjust analyte signals through the linear range of calibration curves. This limitation may be circumvented using less sensitive lines for Fe. Taking into consideration that an assortment of atomic lines of a given element is available in HR-CS FAAS, the feasibility of alternate lines for Fe located at the wings (side pixel registration approach) of the principal line for Fe at 248.327 nm were then evaluated in order to achieve conditions for a fast and multi-element determination of Cu, Fe, Mn and Zn in a single solution of medicinal plants.

The influence of side pixel registration on the calibration plots of Fe was evaluated by measuring absorbance with-

in the 1.0 – 20.0 mg L^{-1} range at 248.333 nm, 248.332 nm, 248.331 nm, 248.329 nm, 248.325 nm, 248.324 nm, 248.323 nm, and 248.321 nm. The main figures of merit (slope, correlation coefficient, limit of detection and relative standard deviation) related to these plots are described in Table I.

Table I. Analytical characteristics of main, secondary and alternate lines for Fe obtained by HR-CS FAAS. R, LOD and RSD are mean linear correlation coefficient, limit of detection and relative standard deviation, respectively.

Wavelength (nm)	Calibration (mg L^{-1})	Slope (A L mg^{-1})	R	LOD (µg L^{-1})	RSD (%)
248.321[a]	1.0 – 20	0.0009	0.9957	270	5.2
248.323[a]	1.0 – 20	0.0027	0.9803	100	3.7
248.324[a]	1.0 – 5.0	0.0094	0.9986	35	2.8
248.325[a]	1.0 – 5.0	0.0186	0.9995	13	2.5
248.327[b]	0.1 – 1.0	0.1006	0.9997	7	2.1
248.329[a]	1.0 – 5.0	0.0184	0.9886	13	3.0
248.331[a]	1.0 – 5.0	0.0080	0.9998	37	4.2
248.332[a]	1.0 – 10	0.0028	0.9916	139	3.6
248.333[a]	1.0 – 20	0.0010	0.9959	330	1.9
252.744[c]	1.0 – 20	0.0136	0.9939	66	1.4

[a] Alternate lines; [b] Main line; [c] Secondary line

Absorbance evaluations at increased distances to the core line of 248.327 nm resulted in lower sensitivity and a higher limit of detection. The upper limits of linear response were 1.0 mg L^{-1} (248.327 nm), 5.0 mg L^{-1} (248.324 nm, 248.325 nm, 248.329 nm, 248.331 nm), 10 mg L^{-1} (248.332 nm), and 20 mg L^{-1} (248.321 nm, 248.323 nm, 248.333 nm).

Considering that linear response curves over a wide-range are attractive for several analytical applications, the lines 248.321 nm, 248.323 nm and 248.333 nm could be employed for Fe determination. The wavelengths 248.321 nm and 248.333 nm furnished similar linearity (R≈ 0.996), but better than that for 248.323 nm (R= 0.980). The RSD of measurements obtained for lines 248.321 nm and 248.333 nm were 5.2% and 1.9%, respectively. These lines allowed limits of detection of 270 and 330 µg L^{-1}, respectively. For comparison purposes, the secondary line at 252.744 nm furnished linear response up to 20 mg L^{-1} (R= 0.994) with good precision (RSD= 1.4%). These findings emphasize the feasibility of measurement at side pixels corresponding to 248.333 nm with good precision and accuracy for Fe determination in plant materials without need of further dilutions. Improvement in sensitivity by the wavelength-integrated absorbance (WIA) approach [9] is also a simple and feasible feature of the HR-CS FAAS technique. In this case, the number of pixels used for detection depends on the atomic peak profile: broader peaks usually require more pixels than narrow peaks. The influence of WIA on sensitivity for Cu, Mn and Zn were studied by measuring atomic absorbance at lines 324.754 nm, 279.482 nm and 213.875 nm, respectively, at 1, 3, 5, 7, and 9 pixels. Improvement in sensitivity was obtained with up to 5 pixels used for detection. The variation of peak volume evaluation for sensitivity enhancement was ineffective for numbers of pixels higher than 5 (Fig.1).

