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BrJAC: In search of recognition

The main objective of a scientific journal is achieving the recognition of the community by the quality of the published works and the contributions of these works to the science and to the society, which is our final target. Although the BrJAC is a relatively new journal (3 years), it already got national and international recognition. In 2011 it was classified as B5 in QUALIS/CAPES score. Also was indexed at CAS-Chemical Abstracts Service and, recently, at Scopus. The next challenge will be the indexation in database of the SciELO and Thomson ISI. This will be a great step towards international recognition and certainly will bring new challenges.

BrJAC maintains its original proposal that is publishing articles, interviews with a senior researcher, letters and point of view. The main focus of the interview is illustrate trajectories, experiences, difficulties and achievements of senior researchers, which these stories serve as encouragement and reference to the younger. With the letter and point of view the objective is the communications and approximation between different audiences. This model adopted by the BrJAC has as aim rapprochement between two readers: the academic researchers and industrial sector (researchers and analysts who work outside of universities). The challenge, particularly for Brazil, is overcome barriers and change paradigms.

However, nothing is possible without efforts and determination. The success of BrJAC depends on the endeavor of all: editorial board, reviewers, authors and the criticism of readers. If any of this character fails in the process, the quality of the final product may be compromised. The publishers are struggling to reduce the total time for the manuscript processing. For this, it is essential the efforts of all editors, reviewers and authors. Additionally, we need more manuscript submissions, with good ideas and quality. This is an important way to build the BrJAC with the quality that we want.

Pedro Vitoriano de Oliveira
Editor

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Summary

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Letter

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

Scientific production in the field of Analytical Chemistry in Brazil from 2002 to 2012

Brazilian researchers in the field of Analytical Chemistry have published their research results (218 publications), such as Article (181) Review (32) Paper and Proceedings (24) in major national and international journals, mainly in the Química Nova (28%), Talanta (11%), Journal of the Brazilian Chemistry Society (8%), Analytica Chimica Acta (5%), Microchemical Journal (5%), Spectrochimica part B Atomic Spectroscopy (3%), Analytical and Bioanalytical Chemistry (2%) and Electroanalysis (2%). The Web of Science Categories shows the following classification of articles published in the last ten years: Multidisciplinary Chemistry (39%), Analytical Chemical (34%), Spectroscopy (8%) and Biochemical Research Methods (4%). Among the subject areas, those that stand out are Chemistry (80%), Spectroscopy (8%), Biochemistry and Molecular Biology (4%) and Electrochemistry (3%). The Institutions that have most published are the University of São Paulo (34%) and the State University of Campinas (20%) followed by the Federal University of São Carlos (11%), Federal University of Bahia (7%) and the Fluminense Federal University (6%). The main Funding Agencies are CNPq (32), FAPESP (20), CAPES (14) and FAPERJ (4). The main countries that cooperate with researchers in Brazil are Spain (12), Germany (8), USA (6) Canada (5), Italy (5) and England (3). Around the world in the same ten-year period 6174 articles were published (Brazil occupies the 11th place, with 3.5% of those articles), and within the web of Science categories the amount of publications is greater within the field of Analytical Chemistry (1837 articles), in relation to Multidisciplinary Chemistry (with 1064 articles). Thus, we believe that, with the arrival of the Brazilian Journal of Analytical Chemistry (BrJAC), the number of both Brazilian and foreign publications will increase, with researchers motivated by the current international prestige shown by Brazil. We also believe that many analytical chemistry researchers who currently publish in the Química Nova and the Journal of the Brazilian Chemistry Society should migrate to BrJAC. I take this opportunity to say that the creation of the National Meeting on Analytical Chemistry (ENQA) and the Brazilian Symposium of Electrochemistry and Electroanalysis (SIBEE), in the early 80's were instrumental in the development of Analytical Chemistry in Brazil. Many of the studies that have been developed only in the major Brazilian centers are now beginning to be disseminated in various corners of Brazil. Special thanks go to researchers Fritz Feigl, Leopoldo Hainberger and Paschoal Senise, as pioneers in the development of Analytical Chemistry in Brazil.

Prof. Dr. Pércio Augusto Mardini Farias
Ex-director of Chemistry Department of the Pontifical Catholic University of Rio de Janeiro
President of the VII National Meeting on Analytical Chemistry

Interview

Roy Edward Bruns is an American chemist who came to Brazil in the 70s to work as a researcher and professor in Universidade Estadual de Campinas (Unicamp), and became the pioneer in chemometrics in our country. In this exciting field of analytical chemistry, Bruns and his team search for mathematical models to predict the activity of substances and to identify metabolite fingerprints. They started at a time when computers were enormous structures occupying entire rooms, and when laboratory instruments did not have an interface with computers. However, in a typical Brazilian way, he was able to overcome difficulties with little resources, and started a highly productive career, with more than 200 publications and 50 post-graduation students supervised.

Professor Bruns has talked with BRJAC about his studies, the history of chemometrics in Brazil, the development of the field since then and the newest mathematical tools, created in the last years. Bruns also explains the role of analytical chemists in the development of research models in the future and shows that there is a large field to explore in analytical chemistry research.

In times of "exportation" of Brazilian scientists to other countries, please, kill our readers' curiosity: what brought you to Brazil? What is the history of your coming to our country and establishing here as a distinguished scientist?

I came to Brazil and started working in the Chemistry Institute of Unicamp (Universidade Estadual de Campinas) in June of 1971. I had several offers for work in the US but not one from a good university where I could form my own research group. In fact I had a NASA fellowship from 1963-1967 to pursue my PhD in chemistry. The US government had created several thousand of these grants to replace scientists and engineers that NASA had hired and removed from the private sector to win the race to the moon during the cold war. By the time I started looking for a job after two years of post-doctoral studies the US had landed on the moon and started phasing out the space program. So I went into the job market at just the same time many ex-NASA scientists and engineers were looking for work. Indeed the market was flooded. I saw an advertisement in "Chemical and Engineering News" for professors in all areas of chemistry at Unicamp and sent my Curriculum Vitae. Needless to say I was excited about coming to Brazil but somewhat doubtful about how this experience would work out.

What is chemometrics and how did you start to work in this area?

Chemometrics is often defined as the use of multivariate methods of mathematics and statistics to optimize the measurement of more reliable data and to extract a maximum amount of useful information from these data. It is a very powerful tool for applications in almost all areas of chemistry and not only analytical chemistry.

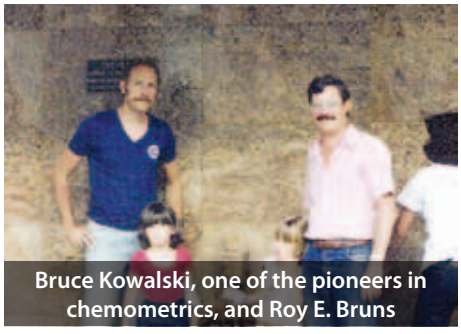
“I went to an American Chemical Society meeting in Chicago in 1976 and ran into a colleague and friend, Bill Dunn. He mentioned that perhaps chemometrics might have a solution to my problem. I had never heard of chemometrics before. In fact the word first started to be used in the early 70’s. Remember the internet did not exist at that time. Bill’s advice was very good.”

Roy E. Bruns with Patricia Kaori Soares, Benício de Barros Neto and Ieda Scarminio

My original and still active research field is the use of quantum chemical techniques to solve problems in molecular spectroscopy. Principally our effort is concentrated on trying to extract information from absolute infrared intensities about electronic structural changes that occur in molecules when they vibrate. So we try to understand why some infrared spectral bands are strong and others are weak. In other words we attempt to model and predict extinctions coefficients of infrared spectral transitions that have important applications in organic qualitative analysis and now, with the use of chemometrics, in quantitative analysis.

In 1975 I was collaborating with Richard Brown and Alfredo Simas on a project that today would be classified as QSAR, Quantitative Structure Activity Relationships. The idea was to use the existing quantum mechanical methods to model and predict the biological activities of members of the chloramphenicol family of drugs used to treat typhoid among other diseases. However it soon became evident to me that we could calculate an extremely large number of theoretical descriptors and by trying hard enough you will find a model that seems to be correct but in the end is useless for designing new drugs. We needed a method of variable reduction before this kind of research would become meaningful. I went to an American Chemical Society meeting in Chicago in 1976 and ran into a colleague and friend, Bill Dunn. He mentioned that perhaps chemometrics might have a solution to my problem. I had never heard of chemometrics before. In fact the word first started to be used in the early 70’s. Remember the internet did not exist at that time. Bill’s advice was very good. Soon I found that principal component analysis could help solve the variable reduction program

and started reading about chemometrics on my return to Brazil. I found that principal component analysis (PCA) consists of a matrix diagonalization technique. I became encouraged about understanding PCA since quantum chemical methods diagonalize an energy matrix. PCA diagonalizes a covariance matrix so instead of combining atomic orbitals into molecular orbitals, which I was used to, I just needed to combine individual chemical measurements or parameters into principal components. It was really the same thing except that the application was different.



Bruce Kowalski, one of the pioneers in chemometrics, and Roy E. Bruns

Why did chemometrics only start to develop in the 1970's? Could chemometrics develop without computers? As a pioneer in chemometric analysis here, can you please tell us a little about the history of this line of research in Brazil?

The ideas upon which many chemometric methods are based were conceived long ago. For example the idea of principal components was first introduced by Pearson in 1901. I soon realized that PCA could only be carried out using computers. At that time microcomputers were starting to emerge in the developed world but were inexistent in Brazil. We all used main frame computers and Unicamp had one of the better ones in Brazil in the late seventies. It had a working memory of 64 kbytes and storage memory of 1 megabyte that was shared by the whole university community. At that time I had become familiar with the work of Bruce Kowalski and his chemometric's group at the University of Washington. One of his students, David Deuwer, had written an extensive FORTRAN program containing chemometric methods that ran on main frame computers. I wrote a letter to Prof. Kowalski introducing myself and asking for a copy of the program. About six weeks later I receive a notice from the post office that a computer tape had arrived. After some haggling with the post office over importation of computer tapes I finally was able to take the tape to the computer center of Unicamp. I had worked with FORTRAN computer programs since 1964, so soon we had the program up and running. The only problem was that the program operated with more than the allowed memory during the day so we were restricted to doing calculations between midnight and 6:00 in the morning. But that was a start.

Then I needed a student to develop a project in chemometrics. I had several students working in theoretical chemistry but I needed a student that was willing to do experimental work but not afraid to tackle computer problems. With the help of my graduate student Mozart Ramos we convinced Ieda Scarminio to accept the challenge. She started her master's work on the chemometric classification of mineral waters in 1979 and defended her dissertation in 1981.

“I soon realized that PCA could only be carried out using computers. At that time microcomputers were starting to emerge in the developed world but were inexistent in Brazil. We all used main frame computers and Unicamp had one of the better ones in Brazil in the late seventies. It had a working memory of 64 kbytes and storage memory of 1 megabyte that was shared by the whole university community.”

“In the 90’s small commercial analytical instruments interfaced with microcomputers started invading most analytical laboratories. Of course this not only changed the way of solving lots of analytical problems but also the attitude of many analytical chemists towards chemometrics.”

The analytical group of CENA headed by Henrique Bergamin in Piracicaba helped us with the experimental work and soon they became enthusiastic about the potential of chemometrics. Along the way Ieda became adept in FORTRAN programming and was soon to become involved in converting the main frame program to microcomputers that were slowly starting to be sold in Brazil.

During this time we were very fortunate to receive the visit of Bruce Kowalski who taught the first short but intensive course on chemometrics in Brazil. Bruce stayed with us for one month and helped in Ieda’s project and the collaborations we had with CENA. The support of our chemometric endeavors from the analytical group at CENA, specially from Elias Zagatto and Antonio Jacintho, was very important. At that time few analytical groups in Brazil and abroad were enthusiastic about chemometrics and microcomputers were not common in the chemical laboratory. With the introduction of microcomputers, the usefulness of chemometrics for solving problems in chemistry soon became recognized.

In the last decades, there was a great increase in technology development and, therefore, computer programming (microcomputer applications). What is the importance of this in chemometrics?

The fact that modern analytical instruments have digital output makes computer-based applications feasible. In the 70’s only large analytical instruments like mass spectrometers and nuclear magnetic ressonance spectrometers were attached to equally spacious computers. In the early 80’s most inexpensive analytical instruments were not interfaced with bench top microcomputers so the bottleneck was transferring data from instrument to computer. One of the earliest efforts to solve this problem was tackled by two graduate students at Unicamp, Celio Pasquini and Mario Cesar Ugulino de Araujo. They interfaced a flame photometer in an analytical laboratory to my 8-bit microcomputer using more than 50 meters of telephone cable. So output from the photometer was sent to the microcomputer that contained a Generalized Standard Addition Method program on a 32 kbyte 8-inch floppy disk. Analytical signals were then transformed to determine calibration models and analytical results.

In the 90’s small commercial analytical instruments interfaced with microcomputers started invading most analytical laboratories. Of course this not only changed the way of solving lots of analytical problems but also the attitude of many analytical chemists towards chemometrics.

Then emphasis in chemometrics turned to microcomputer program development. Very few commercial programs were user friendly and dedicated to microcomputers. In fact our first book on the subject, “Planejamento and Otimização de Experimentos” written in collaboration with Benicio de Barros Neto and Ieda Scarminio, contained a 5¼ inch floppy disk with the chemometric computer progams Ieda had either

written or extracted from the main frame program. The last chapter of the book contained instructions about how to use these programs on the DOS platform. These programs became obsolete with the commercial release of Windows-based programs such as Statistica, Minitab and Design Expert.

It seems that chemistry researchers in the 80's and 90's had to have some talent for problem solving in the area of IT. This was common in other areas, and there are many stories of engineers and physicians spending nights on FORTRAN problems. Has the "Windows" environment changed the way chemists search for solutions in computers? Are the solutions "ready to use" nowadays? What is the profile of young chemists compared to the older researchers?

One of the most famous stories about engineers spending many nights on software problems comes from the Apollo program of NASA. NASA contracted laboratories at MIT to develop software to interface with the gyroscopic guidance system. Development of this computer guidance system was the first contract signed in the Apollo program since it was considered the most difficult part of the whole moon landing effort. This system was the only control for guiding the space capsule when it was on the dark side of the moon. There was an unusally high divorce rate among the engineers working on the software owing, in part, to the tremendous work load thrust upon them.

Most computer programmers in chemistry work on the Linux platform although Windows has a DOS simulator permitting programming in FORTRAN. The biggest problem facing the programmer used to be how to write a program that was efficient at conserving space and saving computing time. This problem was fundamental in getting the first molecular orbital program working in Brazil. The program had a matrix with 10,000 numbers and did not fit in the computer memory so the program could not be compiled and used for computing. Realizing that about 95% of the numbers were zero I was able to replace the matrix with a 500 element vector and their matrix indices reducing the required storage space so the program could be compiled and run. Nowadays the problems of computer time and data storage are not so critical as in those days.

What are the newest mathematical tools being developed in this field? You have published an interesting article on this subject in 2006: has something changed in the last 7 years?

Chemometrics has been active in applying multivariate mathematical and statistical methods that were originally introduced in the statistical or engineering fields. Furthermore chemometricians have developed their own methods. The most applied perhaps is the SIMCA classification method invented by Svante Wold in the 70's. In recent years chemometricians are active in writing computer codes for adapting and developing methods for multivariate curve resolution, imaging analysis and multiway methods.

"In recent years I have become especially interested in multicriteria decision making and have been testing and developing computer applications in this area. Normally, changes in the experimental working conditions that improve some characteristics of a system have adverse effects on others. How can one make a decision on what are the best experimental conditions to approximate a desired result? Computer-based methods seem to be the best way to make this decision."

> **Organization and the governmental agencies of many countries recommend metabolite fingerprinting as a technique of quality control.**

The older PCA method treats a data matrix (two-dimensional data), but now the method development is actively treating data cubes (three-dimensional data or tensors) and hypercubes (four and more dimensional data).

The interaction of chemistry and physics is clear in your publications. However, it is interesting to see that there is also an interaction between chemistry and health sciences investigations, especially nutrition (chromatography of foods, olive oil electrophoresis etc.). Is this a promising new working field for young investigators or is it instead an old path?

My research emphasis has always focused on the modeling of molecular electronic structures for understanding spectroscopic phenomena. Five out of my seven graduate students are currently working in this area. But they also use chemometric techniques to solve problems. Chemometric methods are useful in all branches of science and engineering. We are working on a charge-charge flux-dipole flux model for calculating and understanding infrared intensity results. Molecular vibrations are multidimensional problems and the treatment of intensity parameters derived from this model involves dimensionality reduction and pattern recognition. My work on the chromatography of pesticide mixtures, electrophoresis of olive oil and spectroscopic and chromatographic analysis of phytotherapeutics are all done in conjunction with excellent research groups that are developing research in these areas. My main interest in chemometrics is experimental design and optimization often called DOE. All these research problems require the optimization of a large number of characteristics of the systems being studied. In recent years I have become especially interested in multicriteria decision making and have been testing and developing computer applications in this area. Normally, changes in the experimental working conditions that improve some characteristics of a system have adverse effects on others. How can one make a decision on what are the best experimental conditions to approximate a desired result? Computer-based methods seem to be the best way to make this decision.

What is metabolite fingerprinting and why is it important?

I became involved in metabolite fingerprinting interacting with the research group of Ieda Scarminio at the State University of Londrina. She has studied this subject for quite some time and has been actively publishing experimental results in this field. Secondary metabolites are not involved in normal plant growth, development and reproduction but often have important ecological or long-term functions. They can be characteristic of certain plant species. As such a chromatogram or spectrum or combination of these can be used to find identifying characteristics of metabolites enabling a chemical identification of a plant species. The determination of these fingerprints is important for the quality control of phytoterapeutics, teas and other natural products.

One of the problems with the research on the therapeutic effects of plants is to find the exact substance involved in the relief of symptoms or therapeutic effect. Do you believe that metabolite fingerprinting can help in the research of phytotherapeutics?

The metabolite fingerprint contains identifying features showing the existence of the active substance or substances in the plant extract. Pata de vaca (genus Bauhinia) has several species with similar leafs, flowers and stem appearances but only one is rich in active ingredients against diabetes melitus. The fingerprint guarantees that the Bauhinia being marketed indeed contains significant quantities of active ingredients. So fingerprints are very important for quality control. In fact the World Health Organization and the governmental agencies of many countries recommend metabolite fingerprinting as a technique of quality control.

What is your vision of the future of chemometric analysis? As Sir Maddox would ask: what remains to be discovered?

Such predictions are normally based on linear models and only hold for the very near future. I would expect to see more activity applying metrology to chemometric methods of analysis. This will be important as these methods tend to substitute more costly and time-consuming methods that are now regarded as standard ones. Image analysis will be extended to film analysis to study dynamic processes such as annealing. In general interest will change to study characteristics of time segments rather than of only one instant of it. As such the treatment of multiway data will become more important in analytical chemistry. I do expect increased activity of development of analytical techniques for natural products. The analytical chemist views this area differently than the organic chemist and has a lot to contribute. In experimental design I think multicriteria decision making will become more important in science and engineering.

What is your vision of analytical chemistry research and work field today?

I did my undergraduate, graduate and post-doctoral work in physical chemistry in the United States. At that time work in analytical chemistry was seen to be a subdivision of research in either physical or organic chemistry. This was not the case in Brazil where analytical chemistry has been treated as a co-equal to the areas of inorganic, organic and physical chemistry. In fact analytical chemistry has flourished here, and in some institutions it has more graduate students than the other areas of chemistry. I do feel that analytical chemistry should take on a more general outlook as an information science. In other words it should not only be concerned with method development and substance determination but also with why their results are the way they are. This is being done to some extent by the environmental analytical chemists owing to the immediacy of such questions like climate change. However it should be done in a more general scientific context.

“Image analysis will be extended to film analysis to study dynamic processes such as annealing. In general interest will change to study characteristics of time segments rather than of only one instant of it.”

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Spectrophotometric determination of acetazolamide using a flow injection system with $KMnO_4$ reagent

Maria G. J. Almeida, Marcos F. S. Teixeira, Homero Marques Gomes*
Department of Physics, Chemistry and Biology – Faculty of Science and Technology – University of the State of São Paulo (UNESP) - Presidente Prudente - Brazil

Abstract
A flow injection spectrophotometric method was developed for the determination of acetazolamide, (N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)acetamide), in commercial anti-hypertensive drugs. The new and simple procedure is based on the reaction between sulfonamide groups and permanganate ions in an alkaline medium. The results showed that linear quantification of acetazolamide could be achieved at two wavelengths. A decrease of the permanganate concentration could be measured at 530 nm, while an increase in manganate concentration could be measured at 610 nm, in the concentration ranges from 1.0 x 10^{-5} to 2.5 x 10^{-4} mol L^{-1} and from 2.5 x 10^{-5} to 2.5 x 10^{-4} mol L^{-1}, respectively. The detection limits were 4.5 x 10^{-6} mol L^{-1} (530 nm) and 2.2 x 10^{-6} mol L^{-1} (610 nm). The usual excipients used in commercial drugs did not interfere in the determinations, and the results were consistent with those obtained by a UV reference technique.

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Keywords: Acetazolamide, permanganate, flow injection, spectrophotometric determination.

1. Introduction
The sulfonamide group (-SO_2NH-) is present in many biologically active compounds, including diuretics, antibiotics, insulin-like agents, anti-thyroid hormone agents, antitumoral drugs, and a wide range of other pharmaceuticals [1].

Diuretics are substances that increase the flow of urine, at the same time increasing excretion of sodium and potassium by direct action on the kidneys. This action normally occurs by blocking the absorption of electrolytes associated with water molecules, hence eliminating them from the organism [2-5]. There are two categories of diuretics: those that act directly on the tubules, and those that modify the composition of the filtrate. The first category can be subdivided into four classes. The sulfonamides act on the loop of Henle, while the thiazides, potassium sparers, and carbonic anhydrase inhibitors act on the distal tubules. Diuretics whose action modifies the filtrate, such as mannitol, dilute the urine and increase the volume excreted. Although the different classes of compounds have the same main function, they show distinct mechanisms of action and collateral effects, and are used for different purposes in clinical practice.

Acetazolamide (N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl) acetamide – Figure 1) is a carbonic anhydrase inhibitor that converts bicarbonate present in the body into carbon dioxide, which is more easily reabsorbed. The inhibition results in increased excretion of bicarbonate, and greater elimination of the associated water. Prolonged ingestion of high doses of this type of drug should be avoided, due to the effect on the body's reserves of bicarbonate; for this reason, the use of acetazolamide is now mainly restricted to the treatment of glaucoma, and as an anti-convulsive agent, rather than as a diuretic [1].

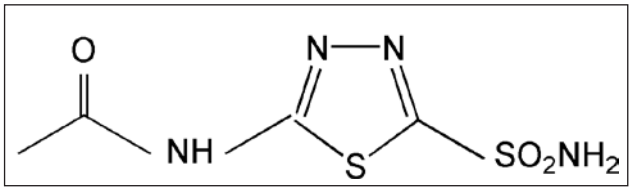


Figure 1. Structural formula of acetazolamide.

In sport, acetazolamide is considered a doping agent, used to enhance athletic performance, and it is therefore prohibited. Since it increases the volume of urine, it reduces (by dilution) the concentrations of other components of the urine, and makes it more difficult to detect prohibited substances [6-8]. Athletes consuming this class of pharmaceutical can experience side effects including dehydration, arrythmia, renal insufficiency, and hypotension. The substance can remain active for several hours, hindering rehydration and potentially leading to further damaging health effects. For this reason, consumption of acetazolamide is banned by the International Olympic Committee (IOC) and the Brazilian National Anti-doping Council (CNAD) [6-8].

There are few techniques for the determination of acetazolamide described in the literature, with chromatographic methods being the most common [9-11]. For ex-

ample, the European Pharmacopoeia [12] recommends liquid chromatography for quantitative analysis of diuretics in pharmaceutical products. However, many of these procedures are expensive, while others are laborious or require specially trained analysts due to the sophisticated instrumentation and complex sample manipulation involved. Another important technique for the indirect determination of acetazolamide is reverse iodometric titration. In this procedure, the sample is reacted with an excess of pyridinium chlorochromate (PCC) in an acidic medium, and the remaining reagent is treated with iodide and titrated to derive the concentration of the drug [13]. The disadvantages of this technique include lengthy analysis times, and the need to dispose of the waste generated (which contains Cr^{3+}). Vladescu and coworkers [14] measured acetazolamide by UV-visible spectrophotometry, employing acid-base equilibrium and knowledge of the pK_a values of the substance. The method was based on analysis of the spectrum obtained for acetazolamide as a function of the pK_a value, and enabled direct determination of the drug in aqueous solution.

In the context of flow injection methods, Fogg and Ghawji [15] studied the determination nitrofurantoin and acetazolamide by amperometric detection with a mercury drop electrode. The acetazolamide was determined in the range 10-70 μg mL^{-1} at -0.85 V vs. SCE in hydrochloric acid medium. The procedure was applied with good accuracy and precision to the determination of the drugs in tablet formulations.

An optical biosensor for the determination of acetazolamide in a flow-through procedure was proposed by Jeronimo *et al*. [16]. This work, the optical biosensor was based on the immobilization of the carbonic anhydrase and used a pH indicator dye. Its application was for the determination of the anti-glaucoma agent acetazolamide by enzyme inhibition measurements. The sensor was integrated in a flow cell and coupled to a continuous flow system operating on a multicommutation and binary sampling approach. Linear response was obtained for acetazolamide concentrations between 1.0 and 10.0 mmol L^{-1}, with a sampling frequency of 22 samples h^{-1} and a detection limit of 0.2 mmol L^{-1}.

Recently, Arruda and collaborators [17] studied a molecularly imprinted polymer for selective catechol extraction followed by its spectrophotometric determination in a flow system. Permanganate solution in acid medium was used as spectrophotometric reagent for catechol determination

In the present work, a flow injection spectrophotometric method was developed for the determination of acetazolamide in pharmaceutical formulations. The procedure involved oxidation of the drug by potassium permanganate ($KMnO_4$) in a basic medium, forming the manganate ion (MnO_4^{2-}). Acetazolamide was then quantified using the reduction in the concentration of permanganate (violet color), measured by absorbance at 530 nm, or by the increase in absorbance at 610 nm caused by the formation of manganate ions (green color).

2. Experimental

2.1. Equipment

The flow injection system consisted of a peristaltic pump (Ismatec IPC4) fitted with different diameter Tygon tubes, 0.8 mm i.d. polypropylene connecting tubes and a T-type acrylic connector. Sample and reference solutions were inserted into the flow system with the aid of a three-piece manual injector-commutator containing two fixed bars and a sliding central bar [18].

A Femto 600 S single beam spectrophotometer was fitted with a 1.0 cm optical path length cell with a volume of 180 μL. Data were collected using a microcomputer running FemWin 670 (version 1.2) software. The comparative technique utilized a UV-visible spectrophotometer (Shimadzu UV 1650), controlled using UV Probe (version 2.21) software.

2.2. Reagents

A 0.1 mol L^{-1} solution of sodium hydroxide was prepared by dissolving 4.0 g of analytical grade NaOH (Aldrich) in approximately 100 mL of recently-boiled deionized water, and diluting to 1000 mL with the same solvent in a volumetric flask.

A standard solution of acetazolamide (1.0×10^{-2} mol L^{-1}) was prepared by dissolving 0.2222 g of the pure compound (obtained from Aldrich) in 0.1 mol L^{-1} NaOH solution to give a total volume of 100 mL. This solution was stable for at least one week at ambient temperature. The standard solution used to generate the analytical curve (in the concentration range 1.0×10^{-5} – 5.0×10^{-3} mol L^{-1}) was prepared by diluting the standard solution with 0.1 mol L^{-1} NaOH.

A stock solution of 0.1 mol L^{-1} potassium permanganate was prepared by dissolving 0.79 g of $KMnO_4$ (Aldrich) in 0.1 mol L^{-1} NaOH, in a 50 mL volumetric flask. Appropriate dilutions were then made using the same solvent.

2.3. Sample preparation

Tablets containing acetazolamide (DIAMOX®, Gemon) were ground to produce a fine powder. Precise quantities of the material were then weighed using an analytical balance, transferred to beakers, and dissolved in 0.1 mol L^{-1} NaOH. The resulting solutions were transferred to 50 mL volumetric flasks, the volumes were made up with the same solvent, and the flask contents were thoroughly mixed. Traces of insoluble excipient were removed by simple filtration, and the filtrates were transferred to 50 mL plastic flasks. Subsequent dilutions were used to obtain theoretical acetazolamide concentrations of 9.0×10^{-5} mol L^{-1} and 5.0×10^{-5} mol L^{-1}, for analysis at the wavelengths of 530 nm and 610 nm, respectively. Quantification of acetazolamide in the sample was achieved using the multiple standard additions method.

2.4. Flow injection analysis system

A simple flow injection system was constructed for the quantification of acetazolamide, as shown in Figure 2.

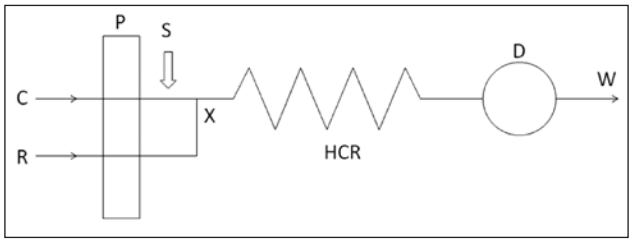


Figure 2. Schematic representation of the flow injection system employed for determination of acetazolamide. C: carrier solution (deionized water); R: oxidant ($KMnO_4$, 2.0 x 10^{-3} mol L^{-1}); P: peristaltic pump; S: sample (acetazolamide solution); X: confluence point; HCR: helical coil reactor; D: detector (spectrophotometer) (λ = 530 and 610 nm); W: waste.

When the valve was switched to the injection position, the sample (S) was transferred to the carrier solution (deionized water). At the point of confluence (X), the analyte was mixed with the $KMnO_4$ reagent (R), with the reaction taking place in the coil (HCR), where the amino sulfonyl groups reacted with the permanganate ions. This caused a reduction in intensity of the violet color, due to a lower concentration of permanganate ions, which consequently reduced the absorbance at 530 nm. This reduction in intensity was proportional to the concentration of acetazolamide present in the solution.

Acetazolamide could also be quantified by the increased intensity of the green color in the medium, measured at 610 nm, caused by the increased concentration of the manganate ion (MnO_4^{2-}) produced by reduction of the permanganate ion.

3. Results and Discussion

3.1. Preliminary tests

Deionized water and 0.1 mol L^{-1} NaOH were tested as carriers, using the two different absorbance wavelengths (530 and 610 nm), maintaining the acetazolamide concentration fixed at 5.0 x 10^{-3} mol L^{-1}, and varying the permanganate concentration between 1.0 x 10^{-4} and 4.0 x 10^{-3} mol L^{-1}. The sample injection volume was 410 µL, the total solution flow rate was 6 mL min^{-1}, and the length of the reaction coil was 130 cm.

Both carrier media were found to be satisfactory, with good repeatability and stability of the signals obtained at the two wavelengths. Deionized water was therefore employed in the subsequent experiments to economize reagent consumption. The absorbance intensity was lower at 610 nm than at 530 nm, as expected since the molar absorption coefficient of the manganate ion is lower than that of the permanganate ion.

3.2. Influence of $KMnO_4$ concentration

The absorbances obtained using an acetazolamide concentration of 5.0 x 10^{-3} mol L^{-1} increased with $KMnO_4$ concentration in the range 5.0 x 10^{-4} - 4.0 x 10^{-3} mol L^{-1}, at both wavelengths (see Figure 3). A $KMnO_4$ concentration of 2.0 x 10^{-3} mol L^{-1} was therefore used subsequently, since it produced a good analytical signal and required a modest amount of the reagent. The medium used ensured provision of the alkaline conditions required for reduction of the permanganate ion by the sulfonamide group.

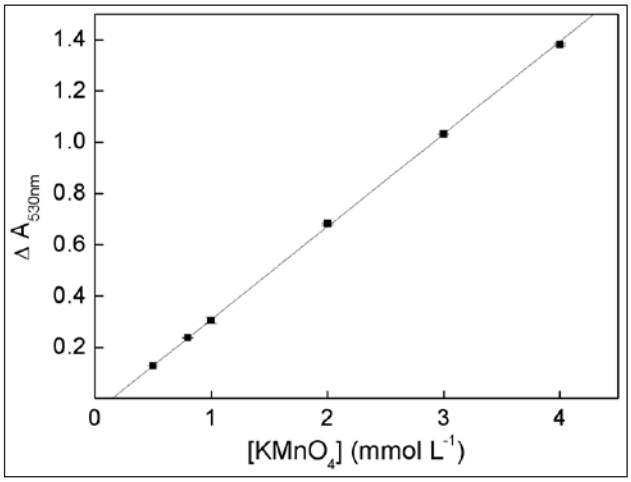


Figure 3. The effect of the potassium permanganate concentration on the analytical signal (n = 3) at λ = 530 nm. Carrier solution: deionized water; acetazolamide concentration: 5.0 x 10^{-3} mol L^{-1} in 0.1 mol L^{-1} NaOH; $KMnO_4$ solution: 5.0 x 10^{-4} - 4.0 x 10^{-3} mol L^{-1} in 0.1 mol L^{-1} NaOH; total solution flow rate: 3.0 mL min^{-1}; sample volume: 410 µL; reaction coil length: 130 cm. The difference in absorbance (Δ A_{530}) was obtained between the analytical signal in the absence and presence of acetazolamide.

3.3. Optimization of chemical parameters

The influence of the concentration of the NaOH solution used to prepare the $KMnO_4$ solution, from 0.09 to 0.3 mol L^{-1}, was studied at both 530 and 610 nm. Constant values were used for the $KMnO_4$ concentration (2.0 x 10^{-3} mol L^{-1}), the acetazolamide concentration (5.0 x 10^{-3} mol L^{-1} in 0.1 mol L^{-1} NaOH), and the total flow rate (4.5 mL min^{-1}), as well as the reaction coil length (130 cm). The absorbances obtained at the different NaOH concentrations lay within a narrow range (0.535 – 0.590); hence the NaOH concentration was maintained unchanged at 0.1 mol L^{-1}. The results were equivalent at both wavelengths (although the absorbances obtained at 610 nm were smaller).