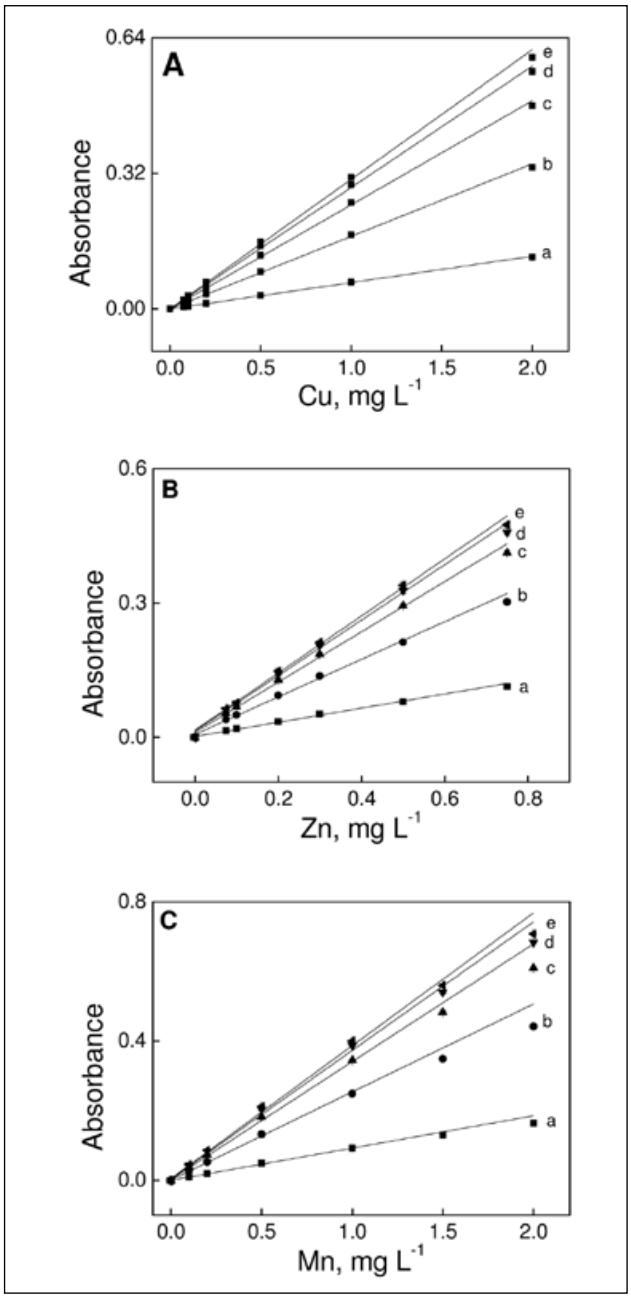

Figure 1. Calibration plots for Cu at 324.754 nm (A), Zn at 213.875 nm (B), and Mn at 279,482 nm (C) built up by HR-CS FAAS employing 1 (a), 3 (b), 5 (c), 7 (d) and 9 (e) pixels for peak volume evaluation.

Calibration plots with suitable linear coefficient correlations were obtained in most situations. The RSD of measurements were in all situations lower than 3%. In general, higher slopes should result lower LODs [13]. When the number of pixels was changed from 1 to 9, the LOD changed from 0.91

to 0.59 μg L^{-1} Cu, 1.94 to 2.59 μg L^{-1} Mn, and 8.16 to 19.56 μg L^{-1} Zn, respectively. The increased LOD for Mn and Zn may be attributed to the contribution of baseline noise during integration. The lowest LOD for Cu (0.12 μg L^{-1}), Mn (1.42 μg L^{-1}), and Zn (8.12 μg L^{-1}) was observed for 5 pixels, 3 pixels and 1 pixel, respectively. With these number of pixels, the corresponding LODs were suitable for determination of analytes in selected medicinal plant samples in this work. After atomic line evaluations, the procedure was applied to the sequential multi-element determination of Cu, Fe, Mn and Zn in medicinal plants. The most sensitive lines of Cu, Mn and Zn were used. For aspiration rates of the nebulizer at 5.0 ml min^{-1}, analytical curves in the 0.025 – 2.0 mg L^{-1} Cu, 0.05 – 2.0 mg L^{-1} Mn, 0.025 – 0.75 mg L^{-1} Zn concentration ranges were always attained with linearities better than 0.998. The determination of Fe was carried out within the 1.0 – 20.0 mg L^{-1} using side pixel registration at 248.333 nm and linear correlation coefficients better than 0.996 were typically obtained.

Accuracy was checked after analyte determinations in two certified plant reference materials (1573a Tomato Leaves; 1547 Peach Leaves). Results for Cu, Fe, Mn and Zn determination were in agreement at a 95% confidence level (paired *t-test*) with reference values (Table II).

Table II. Results (mean ± standard deviation) of determinations (N= 3) of Cu, Fe, Mn and Zn in medicinal plants and certified reference materials by the proposed method.

Sample	Found concentration, μg g^{-1}			
	Cu	Fe	Mn	Zn
Peumus boldus	18.2 ± 1.0	264.9 ± 5.1	136.0 ± 4.4	13.6 ± 0.9
Matricaria chamomilla	12.8 ± 0.7	157.5 ± 4.4	29.9 ± 2.0	30.1 ± 2.2
Baccharis trimera	9.3 ± 0.3	114.7 ± 6.2	101.9 ± 7.2	27.8 ± 1.4
Rhamnus purshiana	11.6 ± 0.4	83.4 ± 7.3	146.7 ± 9.2	8.7 ± 1.8
Equisetum arvense	27.6 ± 0.7	134.2 ± 5.7	85.6 ± 8.2	54.8 ± 3.9
Centella asiatica Urban	40.5 ± 0.2	2157.6 ± 130.8	187.9 ± 6.5	38.2 ± 1.7
Echinodorus grandiflorus	24.1 ± 0.1	1461.9 ± 34.6	289.4 ± 10.4	64.7 ± 2.1
Lippia alba	13.9 ± 0.6	275.3 ± 6.2	152.1 ± 3.1	33.1 ± 2.5
Pimpinella anisum	33.2 ± 1.1	196.1 ± 9.6	11.5 ± 0.2	52.1 ± 1.4
Maytenus ilicifolia	12.1 ± 0.5	353.6 ± 5.4	362.8 ± 12.8	44.2 ± 2.3
Ginkgo biloba	8.6 ± 0.2	830.9 ± 18.4	64.6 ± 2.5	35.4 ± 1.4
Panax ginseng	10.9 ± 0.4	81. 8 ± 7.4	28.0 ± 1.4	12.9 ± 1.0
Annona muricata	29.4 ± 0.2	67.7 ± 4.5	62.6 ± 1.7	9.9 ± 0.4
Casearia sylvestris	18.9 ± 1.1	246.6 ± 2.6	291.2 ± 8.2	35.4 ± 0.2
Mentha spp	22.4 ± 0.2	1727.7 ± 32.8	120.1 ± 1.9	37.4 ± 2.1
Melissa officinalis	22.1 ± 0.1	211.3 ± 3.6	75.4 ± 1.5	44.0 ± 1.0
Bauhinia forficata	24.2 ± 0.1	1129.6 ± 22.7	322.5 ± 1.2	26.6 ± 1.1
Mentha pulegium	32.3 ± 0.2	2432.5 ± 38.6	137.2 ± 1.0	42.6 ± 0.3
Cassia angustifolia	12.4 ± 1.1	254.7 ± 10.4	25.2 ± 1.1	17.4 ± 0.7
Peach Leaves[a]	4.2 ± 0.3	197.4 ± 10.3	92.1 ± 2.4	19.3 ± 0.6
Tomato Leaves[b]	4.0 ± 0.6	329.3 ± 12.3	233.6 ± 7.6	29.8 ± 0.5

a) Certified values (in mg kg^{-1}): Cu (3.7 ± 0.4); Fe (218.0 ± 14.0); Mn (98.0 ± 3.0); Zn (17.9 ± 0.4).
b) Certified values (in mg kg^{-1}) : Cu (4.70 ± 0.14); Fe (368.0 ± 7.0); Mn (246.0 ± 8.0); Zn (30.9 ± 0.7).

Results for Fe using the secondary line at 252.744 nm are in agreement with those obtained for side pixel registration at 248.333 nm. Thus, the side pixel registration approach was feasible to extend the linear working range and determine Fe together with Cu, Mn and Zn in plant extracts. Indeed, 19 medicinal plant samples were analyzed by the proposed method (Table II). The concentrations found for the analytes varied in the 8.6 – 40.5 μg g^{-1} Cu, 67.7 – 2432.5 μg g^{-1} Fe, 11.5 – 362.8 μg g^{-1} Mn and 8.7 – 64.7 μg g^{-1} Zn ranges. These concentrations are comparable to those found in earlier works [14,15]. Accuracy was also checked using recovery tests for sample digests spiked with Cu, Fe, Mn, and Zn. For addition of analytes (0.10 mg L^{-1} Cu, Mn, and Zn plus 1.0 mg L^{-1} Fe; 0.25 mg L^{-1} Cu, Mn and Zn plus 2.0 mg L^{-1} Fe) in digests of *Maytenus ilicifolia* (sample 1) and *Annona muricata* (sample 2), recoveries varied within the 89 – 105% (Cu), 95 – 108% (Fe), 94 – 107% (Mn) and 93 – 110% (Zn) intervals (Table III).