3.4. Optimization of flow injection system parameters

In order to ensure that acetazolamide was the limiting reagent, a parallel experiment (in batch mode) was performed to confirm that the drug concentrations used were compatible with the $KMnO_4$ concentration. Under the flow system conditions employed, a suitable concentration of acetazolamide was 2.5 x 10^{-4} mol L^{-1}, with an injection volume of 572 µL.

In flow injection systems, the solution flow rate exerts

a direct influence on the intensity of the analytical signal. Here, the analytical signal decreased according to total flow rate (in the range 1.5 - 7.5 mL min^{-1}) because the time elapsed between the points of reagent mixing and the arrival at the detector became insufficient to allow the reaction to reach completion. This effect could be compensated by increasing the length of the reaction coil, hence increasing the time taken for the mixture of reactants to reach the detector. However, a longer reaction coil also increased sample dispersion, nullifying the desired effect. The best compromise between absorbance signal and speed of analysis was obtained at a flow rate of 3.0 mL min^{-1}.

At both wavelengths, the analytical signal depended on the sample injection volume (see Figure 4). All other parameters were maintained constant, and the injection volume was varied between 123 and 980 µL. The signal progressively increased up to 817 µL, after which it stabilized. A volume of 570 µL was chosen, representing the best compromise between detectivity, sampling frequency, and reagent consumption.

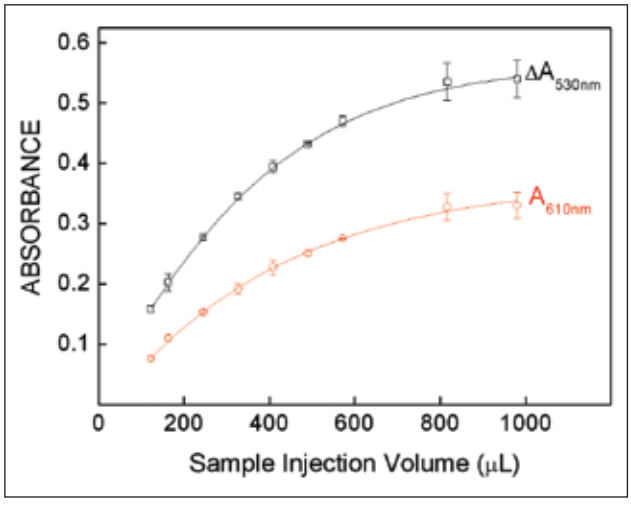


Figure 4. Effect of the sample injection volume on the analytical signal (n = 3) for 2.5 x 10^{-4} mol L^{-1} acetazolamide in 0.1 mol L^{-1} NaOH. Carrier solution: deionized water; $KMnO_4$ solution: 2.0 x 10^{-3} mol L^{-1} in 0.1 mol L^{-1} NaOH; total solution flow rate: 3.0 mL min^{-1}; reaction coil length: 130 cm.

The influence of the reaction coil length was evaluated at 530 nm, using lengths between 15 and 130 cm, with the remaining chemical and physical parameters fixed at the values selected previously. The analytical signal increased progressively with coil length up to 100 cm, reflecting the kinetics of the reaction between acetazolamide and permanganate. At coil lengths exceeding 100 cm, the signal diminished because the influence of sample dispersion was greater than the kinetic effect. When the analytical signal was measured at 610 nm (absorbance of the manganate ion), the best coil length was 80 cm, with a decrease of the signal when the length was further increased, probably because the influence of the sample dispersion zone was more significant than any possible enhancement of manganate production.

3.5. Analytical curve

Fig. 5A illustrates the FI absorbance/time response for different acetazolamide concentrations. At 530 nm, the analytical curve (Figure 5B) was linear in the acetazolamide concentration range 1.0 x 10^{-5} – 2.5 x 10^{-4} mol L^{-1} (ΔA_{530nm} = 0.107 + 669.99 [ACT (mol L^{-1})]; r = 0.9979, where A is the absorbance value), with a detection limit of 4.5 x 10^{-6} mol L^{-1} (3σ/slope).

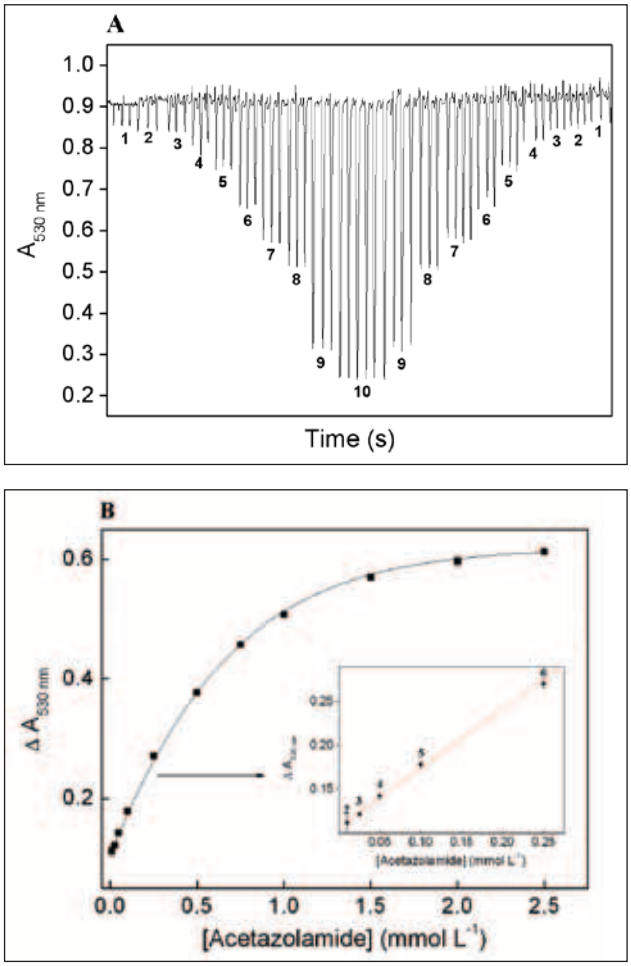


Figure 5. (A) Transient absorbance signals obtained in triplicate for acetazolamide concentrations: 1 – 10 = 5.0 x 10^{-5} - 2.5 x 10^{-3} mol L^{-1}. (B) Analytical curve for acetazolamide (n=6). The insert shows a magnification of the linear concentration range: 2 – 1.0 x 10^{-5}, 3 – 2.5 x 10^{-5}, 4 – 5.0 x 10^{-5}, 5 – 1.0 x 10^{-4}, 6 – 2.5 x 10^{-4} mol L^{-1}. $KMnO_4$ solution: 2.0 x 10^{-3} mol L^{-1} in 0.1 mol L^{-1} NaOH; total solution flow rate: 3.0 mL min^{-1}; sample volume: 571.9 µL; reaction coil length: 100 cm; λ = 530 nm.

The same experiment was conducted using a detection wavelength of 610 nm, for which the analytical curve was linear in the acetazolamide concentration range 2.5 x 10^{-5} – 2.5 x 10^{-4} mol L^{-1} (A_{610nm} = 0.007 + 658.92 [ACT (mol L^{-1})]; r = 0.9988), with a detection limit of 2.2 x 10^{-5} mol L^{-1} (3σ/slope).

3.6. Study of interferences

Evaluation was made of any interference from the excipients present in the pharmaceutical formulation used for acetazolamide determination. The analytical signal obtained for a solution containing 1.0 x 10^{-4} mol L^{-1} of acetazolamide was compared with solutions containing acetazolamide and a five-fold higher concentration of the excipients (see Table I). There was no significant difference between the analytical signals, indicating that there was no interference.

Recoveries of 96.3 - 106 % of acetazolamide from pharmaceutical formulations (n=3) were obtained using the flow-injection procedure. In this study, 1.0 x 10^{-5}; 3.0 x 10^{-5} and 5.0 x 10^{-5} mol L^{-1} of acetazolamide were added to the sample. The recovery results obtained suggest the absence of a matrix effect in the determination of acetazolamide in this sample.

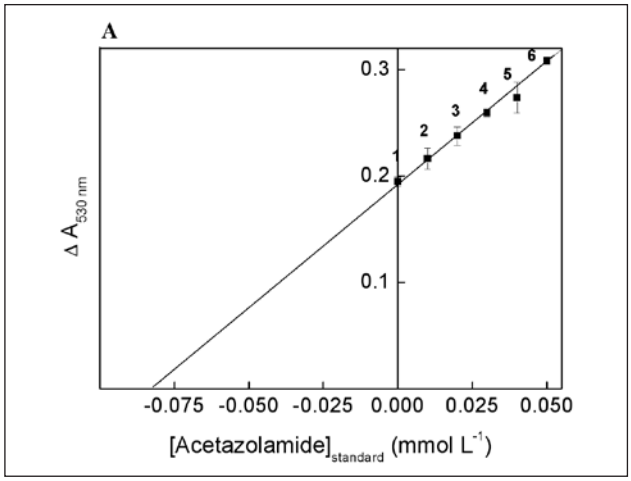
Table I. Influence of excipients as potential interferents in the determination of acetazolamide

Excipient	CE/CA	(%)
NaAMG	5	100
Phosphate	5	100
Starch	5	98.1
MgS	5	99.5

NaAMG: sodium amidoglycolate; **Phosphate:** calcium phosphate; **MgS:** magnesium stearate; **CE/CA:** ratio between the concentrations of excipient and acetazolamide.

3.7. Determination of acetazolamide in pharmaceutical formulations

Acetazolamide was measured in pharmaceutical products using the proposed method at wavelengths of 530 and 610 nm. The UV spectroscopy method was used as a reference technique [14]. Standard additions were employed in both procedures, with acetazolamide concentrations of between 1.0 x 10^{-5} and 5.0 x 10^{-5} mol L^{-1}. The results are shown in Figures 6A and 6B.



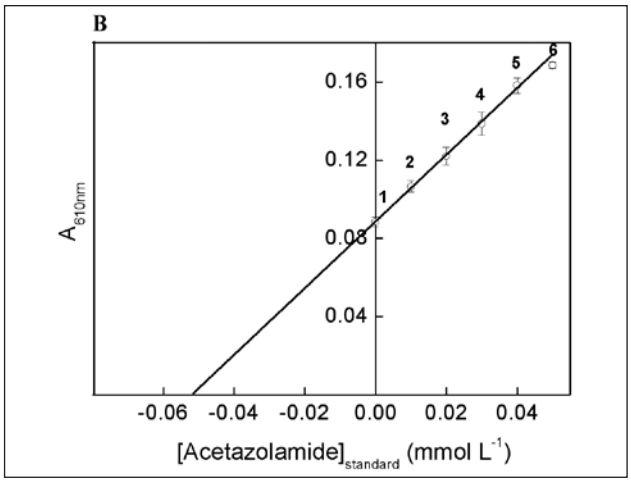


Figure 6. (A) Multiple standard additions curve for determination of acetazolamide in a pharmaceutical formulation (λ = 530 nm) (n = 3). (B) Multiple standard additions curve for determination of acetazolamide in a pharmaceutical formulation (λ = 610 nm) (n = 3)

The values obtained using the proposed and comparative methods are presented in Table II. The results were in agreement at a 95% level of confidence, demonstrating the reliability of the method using the two selected absorbance wavelengths.

Table II. Determination of acetazolamide in a commercial pharmaceutical formulation (tables with a nominal acetazolamide content of 250 mg), using the proposed method and UV spectroscopy technique [14]. (n=3)

Samples	UV (mg/tablet)	Proposed flow procedure (mg/tablet)		E_r (%)
	255 nm	530 nm[a]	610 nm[b]	
A	248 ± 4	251 ± 1	-	+1.2
B	249 ± 5	-	245 ± 3	-1.6

Mean ± standard deviation; 95% confidence level; E_r = relative error of proposed procedure *vs.* comparative method.

[a] $t_{calculated}$ = 1.30
[b] $t_{calculated}$ = 1.38

Conclusions

A flow injection spectrophotometric technique was successfully developed for the determination of acetazolamide, based on the oxidation of the compound by $KMnO_4$ in an alkaline medium, with formation of manganate ions. Quantification of acetazolamide could be achieved using two different wavelengths. At 530 nm, measurement was made of the reduction in concentration of permanganate ions (violet colored). At 610 nm, quantification was based on the increase in concentration of manganate ions (green colored). The results obtained by the proposed method and by a reference technique (UV spectroscopy) were in excellent agreement. The new method showed good precision, reproducibility, and detectivity for both synthetic and commercial samples.

Acknowledgements

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Determination of the antioxidant capacity of sugarcane juice and passion fruit extracts using the spectroscopic DPPH• and the electrochemical CRAC assays

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Abstract

CRAC (ceric reducing antioxidant capacity) is a simple and fast electrochemical method for the evaluation *in vitro* of antioxidant capacity even in complex matrixes. It uses a boron-doped diamond electrode and chronoamperometric measurements that allow quantifying the ability of the studied sample to reduce Ce^{4+} species through a single electron transfer mechanism. The antioxidant capacity of methanol extracts of sugarcane juice and passion fruit pulp plus the standard antioxidant compounds rutin and resveratrol was investigated using the CRAC method. The results were compared with those obtained by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) method, a spectrophotometric assay that determines the antioxidant (radical scavenging) capacity by direct reduction via electron transfer and/or by radical quenching via hydrogen atom transfer. Both the DPPH• and CRAC assays showed convergent results. The passion fruit pulp presented higher antioxidant capacity than the sugarcane juice, in the same experimental conditions.

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Keywords: CRAC assay, DPPH• assay, passion fruit pulp, sugarcane juice.

1. Introduction

The importance of oxidation in the body and in foodstuffs has been widely recognized due to the value of oxidative metabolism in cellular survival. However, active oxygen species such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), which are products of normal metabolism, attack biological molecules leading to destructive and lethal cellular effects by oxidizing cellular proteins, DNA and enzymes, thus shutting down cellular respiration. These damages are responsible for development of degenerative diseases such as cancer, heart diseases and cerebrovascular diseases through multiple mechanisms [1,2].

Antioxidant compounds can interfere with the oxidation processes by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers [3]. The deactivation of radicals can occur by two major mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET). The end result is the same, regardless of the mechanism, but the kinetics and the potential for side reactions differ. Electron and hydrogen atom transfer reactions may occur in parallel, and the dominating mechanism in a given system will be determined by the antioxidant's structure and properties, its solubility and partition coefficient, and by the system's solvent [4].

Natural foods and food-derived antioxidants such as phenolic compounds, carotenoids and vitamins have received growing attention due to their capacity to minimize free radical action. In recent years an increasing number of publications have reported the antioxidant capacity of various plants and foods [5-9].

Sugarcane juice and passion fruit are largely consumed by the population of several tropical countries, including Brazil. Previous studies showed that sugarcane juice and passion fruit are rich in phenolic compounds, mainly flavone *O*- and *C*-glycosides [10-12]. Therefore, the evaluation of these foods has been aimed at their application as possible functional foods. However, due to the complex composition of these materials, separating each antioxidant compound and studying it individually is costly and inefficient, notwithstanding the possible synergistic interactions among the antioxidant compounds in a food mixture [13].

Meanwhile, the total antioxidant capacity assay using a single chemical reaction seems to be rather unrealistic and not easy to come by. In a review by Carocho and Ferreira [14], the authors pointed out that the detection of antioxidant activity as well as specific antioxidant compounds can be carried out with a large number of different assays, all of them with advantages and disadvantages. Thus, the methods for the evaluation of the antioxidant action should be based on the identification of different antioxidant

mechanisms under variable conditions, reflecting the multifunctional properties of antioxidants in both physiologically and food-related oxidative processes [15].

On the other hand, MacDonald-Wicks *et al.* [16] suggest selecting one assay to measure reducing ability that is widely reported and validated to measure the reducing ability. Gülçin [17] considers that the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging capacity assay is an easy and accurate method for use in food constituents. However, the interpretation is complicated when the test compounds have spectra that overlap DPPH$^{\bullet}$ at 515 nm. For example, carotenoids in particular interfere in the measurements [18]. Therefore, another antioxidant capacity assay should be used for complementing the results obtained with the DPPH$^{\bullet}$ assay that is used as the standard assay.

Ferreira and Avaca [19] developed an electrochemical assay using a boron-doped diamond (BDD) electrode for the determination of the antioxidant capacity of several antioxidant standards in ethanol solutions and denominated it the CRAC (ceric reducing antioxidant capacity) assay. The CRAC methodology uses chronoamperometric measurements and the Cottrell equation to monitor the decline of the Ce^{4+} species concentration. Due to the high redox potential of the Ce^{4+}/Ce^{3+} couple, this system allows measurements of the majority of the known antioxidant molecules if a BDD film is used as the cathode. That powerful analytic tool allows determining the antioxidant capacity of complex samples, mainly due to the fact that the chronoamperometric assays do not suffer interference from turbidity and/or colored samples. Besides, the CRAC assay is a simple, fast, and cheap method for the determination of the antioxidant capacity even in complex matrixes. However, to the best of our knowledge, applications of CRAC assay in the study of food samples have not yet been reported in the literature.