Table III. Recovery results for spiked Cu, Fe, Mn and Zn determined in sample digests (N= 3) by the proposed method. RSD of measurements ≤ 3%.

Sample	Spiked, mg L^{-1}				Found, mg L^{-1} and Recoveries, %			
	Cu	Fe	Mn	Zn	Cu	Fe	Mn	Zn
1	0	0	0	0	0.12	3.54	3.63	0.44
1	0.10	1.00	0.10	0.10	0.20 (91)	4.31 (95)	3.99 (107)	0.52 (96)
1	0.25	2.00	0.25	0.25	0.38 (103)	5.98 (108)	3.92 (101)	0.76 (110)
2	0	0	0	0	0.29	0.68	0.63	0.099
2	0.10	1.00	0.10	0.10	0.41 (105)	1.66 (99)	0.69 (95)	0.184 (92)
2	0.25	2.00	0.25	0.25	0.48 (89)	2.81 (105)	0.86 (98)	0.370 (106)

The relative standard deviations (*n*=12) were ≤ 3% for all analytes for digests of *Maytenus ilicifolia* containing 0.12 mg L^{-1} Cu, 3.54 mg L^{-1} Fe, 3.63 mg L^{-1} Mn and 0.43 mg L^{-1} Zn. The limits of detection were 0.12 μg L^{-1} Cu, 330 μg L^{-1} Fe, 1.42 μg L^{-1} Mn and 8.12 μg L^{-1} Zn.

The use of the special instrumental features of the HR-CS FAAS such as WIA and side pixel registration allowed the multi-element determination of Cu, Fe, Mn and Zn in single aliquots of medicinal plant digests without the need of further dilutions of samples. The proposed method based on the HR-CS FAAS technique is simple, fast, and presented accurate and precise results. These findings are also possible in inductively coupled plasma atomic emission spectrometry (ICP OES), but not in atomic absorption spectrometry with line sources.

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Conductometric determination of sulphate ions in hydrated ethanol fuel

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Abstract

The presence of sulphate ions in hydrated ethanol fuel may cause serious damage to automotive vehicles in the form of corrosion. Taking into account the increasing number of cars powered with alcohol or with a mixture of ethanol/gasoline, the importance of new analytical technologies to ensure the quality of fuel becomes fundamental. This work proposes an analytical method, using conductometric titration, to determine the concentration of sulphate ions in automotive alcohol fuel. The results obtained were very satisfactory and showed accuracy and reproducibility, thus indicating that the method can be carried out to quantify sulphate ions in ethanol fuel and has some advantages in comparison to the standard methods, such as being faster and simpler, with a determination limit of about 0.32 mg kg^{-1}.

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Keywords: sulphate ions, hydrated ethanol fuel, conductometry

1. Introduction

The need for renewable fuels and the Kyoto Protocol (which controls greenhouse gas emissions responsible for climatic changes) coming into effect has hastened the processes that brought attention to bioenergy, mainly alcohol. Many countries are gearing towards the addition, or to an increase, of the amount of ethanol in gasoline. In Brazil, flex cars which can run either on hydrated ethanol or gasoline, or a mixture of both, were launched in 2003 and since then the consumption of ethanol fuel has increased. In 2011, the production of anhydrous ethanol in Brazil, added to gasoline, was 8.0 million m^3 plus 19.6 million m^3 of hydrated ethanol [1].

Compared to other fuels, the use of alcohol has several advantages for the environment, since it derives from a renewable source, quite the opposite of petroleum. Other strengths brought by alcohol are that it reduces automotive pollution, mainly carbon monoxide, sulphur oxide, toxic organic compounds such as benzene, lead compounds and it does not affect the ozone layer. In addition, the operational cost is lower and it pollutes less [2].

One of the major problems caused by the use of hydrated alcohol as fuel is its corrosive characteristics. Corrosion is the deterioration of a material due to chemical or electrochemical interaction with the environment, which can be associated with mechanical exertion or not [3]. The main causes of alcohol fuel's corrosion potential are free acidity, dissolved oxygen and the presence of ions - chloride, sulphate and metal ions - which increase the ionic strength of the solution and its loading transfer capacity [4].

The sulphate ions contained in fuel alcohol are due to pH adjustment during production, because of the addition of sulphuric acid in order to avoid the growth of undesirable microorganisms during sugar cane fermentation, which are later swept away during distillation. In addition, their presence may also be attributed to contamination during storage or transportation [5].

The sulphate ion is used by the sulphate-reducing bacteria (BRS) *Desulfovibri and Desulfotomaculum*, in which the sulphate ion acts as an electron receiver instead of an oxygen receiver. Thus, bacteria have a direct participation in the kinetic corrosion process, producing sulphide ions in solution and therefore causing the immediate precipitation of metal ions in alcohol in the form of insoluble metal sulphides, as well as the reduction of sulphate [3]. The sulphide formed by those bacteria is converted to yield elementary sulphur, an extremely iron corrosive substance which is the main component of steel [4].

The specifications for hydrated ethanol fuel are determined by the National Petroleum Agency (ANP), an administrative body responsible for quality control and inspection of automotive fuel in Brazil. The maximum content of sulphate ion allowed is 4 mg kg^{-1} [6] and the quantification must be carried out according to NBR10894 [7] or NBR12120 [8] methods.

NBR 10894[7] describes the determination of sulphate and chloride ions by ion exchange chromatography, whereby approximately 40 g of sample, with an addition of sodium carbonate, are evaporated until it is almost dry.

The remainder is dissolved in deionized water, filtered and injected in a liquid chromatograph equipped with an anionic column for chromatographic analysis. As this method requires a long time for analysis, expensive equipment and the necessary column, as well as, maintenance, it becomes unsuitable to implement as a routine method.

NBR 12120 [8] describes the volumetric determination of sulphate ion in hydrated ethanol fuel. This method uses 100.0 mL of sample to which a sodium carbonate solution is added, followed by evaporation until almost dryness. Then, hydrochloric acid, acetone, nitron and the dimethylsulfonazo III indicator are added. Finally, this solution is titrated under constant agitation with a barium perchlorate solution until the final stage where the change of color from violet to blue occurs. In order to determine the concentration of sulphate, a blank assay needs to be carried out. This method is strenuous, time-consuming and the analysis depends on the subjectivity of the analyst to determine the final stage, which becomes evident when the color of the indicator changes from violet to blue. Moreover, this change has to be detected in a turbid solution, due to the formation of solid $BaSO_4$, which demands high visual precision and impacts the reproducibility of the results.

Apart from the standard methods, the literature describes the determination of sulphate ions in fuel alcohol using capillary electrophoresis [9,10], visible spectrophotometry with dimethylsulfonazo [11], x-ray fluorescence [12], and amperometry with modified electrodes [13]. These methods require sample pretreatment, thus increasing costs and time for analysis, and are subject to contamination and loss of analytes, which make the analytical process rather difficult.

Conductometry is a very responsive and adequate analytical method to quantify ions in a solution, but lacks selectivity [14]. A way of increasing selectivity, which is essential in an analytical method, is by using a conductometric titration, where conductivity is measured as successive parts of reagent, specific for that analyte, are added to the solution, and the final point is determined graphically [14]. The main advantages of conductometric titration, in comparison with volumetric titration, are the determination of the final point even in colored or turbid solutions and that there is no need of a colored indicator, facts that prevent analyst errors during assessment. In addition, conductometric titration can be performed in a non-aqueous environment and it allows simultaneous determination of components in a mixture [14].

Conductometric titration was successfully used to determine total acidity and chloride ion concentration in hydrated ethanol fuel, using $AgNO_3$ as titrant, providing better detectivity and repeatability than the standard methodologies specified by the ANP directive [15].

Thereby, the objective of this work is the development of an analytical method to determine sulphate ions in hydrated ethanol fuel based on conductometric titration with barium perchlorate, leading to the formation of a very poorly soluble salt ($BaSO_4$, $K_{ps} = 1\ 10^{-10}$). This new method does not demand sample pretreatment and provides similar results to those obtained with NBR 12120.

2. Experimental

2.1 Equipment and Reagents

The conductometric measures were performed using Nova Técnica NT-CVM conductivity meter and of Digimed DMC-001M conductometric cell with a constant of 0.1 cm^{-1}. An automatic densimeter from Anton Paar (DMA4500) was used to determine the specific gravity of the samples. A Metrohm E274 piston burette with capacity for 20.00 mL and 0.02 mL divisions was used for titration, and sample evaporation was carried out in a Janke & Kunkel rotating evaporator, model RV0553.