Therefore, the purpose of this study is to evaluate the antioxidant capacity of methanol extracts of sugarcane juice *(Saccharum officinarum L.)* and yellow passion fruit pulp *(Passiflora edulis f. flavicarpa Degener)* and to compare them with standard antioxidant compounds such as rutin and resveratrol using two different antioxidant capacity assays, namely the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging capacity and the ceric reducing antioxidant capacity (CRAC).

2. Experimental

2.1. Reagents and solutions

All the reagents used in the experiments were prepared using chemicals of the highest available purity. Rutin hydrate, resveratrol (3,4´,5-trihydroxy-trans-stibene) and DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent were obtained from Sigma Chemical Co. (St. Louis, Missouri); analytical grade methanol was purchased from Tedia Co. (Fairfield, Alabama); trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, was from Aldrich Chemical Co. (St. Louis, Missouri) and cerium (IV) sulfate tetrahydrate ($Ce(SO_4)_2.4H_2O$) from E. Merck (Darmstadt, Germany).

Methanol stock solutions of resveratrol and rutin were freshly prepared before the measurements in the concentrations of 4.28×10^{-3} mol L^{-1} (9.78×10^{-1} g L^{-1}) and 1.70×10^{-3} mol L^{-1} (1.04 g L^{-1}), respectively, and used as standards of comparison in both assays. A methanol stock solution of trolox was freshly prepared in the concentration of 4.25×10^{-3} mol L^{-1} (1.06 g L^{-1}) and used as standard in the determination of the trolox equivalent (TE), an experimental parameter of the CRAC assay [19].

A DPPH$^{\bullet}$ methanol solution in a concentration of 2.50×10^{-2} g L^{-1} was used in the DPPH$^{\bullet}$ assay. Sulfuric acid (97.99 % m/m H_2SO_4) from Mallinckrodt (Xalostoc, Mexico) was used as supporting electrolyte at a concentration of 0.5 mol L^{-1} while a 1.01×10^{-3} mol L^{-1} stock solution of $Ce(SO_4)_2.4H_2O$ in 0.5 mol L^{-1} H_2SO_4 (CRAC reagent) was used as the oxidant in the CRAC assay. Both solutions were prepared using doubly deionized water with a resistivity greater than 18 MΩ at 25 °C using a Milli-Q system from Millipore (Bedford**,** Missouri).

2.2. Samples

The sugarcane analyzed in this study was of the species *Saccharum officinarum L.* (Gramineae) and the material was collected at the Embrapa – Agropecuária Sudeste plantation in São Carlos, SP, Brazil. The stems were ground to collect the sugarcane juice and afterwards the material was frozen. The passion fruit species used in this study was *Passiflora edulis f. flavicarpa* Degener and the fruits were purchased in the city of São Carlos, SP, Brazil. The fresh pulp was separated from the seeds by sieving, and then the material was frozen.

Sugarcane extracts were prepared by sonication of sugarcane juice (100 mL) and 10 mL of methanol for 5 minutes at room temperature. The samples were centrifuged at 10000 rpm for 20 minutes at 25 °C, and the resultant supernatant was taken to dryness under vacuum and below 50 °C in a previously weighed flask, using a rotary evaporator. The residue was *used for antioxidant assay. The mass of the dried methanolic extract was calculated by difference and aliquots at different concentrations were prepared as described in 2.3.1. The same procedure was followed for preparing the passion fruit pulp methanol extracts, using 30 mL of passion fruit pulp.*

2.3. Antioxidant capacity assays

2.3.1. DPPH$^{\bullet}$ scavenging capacity assay

The DPPH$^{\bullet}$ method reported by Brand-Williams *et al.* [5,20] with some modifications as described later was used to evaluate the antioxidant capacity of the different methanol food extracts under investigation. This method is based on the reaction between the free radical DPPH$^{\bullet}$ and the studied samples followed by monitoring the decrease in DPPH$^{\bullet}$ absorbance in a given time interval or after the steady state was reached for the reaction [19, 20-24]. An

aliquot of 100 µL of methanol extract at different concentrations (35.00 – 195.00 g L^{-1} for sugarcane juice and 1.00 – 80.00 g L^{-1} for passion fruit pulp) was added to 3.9 mL of DPPH• methanol solution. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 1 hour. The same procedure was carried out with methanol solutions of rutin and resveratrol (standard compounds) in a concentration interval of 0.06 – 1.00 g L^{-1}. Rutin was chosen as a standard as it is a low-cost flavonol *O*-glucoside with antioxidant properties [25], and also because only a few of *Passiflora* and sugarcane *C*-glycosylflavones are commercially available as analytical standards. Resveratrol was chosen as one of the standards due to its correlation with the claimed benefits of red wine as a functional food, although the concentration used in this assay was higher than in Brazilian red wine (2.57 mg L^{-1}) [26] or French red wine (5.06 mg L^{-1}) [27].

The decrease in absorbance of the resultant solution was monitored at 515 nm after 1 hour, using a Perkin Elmer UV-Vis model Cary 5G spectrophotometer (Waltham, Massachusetts). A methanol solution of DPPH• in the concentration of 2.50 x 10^{-2} g L^{-1} was used as the blank. The percentage of scavenged DPPH• was calculated using equation 1 [28]:

$$DPPH^{\bullet}_{scavenging}\ \% = (Ao - A_S / Ao) \times 100 \qquad (1)$$

where *Ao* is absorbance of the blank and A_S is absorbance of the sample at 515 nm.

The parameters utilized to define antiradical efficiency were the percentage of scavenging DPPH• and the amount of antioxidant necessary to decrease by 50% the initial concentration of DPPH• (EC_{50}) [28].

2.3.2. CRAC assay

As described for Ferreira and Avaca [19], the CRAC assay consists in the monitoring of the decrease in the initial concentration of Ce^{4+} species after four minutes of reaction with the added antioxidant, using chronoamperometric measurements and correlating it with the antioxidant/reducing capacity through the Cottrell equation [29]:

$$I_t = \frac{nFAC_O^o D_O^{1/2}}{\pi^{1/2} t^{1/2}} = bt^{-1/2} \qquad (2)$$

where: I_t is the instantaneous current at time *t*, *n* is the number of electrons involved in the process, *F* is the Faraday's constant, *A* is the electrode area, C^*_O is the bulk concentration of the electroactive species (normally an oxidized entity), D_O is its diffusion coefficient and *b* is the Cottrell slope. The results were analyzed by means of the linear relationship between *I* and $t^{-1/2}$ (Cottrell lines), which, in turn have a slope for a given antioxidant *b* directly proportional to the remaining concentration of Ce^{4+} species. Keeping all other parameters constant (n, F, A, D and π), the slope variation for experiments with different antioxidants will reveal the variations in Ce^{4+} concentration and consequently the relative antioxidant capacity [19].

To carry out the chronoamperometric assays, 10 mL of CRAC reagent were initially deoxygenated with N_2 for six minutes and then 100 µL of methanol extract solution, having different concentrations, were added, maintaing the stirring with N_2 for an additional four minutes (reaction time). At this point, the measured open circuit potential for the system ($E_{initial}$ ~ 1.29 V) was applied to the electrode for two seconds and then stepped to E_{final} = 0.8 V where the variation of the current with time was recorded during 10 seconds. That same procedure was carried out with methanol solutions of resveratrol and rutin in a concentration interval of 0.06 – 9.78 x 10^{-1} g L^{-1} and 6.10 x 10^{-3} – 3.05 x 10^{-2} g L^{-1}, respectively, and with a 50 x 10^{-6} mol L^{-1} (1.20 x 10^{-2} g L^{-1}) trolox solution used as standard in the determination of the TE.

Preliminary experiments using cyclic voltammetry were carried out to characterize the systems under investigation. Thus, for each sample under investigation (i.e., 195.00 g L^{-1} for sugarcane juice and 80.00 g L^{-1} for passion fruit pulp) 100 µL of methanol extract in 10 mL of supporting electrolyte (0.5 mol L^{-1} H_2SO_4) were cycled in the range between −0.7 V and 1.8 V (100 mV s^{-1} scan rate) after deoxygenating the solution for 10 minutes with N_2.

All measurements were carried out using a model PGSTAT30 potentiostat/galvanostat from Autolab (Utrecht, Netherlands) connected to a computer for data collection and analysis. The electrochemical experiments were carried out in a one-compartment Pyrex® glass cell (30 mL) provided with three electrodes and degassing facilities for bubbling N_2. The reference system was the Ag/AgCl (3.0 mol L^{-1} KCl) electrode and the counter one was a 2 cm^2 Pt foil. The working electrode was a boron-doped diamond (BDD) single-faced plate with an exposed area of 0.36 cm^2 and final boron content of the order of 8000 ppm. The BDD films were produced by Adamant Technologies SA, La Chaux-de-Fonds, Switzerland, on silicon wafers using the hot filament chemical vapor deposition (HF/CVD) technique. Prior to the experiments, the BDD electrode received a pre-treatment at +3.0 and -3.0 V during 15 and 45 seconds, respectively, in a 0.5 mol L^{-1} H_2SO_4 solution to ensure reliable and reproducible results [30].

2.4 Statistical analysis

The results were obtained in tripllcate and were analyzed by variance analysis ($p \leq 0.05$). All the data were processed by using the program Microcal Origin® 7.5.

3. Results and discussion

3.1. DPPH• scavenging capacity assay

In order to evaluate the antioxidant capacity, the method developed by Brand-Williams *et al*. [5,20] was initially followed. The reaction between the free radical DPPH• and

the methanol extracts was monitored by UV-Vis spectroscopy resulting in kinetic curves for sugarcane and passion fruit samples, as shown in Figure 1.

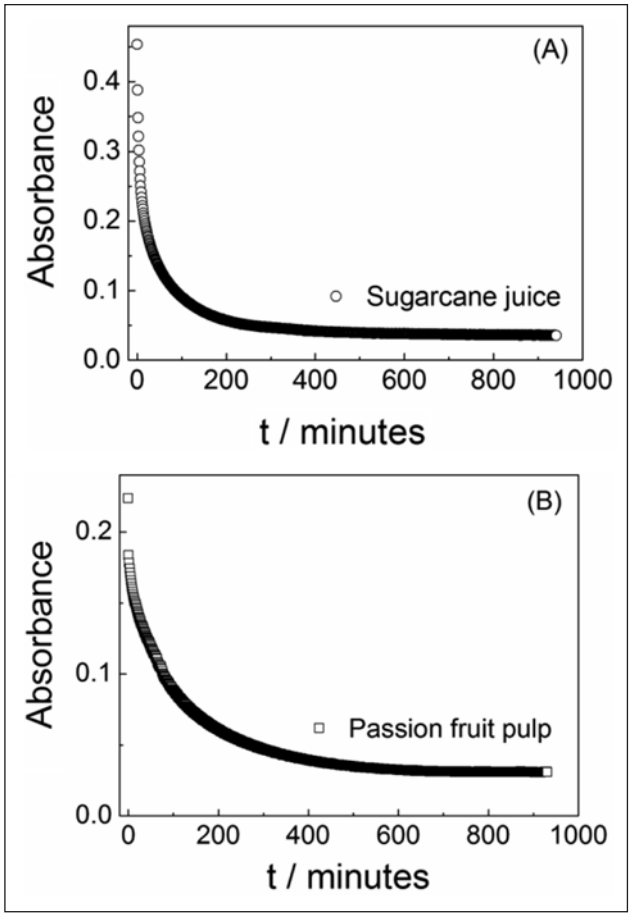

Figure 1. Kinetic curves for the reaction between DPPH• and (A) sugarcane or (B) passion fruit (λ = 515 nm).

These curves show the decrease of the absorbance of the remaining DPPH• as a function of time until the absorbance value become constant. Through the absorbance value of the steady state the percentage of scavenged DPPH• could be calculated to obtain the EC_{50}. However, sugarcane juice and passion fruit pulp extracts showed a very slow decrease of absorbance that resulted in a long reaction time (more than 7 hours) to reach the steady state. Therefore, some modifications in the original procedure of Brand-Williams *et al.* [20] method were needed.

Thus, a modification proposed by Kim *et al.* [25] was used and consists in measurement of the DPPH• absorbance after 1 hour of reaction. Through the absorbance value for each sample, the percentage of scavenged DPPH• was calculated (Table I) to obtain the EC_{50} value, a parameter that characterizes the antioxidant power and is inversely related to the antioxidant capacity. Several tests with different concentrations for each extract were carried out to evaluate the appropriate concentration range to reach the EC_{50} values.

Table I. Results of the DPPH assay for sugarcane, passion fruit, resveratrol and rutin.

Samples	Sample concentration (g L^{-1})	% scavenged DPPH•	EC_{50} ± SD (g L^{-1})	R^2
Sugarcane juice	195.00	70.30	100.80 ± 2.56	0.998
	155.00	61.50		
	135.00	50.90		
	75.00	46.00		
	35.00	36.20		
Passion fruit pulp	80.00	80.39	38.50 ± 2.28	0.998
	60.00	70.05		
	40.00	53.94		
	20.00	26.97		
	1.00	7.00		
Resveratrol (standard)	1.00	80.75	0.30 ± 0.01	0.998
	0.50	65.05		
	0.25	47.90		
	0.12	25.80		
	0.06	10.75		
Rutin (standard)	1.00	94.00	0.10 ± 0.01	0.998
	0.50	94.00		
	0.25	82.30		
	0.12	59.00		
	0.06	35.20		

EC_{50}: amount of antioxidant necessary to decrease by 50% the initial concentration of DPPH•
SD: standard deviation
R^2: correlation coefficient

The antiradical capacity could be evaluated from a graph of the percentage of scavenging DPPH• as a function of antioxidant concentration in the samples (Figure 2). The EC_{50} values were calculated by using the first order decrease exponential equation ($y = y_0 + A_1 . e^{-x/t}$) from each sample's graph. A lower value of EC_{50} indicates a higher antioxidant capacity, thus the comparison of the values showed a higher free radical scavenging capacity of the extracts in the order: passion fruit pulp > sugarcane juice. In the concentration range utilized for the solution of standards (Figure 2), the antioxidant hierarchy was rutin > resveratrol > passion fruit pulp > sugarcane juice.

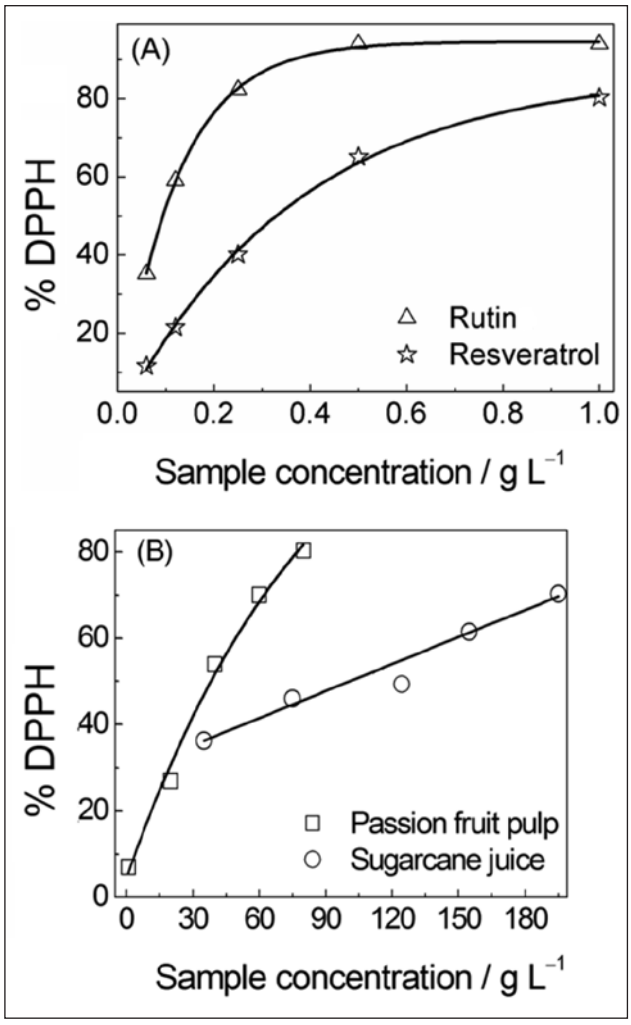

Figure 2. Percentage of DPPH• scavenging against antioxidant concentration for (A) rutin and resveratrol (standards), (B) passion fruit and sugarcane.

3.2. CRAC assay

A preliminary characterization of the systems was carried out by cyclic voltammetry with the BDD electrode in a 0.5 mol L^{-1} H_2SO_4 aqueous solution. The experimental results are shown in Figure 3 where curve 1 is the response of the oxidant (Ce^{4+}) showing both the reversibility of the system as well as the potential value (E_{RED} = 0.8 V) for the chronoamperometric determination of the remaining Ce^{4+} species to be carried out after reaction with the different samples [19]. Figure 3 also shows the irreversible voltammetric response for the methanolic extracts of sugarcane juice (curve 2) and passion fruit (curve 3) under investigation. It is clear from this figure that the reduction process to be carried out at 0.8 V after the reaction between the oxidant (Ce^{4+}) and each sample will not be affected by reduction of the products generated by oxidation of the sample.