The samples of hydrated ethanol fuel were collected from gas stations, stored in amber polyethylene flasks and sealed until analysis. The water to prepare the solutions was deionized, using a Millipore deionizer, Simplicity 185, and the reagents were p.a. quality.

A solution of $Ba(ClO_4)_2$ (0.000670 ± 0.000036) mol L^{-1} obtained through solubilization of salt (Merck) in deionized water and standardized with anhydrous $NaSO_4$ (Quimex) solution was used as standard solution. The dimethylsulfonazo III (DMSA III) indicator solution was prepared by solubilizing 0.076 g Na-DMSA III (Sigma – Aldrich) in 20 mL of deionized water. A saturated nitron solution (Merck) was prepared by dissolution in 5 %(v/v) acetic acid until saturation and it was later filtered. Solutions of Na_2CO_3 at 5 %(m/v), of 0.3 mol L^{-1} HCl and of 0.0002 mol L^{-1} HNO_3 were also prepared, as well as the analytical reagents propanone (Synth) and ethanol (Carlo Erba).

2.2. Experimental Procedures

2.2.1 Conductometric Titration

Parts of 100.0 mL hydrated ethanol fuel, together with 1 mL of 0.0002 mol L^{-1} HNO_3, used as supporting electrolyte, were transferred to a beaker with the conductometric cell submerged and maintained under magnetic agitation throughout the measurements. The analyte was titrated with a $Ba(ClO_4)_2$ solution with conductivity readings (k) after the addition of each increment, at intervals of 30 s for homogenization and stabilization of the measurement.

The final stage of titration was obtained by the crossing of line segments of the corrected conductivity graph (k_{cor}), due to the successive dilution of the sample during titration, as a function of the volume of standard solution added [14]. The five samples were analyzed seven times each, and the concentration of sulphate ion took into consideration volume consumption, standard solution concentration, stoichiometry of the reaction and the specific gravity of the samples, as the result must be shown in mg kg^{-1}.

2.2.2 NBR 12120 - Volumetric Titration

From the sample, 100.0 mL plus 1 mL of Na_2CO_3 at 5 %(m/v) were transferred to a distillation flask and the mixture was evaporated, in a rotary evaporator in a bath at 80 °C, until it was almost dry. While it was still hot, 0.5 mL of concentrated HCl were added and the flask was cooled down to room temperature. Two drops of nitron solution and two of the indicator solution DMSA III were added. The material contained in the flask was titrated, under constant agitation, with the same standard solution used for the conductometric analysis, until it changed from violet to blue. The complete analytical process was carried out in the same manner as in the conductometric titration.

3. Results and Discussion

The conductometric titration curves for this equilibrium (Figure 1) show two upward line segments, as the relationship conductance versus concentration is linear, and the concentration segment presents more inclination than the conductance segment due to the difference in equivalent ionic conductance ($\lambda°$) of the ions in the solution. In this way, final point of the titration can be easily detected by the crossing of the two line segments.

In the development of this method and to determine the most appropriate procedure, some parameters had to be evaluated, such as the amount of sample, standard solution concentration, supporting electrolyte concentration and reading stabilization time, taking into consideration the amount of sulphate ions allowed by law and the results obtained using the methodology proposed by NBR 12120 [8].

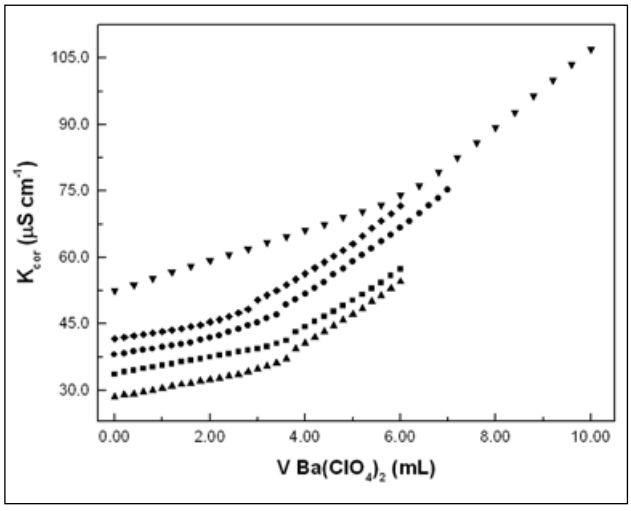

Figure 1. Conductometric titration curves obtained for different samples of hydrated ethanol fuel. (■) sample A; (●) sample B; (▲) sample C; (▼) sample D; (◆) sample E.