For the chronoamperometric assays, Figure 4 shows the experimental results of current decays recorded for Ce^{4+} solutions at different concentrations. These curves are used

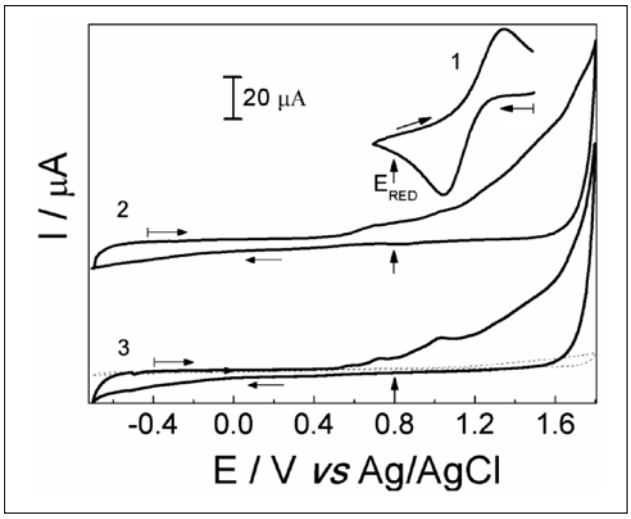

Figure 3. Cyclic voltammograms recorded on BDD at 100 mV s^{-1} for (1) the oxidant (1 x 10^{-3} mol L^{-1} Ce^{4+} in 0.5 mol L^{-1} H_2SO_4) and for 100 µL of methanol extracts of (2) sugarcane juice (195.00 g L^{-1}) and passion fruit (80.00 g L^{-1}) in 0.5 mol L^{-1} H_2SO_4. The dotted line represents the blank responses (0.5 mol L^{-1} H_2SO_4).

to obtain the Cottrell lines (inset A) showing that the values of the slopes depend on the Ce^{4+} concentration. The mathematical dependence of the Cottrell slope (*b*) with the Ce^{4+} concentration is presented in inset B of Figure 4 as obtained by regression analysis of the values of *b* in µA $s^{1/2}$ and *[Ce^{4+}]* in mol L^{-1}:

$$b_{exp} = (0.19 \pm 0.10)\ \mu A\ s^{1/2} + (3.46 \times 10^{4} \pm 1.11 \times 10^{2})\ \mu A\ s^{1/2} / mol\ L^{-1} \times [Ce^{4+}]\ mol\ L^{-1} \quad (3)$$

where b_{exp} is the experimental value of the Cottrell slope and *[Ce^{4+}]* is the concentrations of Ce^{4+} species in solution.

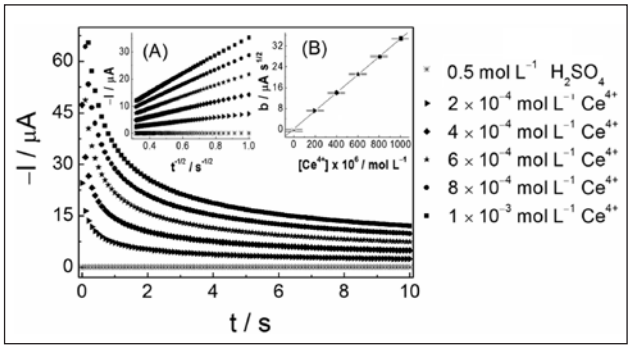

Figure 4. Data plotted as *I* versus *t* (current decays lines) recorded for the supporting electrolyte (0.5 mol L^{-1} H_2SO_4) and for Ce^{4+} solutions at different concentrations. Insets (A) data plotted as *I* versus $t^{-1/2}$ (Cottrell lines) and (B) values of the slopes (*b*) for the Cottrell lines plotted against Ce^{4+} concentration.

The relative CRAC value for a sample corresponds to the concentration of Ce^{3+} formed after a fixed reaction time between pre-established amounts of oxidant (Ce^{4+}) and antioxidants present in the samples followed by comparison with a standard material (e.g. trolox). This can be achieved

using the experimental value of the Cottrell slope for a given sample (b_{AO}) together with Equation 4 and the initial value of the probe (Ce^{4+}) concentration, namely, 1×10^{-3} mol L^{-1}. All this information is condensed in Equation 4:

$$\frac{CRAC\ value}{([Ce^{3+}]/mol\ L^{-1})} = 1 \times 10^{-3} - \frac{b_{AO} - 0.19}{3.46 \times 10^{4}} \quad (4)$$

The TE is obtained from a simple relationship between the CRAC value determined for a given sample and that measured for trolox under the same experimental conditions (Equation 5):

$$TE = \frac{CRAC\ Value_{AO}}{CRAC\ Value_{Trolox}} \quad (5)$$

The slope values (b_{AO}) were measured for each sample. Using b_{AO} values and equations 4 and 5, the CRAC value for each sample as well as the corresponding TE were calculated and are presented in Table II. For pure Ce^{4+} and trolox solutions the experimental values of the Cottrell slope were, respectively, 34.78 ± 0.03 and 31.36 ± 0.26 μA $s^{1/2}$.

Table II. Results of the CRAC assay for sugarcane, passion fruit, resveratrol and rutin.

Samples	Sample concentration (g L^{-1})	b_{AO} (μA $s^{1/2}$)	CRAC value × 10^6 ([Ce^{3+}]/ mol L^{-1})	TE	Slope: TE x Sample concentration (L g^{-1})	Standard error (slope)	R^2
Sugarcane juice	195.00	29.44	154.48	1.56	8.11×10^{-3}	9.70×10^{-4}	0.945
	155.00	30.63	120.15	1.21			
	135.00	31.99	80.85	0.81			
	75.00	32.88	55.06	0.55			
	35.00	34.04	21.73	0.22			
Passion fruit pulp	80.00	10.94	689.33	6.95	6.75×10^{-2}	1.31×10^{-3}	0.998
	60.00	15.37	561.19	5.66			
	40.00	20.21	421.48	4.25			
	20.00	25.19	277.49	2.80			
	1.00	28.94	168.95	1.70			
Resveratrol (standard)	9.78×10^{-1}	19.96	428.62	4.32	4.42	6.75×10^{-2}	0.999
	4.89×10^{-1}	27.11	221.90	2.24			
	2.44×10^{-1}	31.04	108.46	1.09			
	1.22×10^{-1}	32.72	59.82	0.60			
	0.06	33.96	24.00	0.24			
Rutin (standard)	3.05×10^{-2}	15.42	559.79	5.64	1.75×10^{2}	5.19	0.996
	2.44×10^{-2}	18.55	469.32	4.73			
	1.83×10^{-2}	22.04	368.55	3.71			
	1.22×10^{-2}	26.20	248.13	2.50			
	0.61×10^{-2}	29.98	139.16	1.40			

b_{AO}: experimental value of the Cottrell slope for a given sample
TE: trolox equivalent
R^2: correlation coefficient

The TE values presented in Table II can be used for a comparison between the different samples under investigation. Figure 5 shows the TE value for each sample as a function of its concentration in the studied interval. However, in contrast to the DPPH• assay, the CRAC assay does not quantify the concentration of sample required to reduce 50% of the oxidant because, in many cases, it would be necessary to do an extrapolation of the Cottrell lines. Therefore, the comparison was made based on the slope values of the lines shown in Figure 5. This coefficient provides a magnitude of how much a sample is more efficient than another in terms of its antioxidant properties. From the slopes values (rutin: 1.75 x 10^2 L g^{-1}; resveratrol: 4.42 L g^{-1}; passion fruit pulp: 6.75 x 10^{-2} L g^{-1} and sugarcane juice: 8.11 x 10^{-3} L g^{-1}) it is possible to observe the same antioxidant hierarchy determined by the DPPH assay in the concentration range of the samples and standards as shown in Figure 5, namely: rutin > resveratrol > passion fruit pulp > sugarcane juice.

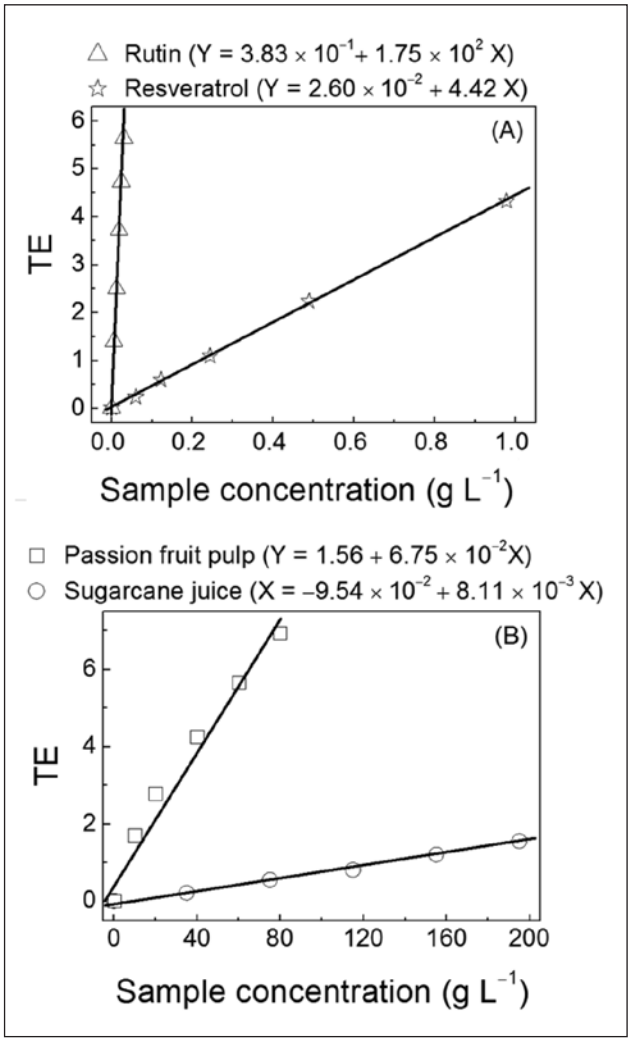

Figure 5. Data plotted as *TE* versus sample concentration recorded for methanol extracts of (A) rutin and resveratrol (standards), (B) passion fruit and sugarcane.

3.3. Comparison between DPPH• and CRAC assays

Originally, it was assumed that the DPPH• assay was driven only by a hydrogen transfer reaction [20] but Foti *et al*. [31] suggest that it also can operate following an electron transfer reaction. Thus, the initial hydrogen transfer occurs very quickly and depends on hydrogen-bond accepting capacity of the solvent while the subsequent electron transfer occurs more slowly [4]. Since methanol, a strong hydrogen-bond accepting solvent is used in the present DPPH radical scavenging capacity assay, the hydrogen abstracting reaction occurs as a main reaction pathway [31]. On the other hand, the CRAC assay is a direct electron transfer test that involves one simple redox reaction between an oxidant and the antioxidant [19]. Therefore, the difference between the antioxidant mechanisms of the two assays can influence directly in the values of the total antioxidant capacity measured for a given sample.

Another factor that can influence the total antioxidant capacity is the hydrogen ion concentration (pH). The pH of the solution is particularly important in electron transfer assays. Under neutral conditions (aqueous solution, pH = 7), radicals of most flavonoids with a catechol structure (pKa ~ 4 – 5) are completely deprotonated and more easily oxidized under these conditions [32]. On the other hand, proton dissociation of phenolic compounds would enhance the reducing capacity of a given molecule while under acidic conditions the reducing capacity may be diminished. Reactions at low pH decrease the ionization potential that drives electron transfer and increase the redox potential, thus causing a shift in the dominant reaction mechanism. The CRAC assay is carried out under extremely acidic conditions since it uses 0.5 mol L^{-1} sulfuric acid as supporting electrolyte [19] while the DPPH assay is carried out in a non-aqueous solvent, where the considerations regarding pH values cannot be applied.

4. Conclusions

The aim of this study was not to determine the best assay but to furnish results that quantify the antioxidant capacity of various samples based on more than one assay. The results indicated that CRAC and DPPH yield convergent results, suggesting that the two assays may be useful models for the *in vitro* evaluation of the antioxidant capacity of complex samples such as passion fruit pulp and sugarcane juice. However, the DPPH• assay indicated an approximately three-fold higher antioxidant capacity of passion fruit pulp extract than that of sugarcane juice extract, while the CRAC assay indicated that passion fruit pulp has an eight-fold higher antioxidant capacity than sugarcane juice extract. The significant difference between the dimensional values of these assays can be attributed to the different experimental conditions of each assay, such as solvent, reaction time and pH. Moreover, these results suggest that SET mechanisms are the main factors responsible for the antioxidant capacity of these samples.

Finally, the difference between the antioxidant capacities of passion fruit pulp and sugarcane juice extracts measured in the two assays is probably attributable to the concentration and structure of the antioxidant compounds in these extracts. Previous studies by LC techniques (LC/UV-DAD, LC/UV/MS and LC/MS) showed the presence of flavones and *C*-glycosyl flavones in passion fruit juice [33] and several *C*- and *O*-glycosyl flavones (mainly tricin derivatives) in sugarcane juice [11,12]. The total flavonoid content in passion fruit (~ 0.16 mg flavonoids mL^{-1} of fruit pulp) was comparable to that of sugarcane juice (~ 0.24 mg total flavonoids mL^{-1}) and orange juice (~ 0.20 mg flavonoids mL^{-1}) [33]. Besides, HPLC microfractionation monitored using DPPH as the detection reagent (thin-layer chromatography monitoring) allowed for the identification of five *C*-glycosyl flavones with radical scavenging activity in sugarcane juice. Quantitative analysis by HPLC-UV/DAD indicated that these compounds correspond to 58.28% of the total flavonoids in sugarcane juice, but the antioxidant ability of sugarcane juice was determined only by the DPPH method (EC_{50} = 100.2 ± 2.6 g L^{-1}) [34].

C- and *O*-glycosyl flavones are probably the main compounds that may be correlated with the antioxidant activity of passion fruit pulp and sugarcane juice, but these samples also contain minor compounds (carotenoids, in passion fruit juice; polyphenolic compounds and other flavonoids) that possibly also contribute to their antioxidant capacity. Due to the overall findings reported here, other chemical studies are being conducted in our laboratories. The findings reported here also indicate the need for detailed nutritional or physiological studies to evaluate the potential of these plants as functional foods.

Acknowledgments

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Methodology for determination of vegetable oils in diesel by capillary gas chromatography

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Abstract

With the mandatory introduction of biodiesel in diesel in Brazil, an emerging problem is the contamination and/or adulteration of diesel with vegetable oils. This work describes a new method for the detection of vegetable oils in diesel by high temperature capillary gas chromatography. Tests indicate a complete separation of the triacylglycerols, present in oils, from the components of diesel and biodiesel and quantification gave more promising results with internal standardization using tricaprin as the internal standard. Evaluation of some parameters of validation showed very satisfactory results such as relative standard deviation of about 6%, linearity of the method from 0.5 to 3.0 %w/w with determination coefficient higher than 0.99 and measurement uncertainty about 15 % for the samples analyzed. The paper also describes details for calculating uncertainty associated with the gas chromatography.

It is necessary to emphasize that this method uses the same configuration used in international standards methodologies focused on the analysis of free and total glycerol, since same injector, same column and same detector are used here, which could make quality control faster and cheaper in laboratories that analyze diesel Bx and biodiesel.

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1. Introduction

The Law 11097/2005 [1] determined the addition of biodiesel to petrodiesel and established 2 % (v/v) as mandatory in Brazil from January 2008. This content has increased in the energetic matrix in Brazil. Often Bx is used to designate the content as % v/v of biodiesel in diesel. This way, it is used B0 for pure diesel, B2 for blends of 2% biodiesel in petroleum-based diesel. Thus, B5 means blends of 5 % biodiesel in diesel and so on.

Biodiesel is defined as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats [2] and it is obtained mainly from transesterification reactions of vegetable oil or animal fat with alcohol (more commonly methanol and ethanol) [3]. At the moment, the National Agency for Petroleum, Natural Gas and Biofuels (ANP), the regulatory agency responsible by the quality control of fuels in Brazil, has as one of its main challenges to ensure the content and quality of biodiesel in diesel Bx. Furthermore, an emerging problem can be the irregular use of non transesterified vegetable oil instead of using biodiesel to blend with the petrodiesel. Different from biodiesel, vegetable oils are composed mainly of triacylglycerols, which can cause problems to diesel engines due to their higher viscosity [4,5].

For quality control, the main methods described to analyze the content of biodiesel in diesel do not consider any contamination with vegetable oils [6-8]. Foglia *et al.* [9] described a methodology for determining biodiesel and triacylglycerols in diesel by HPLC, but the main focus of this paper is on the quantification of biodiesel. In another paper Oliveira *et al* [10] reported the determination of vegetable oils in diesel by spectrometry, using Raman and NIR and, in addition, using chemometrics.

From the above considerations, this work reports the development of a methodology for determining vegetable oils in petrodiesel by high temperature gas chromatography without the use of chemometric tools. It is important to emphasize that *on-column* injection was chosen since it has been described that this is the most recommended for injections of heavy organic components in order to avoid discrimination in the injector [11,12]. Furthermore, this paper describes a validation of the methodology, which includes accuracy, repeatability and measurement uncertainty.

2. Experimental

2.1 Materials

n-Heptane, ≥ 99.5 % (v/v) was purchased from Vetec and used without further purification. Different samples of diesel A (diesel without biodiesel) with different content limits of sulphur ((i) with 1800 mg/kg of sulphur, ii) with 500 mg/kg and iii) 50 mg/kg)) were provided by Global Distribuidora (Brasília) and a refinery from Petrobras (REGAP). Samples of diesel B (containing biodiesel) were obtained from the gas stations in Brasilia. Commercial soybean and

cottonseed oils and refined soybean oil (from Brasil Ecodiesel) were used as sources of vegetable oils.

The studies were carried out using an Agilent 6890N gas chromatograph with a flame ionization detector (FID), a cool *on-column* injector and the software Chemstation. The capillary column used is coated with a phase of 5%-phenyl-95%-dimethylpolysiloxane for use at high temperature (DB-5HT) having dimensions of 15 m × 0.32 mm × 0.10 μm.

2.2 Methods

Preliminary tests with samples containing 1.0% to 3.0% (v/v) of soybean oil in diesel B0, B2 and B5 determined the best analytical conditions. The samples needed to be diluted (20 mL of *n*-heptane added to 100 mg of the diesel sample) and analyzed under the following GC conditions: initially a temperature of 150 °C for 15 minutes, being raised to 360 °C at a rate of 20 °C/min and then kept for 9.5 minutes, with a total run of 35 minutes. Helium was used as the carrier gas at a constant flow of 3.0 mL/min. The detector was operated at 380 °C with H_2 flow at 45 mL/min, air flow at 450 mL/min and nitrogen flow (make up gas) at 50 mL/min. The *on-column* injector followed the programming of temperature of the oven with 0.5 μL of injected volume.

The analytical curve was obtained with triolein and tricaprin (internal standard) in the range of 0.50 mg to 3.0 mg of triolein with a constant amount of tricaprin (2.0 mg). The solutions for the curve were prepared by transferring volumes (using calibrated micropipettes) from stock solutions of tricaprin (8.064 mg/mL) and triolein (5.012 mg/mL) to vials of 25 mL and then adding 20 mL of *n*-heptane. The unit used to quantify triacylglycerol content was %w/w.

The validation was done by evaluating the linearity of the method , repeatability, accuracy and measurement uncertainty.

3. Results and Discussion

3.1. Establishment of chromatographic conditions and quantification

As a first step, tests using samples prepared to have soybean oil in diesel in concentrations ranging to 1.0% to 3.0% (v/v) determined the best chromatographic conditions as well as the necessity to dilute the samples in order to avoid carryover in the gas chromatographic system. This effect can be caused by the accumulation of heavy organic compounds, i.e., compounds with high boiling points, in the column, which introduces signals of the triacylglycerols (main components of the vegetable oils) in subsequent analyses (Figure 1 shows the carryover as a consequence of the injection of the pure solvent *n*-heptane after injection of samples). Even when tested with a higher final temperature of the oven (380 °C) carryover was observed. Thus, it was determined that the best condition is to dilute about 100 mg of the diesel sample with 20 mL of *n*-heptane. For higher contents of vegetable oils (from 5 % v/v) carryover is observed due to the higher concentrations of the heavy organic triacylglycerols. In this case, it would be necessary to use a higher dilution for the calibration curve and also for the samples.

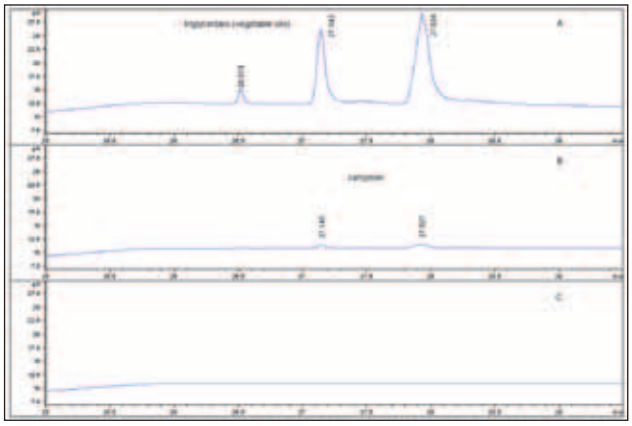

Figure 1. Chromatogram showing carryover for analysis. 0.5 µl of injected volume of a sample of diesel A containing 1% soybean oil (A), 0.5 µl of injected volume of *n*-heptane analyzed after injection of diesel sample (B) and 0.5 µl of injected volume of *n*-heptane analyzed before injection of diesel sample (C)

Figure 2 shows the same diesel sample containing different contents of vegetable oil, which indicates the clear increase of the peaks of the triacylglycerols.

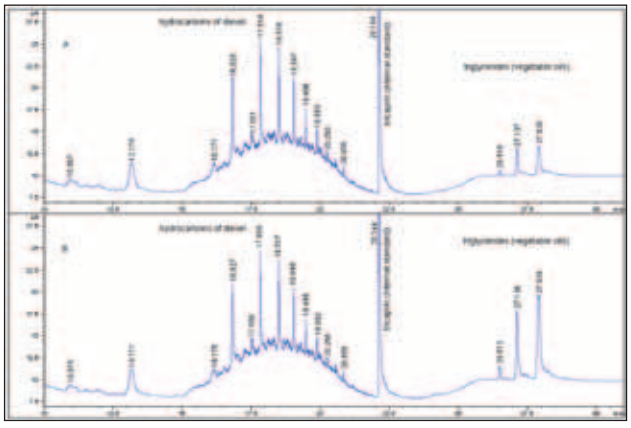

Figure 2. Sample of diesel A containing 1% of soybean oil (A) and sample of diesel A containing 2% of soybean oil (B)

Figure 3 shows a comparison of samples of diesel B (with biodiesel) contaminated with a trace and with 3% v/v of vegetable oil. It also shows a chromatogram of diesel A (without biodiesel) without vegetable oil. Therefore, we can assert that the method is not affected by the different concentrations of biodiesel in diesel (B0, B2, B3, B5, B20, etc). Moreover, we do not expect effects caused by the use of diesel with low contents of sulphur, since it does not contain dye, although we have only tested coloured diesel, which contains a higher content of sulphur.

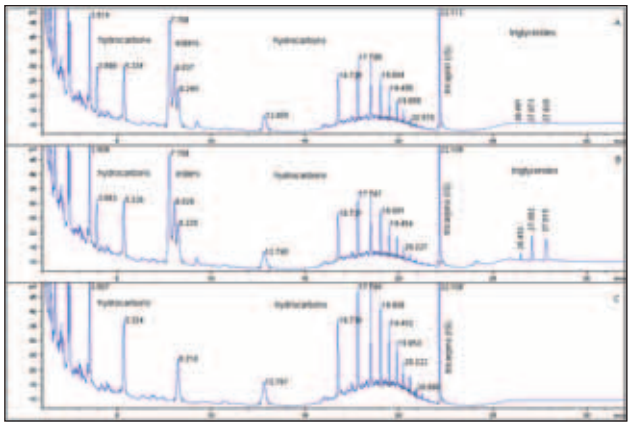

Figure 3. Sample of diesel B (with biodiesel) and addition of less than 0.5 % v/v of soybean oil (A), sample of diesel B (with biodiesel) and addition of 3 % v/v of soybean oil (B) and sample of diesel A (without biodiesel) and without addition of vegetable oil (C). Esters at about 7.8 min indicates biodiesel while the triacylglycerols in the range of 26.5 to 28 min are characteristic of vegetable oils. Other compounds in the chromatograms are hydrocarbons in the diesel.

An evaluation of Figures 2 and 3 indicates the high selectivity of this method as an unequivocal methodology to identify the presence of vegetable oils in diesel. This happens due to the fact that the chromatographic region of triacylglycerols does not contain any component of diesel and biodiesel to interfer in the detection of vegetable oils.

On quantification, this work tested both external standardization and internal standardization. The first approach tested was the external standardization by using different concentrations of soybean oil in diesel.

The problem with external standardization was on the composition of the sample analyzed. For samples containing different oils (for example, a sample contaminated with cottonseed quantified using curves prepared with soybean oil) the results varied. It was determined that this problem could be minimized by preparing an external calibration by using a triacylglycerol, for example triolein. In this case, the quantification was done by the sum of the triacylglycerols identified in the chromatogram (Figures 2 and 3). However, the composition of the diesel matrix could lead to variations in the baseline, which gave bad results, limiting the external standardization, mainly in terms of precision and accuracy, for a complex matrix constituted by diesel, biodiesel and vegetable oil.

Internal standardization was done with triolein as reference component for the quantitation of triacylglycerols and tricaprin as internal standard in the range of 0.5 mg to 3.0 mg. The quantification of vegetable oils was done by the sum of the triacylglycerols identified in the chromatograms (Figures 2 and 3). The following equation was obtained:

$$A_{triolein} / A_{tricaprin} = 0.612 \times (m_{triolein} / m_{tricaprin}) - 0.0808 \quad (1)$$

with R^2: 0.996, where *A* is the area of the component and *m* is the mass of the component (in g).

Then, the final result is obtained in %w/w by dividing the result in mass provided by the curve by the mass of the sample.

3.2. Validation of the method

Validation is used in order to ensure that a new method provides reliable data [13-15]. The first parameter of validation evaluated was linearity. The results are summarized in Table I and are based on the results of residues and ANOVA. Since $F > F_{crit}$, linearity is acceptable. Furthermore, as shown in Figure 4, a plot of residues as a function of $m_{triolein} / m_{tricaprin}$ does not show any trend. Then, the linear model is considered as valid.

Table I. Evaluation of the linearity of the method

m_{triol}/m_{tricap} (x)	A_{triol}/A_{tricap} (y)	$(y_i-<y>)^2$	$y_{predicted}$ [b]	Residues (y_i-y_{pred})	Residues^2
0.24861111 quares	0.085932	0.107652	0.071487	0.014446	0.000209
0.4970231	0.226740	0.035079	0.223623	0.003117	9.713E-06
0.994444	0.485911	0.005166	0.528260	-0.042349	0.001793
1.491666	0.857562	0.196715	0.832776	0.024786	0.000614
$<x> = 0.8079$[a]	$<y> = 0.4140$[a]				
	$\Sigma(y_i-<y>)^2 = SQ$	0.34461268		0.002626175	
	$SQ = MQ$[c]	0.34461268		0.001313088	
[d] $F = MQ_{regression}/MQ_{residues} = 0.3446/0.001313$. Then, $F = 262.44$					

[a] <x> and <y> are the means; [b] Each value of $y_{predicted}$ is obtained by doing $0.612 \times (m_{triolein} / m_{tricaprin}) - 0.0808$, where a (slope) is 0.612 and *b* (intercept) is - 0.0808; [c] MQ of residues was SQ/2, because the degrees of freedom of residues is 2 in this case; [d] $F_{crit} = 38.51$ (bimodal distribution).

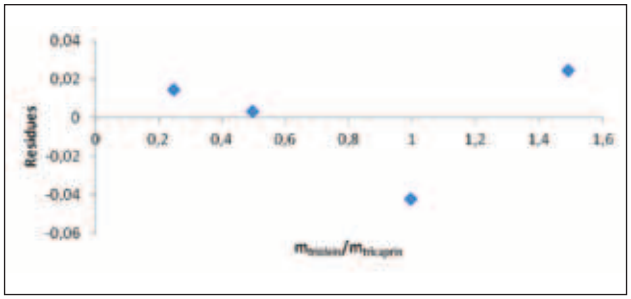

Figure 4. Plot of residues as a function of the $m_{triolein} / m_{tricaprin}$ ratio

For the next step of validation, two samples were gravimetrically prepared by the addition of soybean oil in diesel B by one analyst (analyst 1). The validation parameters were tested by a different analyst (analyst 2). Precision represents the dispersion of results obtained from independent analyses. This parameter was estimated from repeatability, which was done by 5 successive analyses (performed with samples of two different concentrations of vegetable oil in diesel and on different days) and measured by the RSD%, whose expression is the following:

$$RSD\% = (SD/\langle x \rangle) \times 100$$

where SD is the standard deviation and <x> is the mean value for the measurements [14].

The results are shown in Table II and indicate values of RSD % of about 6 %, which can be regarded as satisfactory.

Table II. Results for repeatability studies

Sample 1	
Replicate	**Triacylglycerols content (%v/v)**
1	0.75
2	0.82
3	0.85
4	0.82
5	0.90
Average	0.83
Standard Deviation	0.055
RSD%	6.6

Sample 2	
Replicate	**Triacylglycerols content (%v/v)**
1	2.12
2	2.23
3	2.24
4	2.31
5	2.49
Average	2.28
Standard Deviation	0.137
RSD%	6.0

Measurement uncertainty can be considered as the most important single parameter that describes the quality of measurements. This is because uncertainty fundamentally affects the decisions that are based upon the measurement result [16].

The uncertainty was calculated in this work according to the procedures described in The Eurachem Guide [16] and following methodology previously used in the laboratory for determination of measurement uncertainty of methanol in biodiesel [17] by liquid injection [18].

Detailed evaluation indicated that the main sources of uncertainty in this method are the analytical curve (due to linear regression) and repeatability (Figures 5 and 6). After determining the separate contributions as standard deviations, standard uncertainty, the combined standard uncertainty (μ_c) is obtained as the square root of the total variance, from the combination of all components written as standard deviations. To have an analytical result, the expanded uncertainty (U) is obtained by multiplying μ_c by *k* (coverage factor is 2 for 95% of confidence and an infinite number of degrees of freedom [16,19]. Figure 6 shows a worksheet used to make these steps in order to obtain the expanded uncertainty (U).

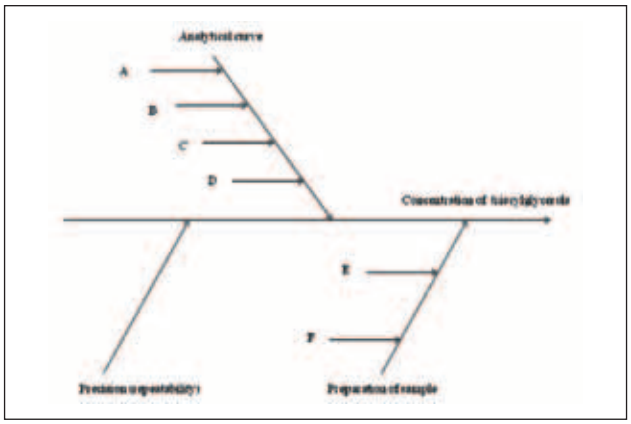

Figure 5. Cause and effect diagram considering the sources of uncertainty for determination of vegetable oil in diesel by gas chromatography. Only the sources that directly affect the final result (concentration of triacylglycerols) are described. Letters A to F are the sources that do not directly affect the concentration, such as linear regression, purity of triolein and tricaprin and calibration of the scale.

Results obtained for expanded uncertainty were the following:

- Sample 1: 0.1406 %w/w

- Sample 2: 0.2886 %w/w

For accuracy, this was tested from the measurement of recovery for the same two samples used for repeatability and uncertainty. Results are tabulated in Table III.

Table III. Recovery studies

Sample	Content of vegetable oil expected	Content of vegetable oil obtained (%w/w)
Sample 1	0.99	0.83 ± 0.14
Sample 2	2.16	2.28 ± 0.29

Although the sum of the value of Sample 1 with its value of uncertainty is lower than the expected result, the method can be considered satisfactory because it does not consider the uncertainty in the preparation of the two samples, but only the uncertainty of the analytical method. Furthermore, the uncertainties range from 12.7 % to 16.9 %, which is similar to the results found in a recent paper [20] (13.9 % to 18.6 %) that compared internal and external standardization for determination of ammonium in the cigarette smoke by ion chromatography. This paper also demonstrated that the uncertainty is lower for the internal standardization when compared to external standardization.

4. Conclusions

This work presents a new methodology to determine vegetable oils in diesel by gas chromatography by employing on-column injection and flame ionization detection. This instrumentation is similar to that used to determine free glycerol and acylglycerols in biodiesel [21].

Symbol	Sources of uncertainty			Distribution	Div	Coefficient sensitivity c_i		Standard uncertainty u_i [ ]	Degrees of freedom ν_i	% Contribution
	Name	Value (+/-)	Unit			Value	Unit			
$u_{Res.sca.\ Samp}$	sample - uncert. resolution of the scale	0.00005	*g*	normal (k)	2.00	1.99E-03	*1/g*	0.00000005	infinite	0.00
$u_{Cal.scal.samp}$	sample - uncert. calib scale	0.00030	*g*	retangular	1.73	1.99E-03	*1/g*	0.00000034	infinite	0.00
$u_{Res.scal.IS}$	tricaprin - uncert. Resol scale	0.00005	*g*	normal (k)	2.00	3.91E-01	*1/g*	0.000010	infinite	0.00
$u_{Cal.scal.IS}$	tricaprin- uncert. calibr scale	0.00030	*g*	retangular	1.73	3.91E-01	*1/g*	0.000068	infinite	0.00
$u_{Pur.IS}$	tricaprin - uncert purity	5.10E-05	*g*	retangular	1.73	3.91E-01	*1/g*	0.000012	infinite	0.00
$u_{Pur.Triol}$	triolein - uncert purity	5.10E-05	*g*	retangular	1.73	3.91E-01	*1/g*	0.000012	infinite	0.00
$u_{Det.curv}$	uncert curve - regression	0.04530	*% w/w*	normal (k)	1.00	1.00E+00	-	0.045300	infinite	9.86
u_{Repe}	uncert repeatability	0.13700	*% w/w*	normal (k)	1.00	1.00E+00	-	0.137000	9	90.14
										100.00
								0.0208		

Combined standard uncertainty	Distribution	Degrees of freedom (ν_{eff})	Coverage factor (*k*)	Expanded uncertainty (*U*) (95% of confidence)	Unit
0.144295	**normal (k)**	**infinite**	**2.00**	**0.2886**	**% w/w**

Figure 6. Detailed worksheet used for calculation of the expanded uncertainty for sample 2

The parameters evaluated in the validation were linearity (in the range of 0.5 to 3.0 %w/w), repeatability (n = 5 and RSD% about 6%), accuracy (recovery) and measurement uncertainty. This was based on Eurachem procedures and can be an important example for evaluation of measurement uncertainty in analyzes of non-volatile organic compounds by gas chromatography.