It was decided that the amount of sample would continue to be 100.0 mL due to the low concentration of sulphate ions in the samples, so as to obtain a larger amount of standard solution and, consequently, appropriate accuracy. This amount also meets the operational needs of conductometric titration, as the format of the conductometric cell requires a larger volume for complete immersion and more initial volume of the solution to minimize the effect of successive dilutions during titration. The use of a large amount of sample makes prior blank titration unnecessary, thus simplifying the analytical process.

To optimize the concentration of the standard solution and obtain well defined titration curves and accurate volumes, several solutions of $Ba(ClO_4)_2$ were prepared in concentrations ranging from 10^{-4} to 10^{-2} mol L^{-1}. The most appropriate concentration was 5 x 10^{-4} mol L^{-1}, because the final stage was easily detected and it was quite accurate, even in samples with lower sulphate contents.

Due to the low conductivity of the environment, approximately 200 μS m^{-1}, the conductivity readings took some time to stabilize, affecting the reproducibility of the results. Therefore, the addition of a supporting electrolyte was necessary to improve electrical conductivity and thus reduce stabilization time. Because of its solubility and the fact that it does not interfere with titration equilibrium, the addition of an HNO_3 solution was chosen as the best option. Nevertheless, due to the high equivalent ionic conductance of H^+ ion, the amount of this component has to be small, because in larger amounts of supporting electrolyte the conductivity of the solution would over increase, making the conductivity variations provided by the titration equilibrium imperceptible, as the components involved have low equivalent ionic conductance.

Additions of 1.0 mL from solutions of different concentrations of HNO_3, ranging from 10^{-5} to 10^{-3} mol L^{-1} were performed. The ideal amount was 1.0 mL from a solution of 1 x 10^{-4}, which enabled quick stabilization of readings (30 s) and easy identification of the titration final stage, with no risk of contamination nor need of blank titration.

The best conditions were used to determine sulphate ions in five samples of alcohol fuel. The corrected conductivity as a function of the titrant volume of the analyzed samples (Figure 1) showed that all samples presented well-defined and reproducible curves, providing sulphate ion concentrations with low relative deviations, average of 2.4%, as shown in Table I.

Table I. Concentration of sulphate in hydrated ethanol fuel samples, obtained using different methods, with the corresponding confidence interval of 95% and relative errors.

Sample	Conductometry (mg kg^{-1})	Relative Error (%)	NBR 12120 (mg kg^{-1})	Relative Error (%)
A	1.651 ± 0.057	3.4	1.735 ± 0.088	4.5
B	1.475 ± 0.020	1.3	1.581 ± 0.061	3.6
C	1.587 ± 0.058	3.7	1.573 ± 0.046	2.7
D	3.156 ± 0.042	1.3	3.259 ± 0.075	2.1
E	1.393 ± 0.030	2.1	1.375 ± 0.080	5.4

The t-test [16] was carried out to compare both techniques, and the t_{calc} value obtained (1.857) was lower than the tabled one (2.776), showing that both techniques are not significantly different with 95% confidence level and demonstrating the accuracy of the proposed method.

Table I shows that the results obtained using the proposed method were accurate and the repeatability had acceptable errors, mainly within the range established by law (4 mg kg^{-1}).

The determination and detection limits of both methods were calculated using ***LD = 3 x Sd*** and ***LQ = 10 x Sd*** [16], where *Sd* is the standard deviation of the sample with the lowest concentration. Therefore, the limits of detection (0.096 mg kg^{-1}) and determination (0.32 mg kg^{-1}) for the proposed method were lower than the values obtained for NBR12120 (0.24 and 0.80 mg kg^{-1}, respectively) thus showing more detectivity with the conductometric method.

4. Conclusions

The proposed method for the determination of sulphate through conductometric titration was fast, versatile, economically feasible and simple to carry out and does not require sample pretreatment. This approach may also be adopted for turbid and colored solutions and the quantification may be done in several concentration ranges of sulphate in alcohol fuel. The results obtained using conductometric titration were more accurate than those obtained following NBR12120. The values obtained for detection (0.096 mg kg^{-1}) and determination (0.32 mg kg^{-1}) limits were also low and were reached in approximately 10 minutes of analysis, as opposed to 50 minutes for the NBR12120 method.