5. Acknowledgments

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Photometric determination of total anthocyanins in fruits

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Abstract

Anthocyanins (ACY) are phenolic compounds responsible for the bluish red color of several typical flowers and fruits. This work presents a proposal for quantifying ACY with low cost procedures, considering the new perspectives of using these dyes in diverse industrial applications. A direct photometric method for total ACY quantification using a low cost photometric device with a light sensitive detector was developed. Extracts of juçara (*Euterpe edulis Mart*), grapes (*Vitis vinifera L. 'Brazil'*) and jaboticaba (*Myrciaria cauliflora*) were analyzed and the concentrations obtained, in mg of anthocyanins per 100 g fruit, were: 956±43, 36.0±0.9 and 32.9±1.3 respectively. No statistically significant difference (95% confidence) between these results and those obtained by the official method involving spectrophotometric measurements was observed.

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Keywords: anthocyanins; quantification; photometric device; flowers and fruits.

1. Introduction

The chromophoric agents responsible for the color of flowers, fruits, leaves and roots are grouped into three major classes of dyes: porphyrins, carotenoids and flavonoids. Anthocyanins (ACY) are the main colorants of the flavonoid class and are responsible for colors including orange, pink, red, violet and blue [1].

ACY are mostly obtained from natural sources like fruits and vegetables [2]. The characterization of ACY extracts and the use of new ACY sources have been described for fruits such as jaboticaba, a native Brazilian fruit, and juçara [3], with prospects of several applications, including some of industrial interests. Currently, there is an increasing interest in using ACY for several purposes, mainly in the food and cosmetic industries, where they can be applied mainly as red dyes, whose stable natural options are limited, compared to synthetic versions which are often discouraged due to their toxicity [1-5].

In addition to the ACY color attributes, the interest in using these compounds has been intensified because of their possible health benefits. Scientific reports indicate the use of ACY to control blood pressure, diabetes and hypoglycemia. ACY are also promising agents in preventing degenerative diseases such as cancer and cardiovascular ones, probably due to their antioxidant properties [6-7].

The total concentration of ACY in crude extracts is principally determined by UV-Vis spectrophotometry. The differential pH method described by Fuleki and Francis [8-9] is an important method that constitutes the basis of the official method for total ACY quantification in concentrated fruits extracts [10].

New perspectives for industrial uses of ACY stimulate this analytical proposal to simplify their quantification in fruit extracts [11]. Prior work by one of the authors [12] inspired the quantification of ACY using a photometric device [13], characterized by simplicity and low cost.

2. Experimental

2.1. Samples, reagents, solutions and instrumentation

The fruits used as ACY sources were: juçara (*Euterpe edulis Mart*), grapes (*Vitis vinifera L. 'Brazil'*) and jaboticaba (*Myrciaria cauliflora*). Fresh fruits were obtained from specialized farmers of São Paulo State (Brazil) and were frozen in bottles (700 g) at approximately -11 °C until being used. To carry out the experimental procedures, portions of the samples were removed from the freezer and thawed at room temperature.

Reagents were all analytical grade. Anhydrous sodium acetate, hydrochloric acid (36.5 to 38.0 %), potassium chloride and ethanol (99.5 %) were purchased from Labsynth (Diadema, SP, Brazil). Deionized water was obtained from a Millipore Milli-Q Plus deionizer.

A solution of pH 1.0 was prepared by mixing aqueous solutions of 0.2 mol L^{-1} HCl and 0.2 mol L^{-1} KCl and a solution of pH 4.5 was prepared from the mixture of solutions of 2.0 mol L^{-1} CH_3COONa and 0.2 mol L^{-1} HCl, following the pH adjustment using a combined glass electrode.

A TE-8D Templette Techne ESRB-11 thermostatized bath was used for extraction procedures. The Pharmacia Biotech Ultrospech 2000 spectrophotometer and quartz cells of 1.0 cm optical path (Q4-Biocel) were used for spectrophotometric measurements. The photometric device and Duran glass tubes (solution holder for the device) were used for the photometric measurements.

2.2. ACY extraction

Crushed fresh fruits were mixed without stirring with a 94 % (v/v) aqueous ethanol solution in the ratio 1:3 (w/v) mass of fruit (g) to solvent volume (mL), at 55 °C for 30 minutes. Stable and highly concentrated extracts can be obtained under these conditions [14]. The mixtures were filtered using quantitative paper (14 µm, Qualy) and then dried under 6 L min^{-1} air flow for 24 hours [11].

2.3. Photometric data

The photometric device works on the principle of taking electrical resistance readings that are proportional to the photometric measurements. The device (Figure 1) consists of a Teflon® Graphite cell with a Light Dependent Resistor- LDR (5 mm diameter) as a photosensitive detector connected to a multimeter for measurements of resistance and a white light source. Five minutes are required to stabilize the device before the start of the measurements, checking blanks before every reading.

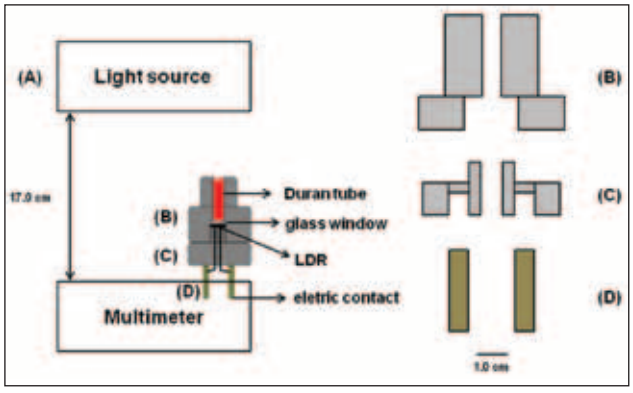

Figure 1. Scheme of the photometric device (A) and the Teflon® Graphite cell details in scaled zoom (B, C and D), adapted from [12].

The resistance readings represent the photometric measurements of the solution contained in the cell and, in this case, they are proportional to the total ACY concentrations in the extracts, analogous to the absorbance obtained from spectrophotometric measurements, used in the official method for total ACY quantification that recommends absorbance readings at 510 and 700 nm of the ACY extract at pH 1.0 and pH 4.5 [10]. So, first, in order to find a relationship between the absorbance measurements at these wavelengths and the photometric measurements, a series of successive dilutions of ACY stock solutions was carried out. The ACY stock solutions were obtained by direct dissolution of the dry extract of each fruit using solutions of pH 1.0 and pH 4.5, separately.

Aliquots of 1.0 mL of each different diluted pH solution were transferred to a Duran glass tube for reading the electrical resistance using the photometric device, exposed to a 15 W fluorescent lamp (Osram) according to Figure 1.

Absorbance measurements at 510 and 700 nm of these solutions were performed in a quartz cell of 1.0 cm optical path using the spectrophotometer.

A linear relationship between resistance and absorbance values was found for each fruit extract solution. Proportionality constants (P) were obtained and used to calculate the total ACY concentration from the photometric measurements.

2.4. Official method

The official method for total ACY quantification [10] considers the structural transformations of ACY with pH variation and expresses the concentration of total ACY as cyanidin 3-glucoside (cy 3-glu), which is the most common anthocyanin present in fruits [15]. Some fruit extracts may contain suspended material, which can cause light scattering and can be corrected by measuring the absorbance at 700 nm according to the official method. The 510 nm absorbance represents the absorption of the flavylium cation, the predominant form of ACY in acidic medium. The reading at pH 4.5 is used to correct the error caused by the possible presence of other colored compounds since ACY are colorless at this pH.

A known mass of dry extract of each fruit was dissolved with deionized water in a 50 mL volumetric flask. In sequence, aliquots of ACY extract (100 µL for juçara and 5.0 mL for grape and jaboticaba) were diluted to 10.00 mL with solutions of pH 1.0 and pH 4.5, separately. The absorbance measurements at 510 and 700 nm were taken for each ACY extract solution at the two different pH values using the respective solutions of known pH as blanks.

2.5. Photometric method

This proposed method is based on photometric measurements that are the resistance of the LDR detector positioned under a 3.3 cm column containing the colored sample solution (1.0 mL of solution in a Duran glass tube) and illuminated with the fluorescent lamp as a source of direct and continuous white light, as illustrated in Figure 1.

For spectrophotometric measurements, adjustments are required such as the initial calibration of 0 and 100% transmittance calibration. Photometric measurements also require initial adjustments. For the photometric measurements, solutions of known pH were used as blanks for resistance checking values before every working solution measurement. In practice, the cell was initially filled with the blank solution for its photometric reading, followed by the photometric reading of the solution of interest.

3. Results and discussion

According to the official method for total ACY quantification [10], solutions of the extracts must be diluted separately with solutions of pH 1.0 and 4.5 for absorbance measurements at the respective wavelengths to obtain a combined absorbance value, A_c, expressed in Equation 1. A_c is related to the concentration (C) of total ACY in the diluted extract by Beer's law, expressed as cyanidin 3-glucoside (by its molar absorptivity), as indicated in Equation 2.

$$A_c = (A_{510nm} - A_{700nm})_{pH1.0} - (A_{510nm} - A_{700nm})_{pH4.5} \quad (1)$$

where: A = absorbance measured at wavelength indicated in the subindex, A_c = combined absorbance.

$$A_c = \varepsilon_{(cy3\text{-}glu)} \times b_1 \times C \quad (2)$$

where: $\varepsilon_{(cy\ 3\text{-}glu)}$ = molar absorptivity of cyanidin 3-glucoside (26,900 L mol^{-1} cm^{-1} in aqueous solution at pH 1.0); b_1 = optical path (cm) of spectrophotometric cell; C = concentration of solutions (mol L^{-1}) of extract diluted in pH 1.0 and 4.5.

It is necessary to correlate the concentration (C) of these dilute solutions at pH 1.0 or 4.5 with the concentration of the solution obtained by dissolving the dry extract (C_i).

Since the total ACY concentration is usually expressed in terms of mass and not the quantity of matter, C_i mol L^{-1} must be converted to mg L^{-1} $C_{i(mg-L^{-1})}$, considering the molar mass of cy 3-glu and a factor of 10^3 to adjust the units. Considering that $C_{i(mg-L^{-1})}$ is the quotient between the total ACY mass in mg (m_i) and the volume of the initial solution, it may be obtained from m_i, the mass of ACY present in the portion of the dry extract mass (m_p) used to produce the stock solution. Thus, the total mass of ACY (m_t) present in the mass of dry extract can be calculated from Equation 3.

$$m_t = \frac{m_e \times m_i}{m_p} \quad (3)$$

where: m_t = total mass (mg) of ACY present in the dry extract; m_e = mass (g) of the total dry extract obtained after extraction; m_i = ACY mass (mg) contained in the stock solution; m_p = mass (g) of dry extract used to prepare the stock solution.

The total mass of ACY (m_t) is related to the mass of fruit initially used for the extraction (m_f) and the total concentration of ACY (C_t) in this fruit. To express the concentration of total ACY in terms of mg ACY in 100 g of fruit, it is necessary include the factor 100 as shown in Equation 4.

$$C_t = \frac{m_t \times 100}{m_f} \quad (4)$$

where: C_t = concentration of total ACY expressed as mg per 100 g of fruit; m_t = total mass (mg) present in all ACY dry extract obtained; 100 = factor used to express concentration of 100 g of fruit; m_f = mass (g) of fruit used in the extraction.

Equation 5 emphasizes the dilution of the extract, the mass and volumes used in the preparation of the solutions and of the initial extract to obtain the concentration in mg of ACY per 100 g of fruit.

$$C_t = \frac{m_e \times A_c \times V_f \times V_i \times M \times 10^5}{m_p \times m_f \times V_a \times \varepsilon_{(cy\ 3\text{-}glu)} \times b_1} \quad (5)$$

where: C_t = concentration of total ACY expressed as mg of ACY per 100 g of fruit; m_e = mass (g) of total dry extract obtained after extraction ; m_p = mass (g) of dry extract used for preparing the stock solution; m_f = mass (g) of the fruit used in the extraction; A_c = combined absorbance; V_a = volume of aliquot taken for dilution (mL); V_f = total volume of the diluted solution at pH 1.0 and 4.5 (mL); V_i = total volume of stock solution of extract (L); M = molar mass of cy 3-glu (449.2 g mol^{-1}); $\varepsilon_{(cy\ 3\text{-}glu)}$ = molar absorptivity of cy 3-glu (26,900 L mol^{-1} cm^{-1} in aqueous solution at pH = 1.0); b_1 = optical path (cm) of spectrophotometric cell.

For the photometric measurements, the arguments of Rossi *et al.* [12] are assumed to correlate the variation of the electrical resistance of the LDR detector with the concentration of an analyte in a colored sample solution as expressed in Equation 6, including an appropriate adjustment for considering cy 3-glu as the absorbing agent.

$$(Ri - Rb) = P \times C \times \varepsilon_{(cy\ 3\text{-}glu)} \times b_2 \quad (6)$$

where: Ri = resistance (ohm) of LDR with a sample solution; Rb = resistance (ohm) of LDR with blank; $\varepsilon_{(cy\ 3glu)}$ = molar absorptivity of cy 3-glu (26,900 L mol^{-1} cm^{-1}); P = proportionality constant (ohm); b_2 = pathlength (cm) of photometric device; C = concentration (mol L^{-1}) of the diluted extract solution.

A direct relation between the variation of resistance (Ri-Rb) and the combined absorbance (A_c) is assumed and the combination of Equations 2 and 6 results in Equation 7.

$$(Ri - Rb) = \frac{P \times b_2}{b_1} \times A_c \quad (7)$$

The experimental results fit a linear adjustment for the resistance data (Ri-Rb) with absorbance for each fruit extract, according to Equation 7. The proportionality constant, P, can be obtained from Equation 8.

$$P = \frac{S}{b_2} \times b_1 \quad (8)$$

where: P = proportionality constant (ohm); S = slope; b_2 = pathlength of photometric device (cm); b_1 = optical path of spectrophotometer (cm).

Table I. Experimental photometric and spectrophotometric data.

Fruit	Spectrophotometric measurements Official method for quantifying total ACY [10]					Resistance measurements (0hm)
	pH 1.0		pH 4.5		A_c	pH 1.0
	510 nm	700 nm	510 nm	700 nm		$R_i - R_b$
juçara	1.31 ± 0.03	0.015 ± 0.003	0.17 ± 0.02	0.017 ± 0.003	1.14 ± 0.04	4.0 ± 0.1
jaboticaba	1.756 ± 0.003	1.137 ± 0.003	0.433 ± 0.003	0.161 ± 0.002	1.347 ± 0.005	6.4 ± 0.2
grape	2.82 ± 0.02	0.68 ± 0.01	1.023 ± 0.001	0.680 ± 0.001	1.79 ± 0.02	16.3 ± 0.3

A_c =combined absorbance; R_i = resistance of LDR with sample solution; R_b = resistance of LDR with blank; 6 replicates.

It was necessary to find the best way to correlate the photometric and spectrophotometric data, since the official method involves quantification of absorbance measurements at two different pH values and two different wavelengths [10]. The photometric measurements for the ACY solutions at pH 4.5 were constant and varied at pH 1.0, so we chose the measurements of the solutions at pH 1.0. Several options to fit the photometric and spectrophotometric data of Table I were tested and the best results are present on Table II, including the proportionality constant values used in calculations.

Table II. Proportionality constant (P) values and linear fittings parameters (7 data points).

Fruit	P (0hm)	Linear fit	Regression Coefficient
juçara	1.10 ± 0.08	$R_i - R_b = 3.64 \times A_c + 1.18$	0.9875
jaboticaba	1.48 ± 0.04	$R_i - R_b = 4.88 \times A_c - 0.08$	0.9999
grape	2.74 ± 0.13	$R_i - R_b = 9.04 \times A_c - 0.53$	0.9937

A_c =combined absorbance; R_i = resistance of LDR with sample solution; R_b = resistance of LDR with blank; 6 replicates.

The concentration of total ACY can be calculated with resistance measurements similar to that described for the official method.

Substituting all known values and constants in Equation 9 to calculate the total concentration in mg of ACY/100 g of fruit expressed as cy 3-glu, from measurements with the photometric device for each fruit studied.

$$C_t = 5.10 \times 10^2 \times \frac{(Ri - Rb) \times m_e \times V_f \times V_i}{P \times m_p \times m_f \times V_a} \quad (9)$$

where: C_t = total concentration of ACY expressed in mg of ACY/100 g of fruit; m_e = mass (g) of the total dry extract obtained after extraction; m_p = mass (g) of dry extract used to prepare the stock solution; m_f = mass (g) of the fruit used in the extraction; Ri = resistance (ohm) of the LDR with the solution; Rb = resistance (ohm) of the LDR with blank; P = proportionality constant (ohm); V_a = volume (mL) of aliquot taken for dilution; V_f = total volume (mL) of the diluted solution at pH 1.0 and 4.5; V_i = total volume (L) of stock solution extract.