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Point of View

Analytical chemistry: what is the profile of the professional?

The demand for chemical analysis is increasing in modern society. It is not possible, for example, to imagine that the medicine, food production, and security would be able to function without the information provided by such analyses. Ensuring the composition of drugs, the presence of vitamins, and the absence of pesticides in food are issues encountered daily by analytical chemists. When they leave the laboratory, the results of analyses form the basis of decisions that can lead from disease to health, turn a suspect into a criminal, or transform tons of food into tons of waste (the inverse of these processes is also possible). It is not possible to conceive of a modern, developed country that lacks high quality resources in analytical chemistry. The quality of an analytical result depends on the analyst, in the same way that a good photograph requires a competent photographer; it is not merely related to the value and sophistication of the equipment employed to perform the analysis. A good analyst must not only possess extensive knowledge of chemistry, but should also be able to act in a strictly ethical fashion in relation to the results of his/her work. The current dilemma in University education relates to the question: What is needed to train professionals possessing such a profile? Recent trends in analytical chemistry have demonstrated a successful transition from macro (classical) to micro (instrumental) analysis. Most often, the teaching of analytical chemistry has accompanied this trend, and classical analysis is seen as antiquated. However, instrumental analysis cannot always provide high precision and accuracy when the analyte is present at levels higher than 1% in the sample. The teaching of classical methods of analysis is being compressed in the curricula of Chemistry courses. It would not be surprising if, in the near future, there emerges a high demand in the Brazilian labor market for chemists with experience in determining, for example, the percentage composition of a ceramic material or a metal alloy. In the United States, an alert concerning the lack of analysts with this type of experience was presented by Charles M. Beck II in 1994 (*). On the other hand, how can the teaching of analytical chemistry cope with the basic theory and exponential growth of techniques required for the micro-scale measurements needed to resolve analytical problems in the modern world? Finally, it is necessary to include the development of an ethical professional attitude. The analyst must clearly understand the analytical problem, and take steps to ensure that all steps of the analytical procedure, from sampling to the final result, remain under his/her control. The analyst should be aware that the analytical result should only be used within the context of the problem under investigation, considering the uncertainties of the measurement. The task of training professionals with the quality, and in the quantity, that society needs is difficult, and there is no ready solution. An answer could lie in a bold "new" teaching model.

Arnaldo Alves Cardoso
Full Professor Analytical Chemistry Department, Chemistry Institute, UNESP

(*) C. M. Beck II, Anal. Chem.(1994) 66, 224A-238A .

View of a Brazilian Industry in Analytical Instrumentation

In recent years, there has been a growing interest devoted to the development of Automation in Analytical Instrumentation, with a prominent role with regard to products quality assurance in the environmental area, metrology, and production of reference materials.

Digimed has now more than three decades' experience in analytical instrumentation, developing equipment in conjunction with several universities for sensors nationalization. In 1990, made its first potentiometric sensor, fully developed and manufactured in-house, including the preparation of ion-sensitive glass, special ceramic, which allowed the development of other sensors, thus closing the "cycle of potentiometry". Background was obtained, so cells were developed for galvanometric measurement of dissolved oxygen, and oxidation reduction potential sensors. In 1995, manufacture of the amperometric family was started with the conductivimeters, and different types of conductivity cells of various materials. Spectrophotometers, colorimeters, flame photometers with wide application in Process and Laboratory, started in 2000. This technology was used in nephelometric equipment, thus creating a turbidimeter line for water. The sensor technology of metal rings was developed in 2005, and applied to the concept of the zeta potential of particles, allowing the development of monitor coagulant, widely used in water treatment plants (WTP).

The company holds the ISO 9001-2008 certification, and in 2007 began in the Metrology area, investing and implementing ISO17025 laboratories for Environmental Analysis, and Physical-chemical Laboratory for pH Calibration and Conductivity and, also as a reference material producer for pH solutions.

The local production of analytical instruments became possible because the Business Integration University, and the absorption by the industry of postgraduate professional to work in Research & Development Departments. Collaborative arrangements between universities and companies are starting to become deeper and broader in Brazil. A closer relationship between companies and universities, will optimize the technology development in the field of Analytical Instrumentation.

Francisco Medina
Director of Digimed Instrumentação Analitica

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