The results obtained by the proposed method with photometric measurements and the official method [10] do not show statistically significant differences with 95% confidence (Snedecor F test and paired Student *t* test) for all fruits studied, as indicated in Table III.

Table III. Experimental results of total ACY concentration.

Fruit	Concentration of total ACY (mg ACY per 100g of fruit)		Statistical tests [16]*	
	Official Method	Proposal Method	$t_{calculated}$	$F_{calculated}$
juçara	984 ± 29	956 ± 43	-1.34	2.20
jaboticaba	34.0 ± 0.1	32.9 ± 1.3	-2.13	0.01
grape	35.7 ± 0.3	36.0± 0.9	0.76	0.15

*$t_{critical}$ = 2.23; $F_{critical}$ (two tailed) = 5.05; (p=0.05) [16].

4. Conclusions

The proposed method gives the total ACY concentration using fruit extracts conditioned only at pH 1.0 and a simple and inexpensive photometric device, potentially useful for exploring new ACY sources in areas of high biodiversity and poor laboratorial infrastructure.

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Evaluation of a modified QuEChERS method for the extraction of insecticide and fungicide residues in processed peaches

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Abstract

This paper reports the evaluation of a modified QuEChERS method with determination by liquid chromatography coupled with diode array detector for the simultaneous determination of fungicides (azoxystrobin and dichloran) and insecticides (malathion and fenthion) in processed peaches. To evaluate the linearity, recovery, process efficiency and matrix effect, three calibration sets consisting of a curve in neat solvent, matrix matched calibration curve and working curve, were prepared. Good linearity with correlation coefficient (r) higher than 0.99 was obtained for all compounds. The recoveries varied from 75 to 118% with relative standard deviations (RSD) from 0.4 to 19.9%. For the inter-day precision, the recoveries varied from 78 to 104% while the RSD ranged from 1.5 to 13.1%. The limits of detection varied between 0.03 and 0.6 mg kg^{-1} and the limits of quantification ranged between 0.1 and 2.0 mg kg^{-1}. Thus, the proposed method is robust, rapid and selective, with a simple sample preparation procedure that can be used for the convenient and effective determination of the analytes in processed peach samples.

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1. Introduction

Peaches and nectarines are widely cultivated in the world. Globally, China is by far the largest producer of peaches and nectarines, accounting for approximately 50% of the world production, followed by Italy, Spain and the USA [1]. In Brazil, peaches are produced from the northeast to the extreme south of the country. Rio Grande do Sul state, which yielded 129,295 tons in 2011, stands out as the largest producer in the southern region. Pelotas city is one of the largest production centers, totaling about 24,000 tons and the most important supplier of processed peaches [2-4].

Peaches are frequently exposed to the attack of insects and fungus, a fact that results in significant harvest losses and decreased fruit quality [5]. In Brazil, the peach culture has recorded the presence of 43 active substances that belong to several chemical groups with different toxicities [6,7]. One of the most common routes of pesticide exposure in consumers is via food consumption [8]. Combined exposures to different pesticide residues could therefore occur as a consequence of intake from a single food item containing multiple residues or from several food items containing one or more residues. The consequence of such combined exposure has raised concerns amongst both consumers and regulators. Thus, processed foods have been under investigation for pesticide residues [9,10,11].

Processed peaches are rich in sugars and carbohydrates [12]. Therefore, they are considered a complex matrix, which can make the extraction procedure difficult, interfere in the detector response, affect the analysis precision and method selectivity and generate a high matrix effect [13,14].

For the determination of pesticide residues in food samples, chromatographic techniques have been used [15,16,17]. These compounds are usually analyzed by gas chromatography (GC) and/or liquid chromatography (LC) coupled to various detection systems. LC with conventional UV detection, diode array detector (DAD) or fluorescence detection, often combined with tandem mass spectrometry (MS/MS), are the preferred techniques for polar and thermally labile pesticide detection [18]. According to the literature, the use of LC-MS/MS has been proposed to determine pesticides. These analytical techniques are more specific and sensitive, but they are not accessible in most research laboratories because of the high cost of the equipment [19]. However, LC, coupled with a DAD detector, is a

complementary technique which supplies the spectra of the compound, thus, providing an additional way to confirm peak identity, to check peak purity and the quantity of the compounds [20]. Besides, sample preparation techniques are a fundamental step to ensure the efficiency of analytical procedures, especially in the analysis of trace compounds in foods. Several techniques have been developed for pesticide residue extraction from a wide range of foodstuff and other agricultural products, such as liquid–liquid extraction (LLE) [21,22], matrix solid-phase dispersion (MSPD) [23], accelerated solvent extraction (ASE) [24,25], ultrasound extraction (USE) [26], supercritical fluid extraction (SFE) [27,28], solid-phase microextraction (SPME) [29], dispersive liquid-liquid microextraction (DLLME) [30] and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) [31,32]. The QuEChERS method, developed by Anastassiades and collaborators in 2003, has been used as an alternative for sample preparation aiming at the analysis of multiple pesticide residues in fruits and vegetables because of its simplicity, low cost, relatively high efficiency and minimal number of steps [33,34].

The basic concept of QuEChERS is that the procedure is a template that can be adapted to users' needs, with methods that use acetate buffering (AOAC Official Method 2007.01) [35,36] or citrate buffering (CEN Standard Method EN 15662) [37].

The aim of this study was to evaluate a modified QuEChERS method with determination by LC-DAD for the simultaneous determination of fungicides (azoxystrobin and dichloran) and insecticides (malathion and fenthion) in processed peaches.

In this study, the extraction method reduces the use of reagents and does not require the clean-up step. In addition, a detector that is common to most laboratories is employed; thus, the cost is decreased by comparison with a mass spectrometer.

The developed method was subsequently applied to the determination of insecticide and fungicide levels in processed peaches.

2. Experiment

2.1. Chemicals

Azoxystrobin, dichloran, fenthion and malathion analytical standards (purity>99%) were supplied by Sigma Aldrich (Steinheim, Germany). The chemical structures of the pesticides under investigation and their physicochemical properties are listed in Table I.

Anhydrous magnesium sulfate was purchased from J.T.Baker (Phillipsburg, NJ, USA), HPLC grade methanol and acetonitrile were supplied by Mallinckrodt Baker (USA). Water was purified with a Direct-Q UV3® (resistivity 18.2 MΩ cm) water purification system (Millipore, Bedford, MA, USA).

2.2. Pesticide selection

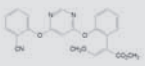
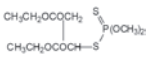
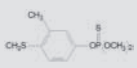
The pesticides were selected among 43 active ingredients whose use is authorized for peach cultivation by *ANVISA* (*Agência Nacional de Vigilância Sanitária*) [7] and *Emater* (*Empresa de Assistência Técnica e Rural*) [4]. These agencies have provided information about the most commonly used pesticides in peach production in the region under study. The availability of standards in the laboratory was also taken into account.

Dichloran and azoxystrobin, moderately toxic fungicides, are often applied to peach crops. Fenthion and malathion are organophosphate insecticides which are toxic to animals and humans [39]. Besides, malathion has been frequently detected in different kinds of food [40,41,42,43,44].

2.3. Instruments

Separation was performed using an LC apparatus consisting of a Waters 600 pump, associated with a Waters 2996 photodiode array detector, Rheodyne 20 µL loop injector, connected to Empower PDA software for data acquisition. The analytical column was operated at room temperature.

Table I. Pesticides under investigation and physicochemical properties[38]

Pesticide	Chemical structures	Molar Mass (g)	Chemical group	CAS number	Toxicity[a]	K_{ow}	Solubility in water (mg L^{-1})	Vapor Pressure (mPa)
Dichloran (fungicide)		207.0	Aromatic hydrocarbon	99-30-9	III	2.8	6.3	0.16
Azoxystrobin (fungicide)		403.4	Strobilurin	131860-33-8	III	2.5	6.0	1.10 10^{-7}
Malathion (insecticide)		330.4	Organophosphate	121-75-5	III	2.7	145	5.3
Fenthion (insecticide)		278.3	Organophosphate	55-38-9	II	4.8	4.2	0.74

[a]II- Highly toxic; III- Moderately toxic

The identification of the pesticides in the samples was accomplished on the basis of their retention times. The DAD spectrum of the compounds in the standard solutions and the DAD spectrum of the detected peaks in the samples were also compared, as well as realizing peak purity tests.

2.4. Preparation of solutions and mobile phases

Individual pesticide stock solutions containing 1000 mg L^{-1} of the target compounds were prepared in methanol and stored at -18 °C. Intermediate working standard mixtures in acetonitrile, containing 100 mg L^{-1} and 10 mg L^{-1}, were prepared and used for spiking samples and for preparing the analytical curves. Working standard solutions were prepared monthly, while the dilutions used for the analytical curves were prepared daily. The mobile phase was filtered through a nylon membrane with 0.45 µm pore diameter (Millipore, Brazil) and degassed for 30 min in an ultrasonic bath before use.

2.5. Peach samples

Peach samples, which were used for method validation, were bought in a local market. The samples for the method applicability were collected considering a percentage of 20% of all cans of processed peaches found on the shelves, from different manufacturers.

The samples were produced in Pelotas city, main producer and supplier of processed peaches in Rio Grande do Sul state, Brazil.

The liquid contained in the cans of the processed peaches was drained; the processed peaches were crushed and homogenized in a food processor (Mega Master Plus RI 3170) and stored at -18 °C.

2.6. Modified QuEChERS sample preparation method

A modified QuEChERS method was employed for the extraction of pesticides from the processed peach samples. Ten g of the crushed and homogenized sample was weighed in a 50.0 mL PTFE centrifuge tube and spiked with the standard solution (except for the matrix matched calibration, in which analytes were added after the QuEChERS procedure and in the blank matrix extract). For the extraction, 10.0 mL acetonitrile was added and hand-shaken for 15 s; after that, it was shaken vigorously in a laboratory shaker (Certomat MV-B Braun) for 1 min. Afterwards, to induce phase separation and pesticide partitioning, 4 g anhydrous magnesium sulfate was added; the mixture was immediately hand-shaken for 15 s and, then, vigorously in a laboratory shaker for 1 min. Finally, it was centrifuged at 5,000 rpm for 3 min [31]. Twenty µL of the extracts was then injected into the LC-DAD system. All experiments were performed in triplicate.

2.7. Validation parameters

The method was validated following the EU established guidelines on quality control procedures for pesticide residues analysis, according to the EU Document nº 12495, and the Brazilian guidelines recommended by the Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO) [46]. The method was validated by evaluating limits of detection (LOD) and quantification (LOQ), linearity, accuracy, intra-day (RSD_i) and inter-day precision (RSD_{ip}), matrix effect (ME) and process efficiency (PE).

3. Results and discussion

3.1. Optimization of the chromatographic separation

To determine the optimum conditions of separation, three columns were tested: Spherisorb ODS2, 5 µm, 80 Å (150 × 4.6 mm), Waters; Kromasil C18, 3.5 µm, 100 Å (10 × 4.6 mm), Hichrom; and Hypersil BDS C18, 5 µm, 120 Å (250 × 4.6 mm), Thermo Scientific.

Different mobile phases comprising several combinations of methanol, acetonitrile and purified water were tested to provide sufficient separation. In the search for a condition that would enable good separation of the compounds, selectivity and retention times, several mixtures of methanol, acetonitrile and ultrapure water were tested. A mixture using 65% content of methanol was not satisfactory because the first compound was detected after a period of around 10 min. Since run time is very important, the target was a shorter run time. By using gradient elution, many oscillations were observed in the baseline, as well as co-elution of some compounds with some matrix components.

By employing elution in the isocratic mode, programming the flow rate during the analysis and using the Hypersil BDS C18 analytical column, the peaks showed good resolution, acceptable retention times and selectivity. The effective separation of the peaks in the chromatogram was achieved when the mobile phase composition was acetonitrile:water (60:40, v/v). The flow rate programming was 0.8 mL min^{-1} for 8 min; then, increased to 1.0 mL min^{-1} up to 16 min and decreased to 0.8 mL min^{-1} up to 18 min.

Because the samples contain a relatively high concentration of sugars and carbohydrates that produce high DAD response, some substances of the matrix were detected in periods before 7 min, but these interferences did not influence the response of the compounds. DAD was operated recording the spectra of the compounds from 190 to 400 nm, simultaneously. Quantification was carried out with DAD detection at 230 nm for azoxystrobin, dichloran and malathion and 250 nm, for the insecticide fenthion.

3.2. Validation parameters

The limit of detection was estimated considering the LOD as 3 times the baseline noise and the LOQ as the concentration that produced a signal 10 times the baseline noise, in a period close to the retention time of the analyte [47]. The LOD values for the pesticides under study were 0.03 mg kg^{-1} for azoxystrobin, dichloran and fenthion and

0.6 mg kg^{-1} for malathion. The LOQ values were 0.1 mg kg^{-1} for azoxystrobin, dichloran and fenthion and 2.0 mg kg^{-1} for malathion (Table II).

Table II. Limit of Detection (LOD), Limit of Quantification (LOQ) and Maximum Residue Limit (MRL) for selected pesticides in peach in mg kg^{-1}

Pesticides	LOD	LOQ	MRL (ANVISA)[7]	MRL (CODEX-EUA)[48]	MRL (European Commission)[49]
Dichloran	0.03	0.1	20	7	0.1
Azoxystrobin	0.03	0.1	0.5	---	2.0
Malathion	0.6	2.0	6.0	---	0.02
Fenthion	0.03	0.1	0.05	---	0.01

According to Table I, malathion has few chromophore groups in its structure, a fact that makes the UV absorption lower than the absorption of the other compounds, resulting in a higher LOQ. In almost all cases, LOQ values were below the Maximum Residue Limits (MRLs) established by the *Agência Nacional de Vigilância Sanitária (ANVISA)* for peach (fresh fruit). Is it important to highlight that there are no limits in the European, American and Brazilian legislations for processed peaches, only for the fresh fruit. Among the pesticides under study, only dichloran has MRL established in the CODEX alimentarius (Table II).

To assess the linearity, recovery, process efficiency and matrix effect, three sets were prepared, as described below [13]. In each set, five concentrations levels were used, corresponding to 0.1, 0.5, 1.0, 2.5 and 5.0 mg kg^{-1} for the compounds dichloran, azoxystrobin and fenthion and 2.0, 4.0, 6.0, 8.0 and 10.0 mg kg^{-1} for malathion.

Set 1 – preparation of the analytical curve in solvent; the standard solution containing the mixture of four analytes was directly added to acetonitrile; based on the collected data, the overall chromatographic system and detector performance can be assessed.

Set 2 – the sample extract after the QuEChERS step was spiked with the standard solution at the same levels as in set 1. The relationship between the values of the peak areas of the analytes obtained in set 1 and 2 reveals if there is a matrix effect acting on the quantification of the analysis.

Set 3 – the samples were spiked in five different concentration levels before the extraction with the QuEChERS method.

3.1.1 Linearity

For all compounds, analytical curves prepared in sets 1, 2 and 3 showed correlation coefficients higher than 0.99; thus, results were satisfactory.

3.1.2 Accuracy (R%) and precision (intra-day and inter-day)

The accuracy of the analytical method was expressed in terms of recovery (R%) and precision, in terms of intra-day (RSD_r) (n=9) and inter-day precision (RSD_{ip}) (n=9). The recovery was determined by comparing the mean peak areas of each compound obtained in set 3 to those obtained in set 2 (equation 1), in three concentration levels selected from the linear range of each one of the pesticides under study (low, medium, high) and injected into the chromatographic system in triplicate.

$$R(\%) = \frac{set\ 3}{set\ 2} \times 100 \quad (1)$$

The recovery determined by the relationship shown in Equation 1 is a "true" recovery value that is not affected by the matrix [13].

Intra-day was expressed as relative standard deviation (RSD_r) and was evaluated by carrying out the extraction and analysis of spiked samples on the same day and by the same analyst. Each spike level was extracted in three replicates and each extract was injected three times. To evaluate the inter-day precision of the method, different days and operators were used, only for spiked levels of 0.1 and 1.0

Table III. Working curve, recovery (R%), intra-day and inter-day precision of the selected pesticides in spiked processed peach samples

Analyte	Working curve (n=3)		Recovery and intra-day precision (n=9)			Inter-day precision	
	analytical curve	r	Spike Levels (mg kg^{-1})	R%	RSD_r (%)	R%	RSD_{ip} (%)
Dichloran	$y = 3.30\ 10^{+4}x + 1.45\ 10^{+3}$	0.998	0.1	105	19.9	83	11.0
			0.5	102	4.2	----	----
			1.0	75	4.0	87	2.3
Azoxystrobin	$y = 6.53\ 10^{+4}x + 2.09\ 10^{+3}$	0.999	0.1	84	2.2	84	13.1
			0.5	101	2.7	----	----
			1.0	77	2.6	87	2.2
Malathion	$y = 5.12\ 10^{+3}x - 4.38\ 10^{+3}$	0.992	2.0	118	11.1	98	2.1
			4.0	83	3.8	----	----
			6.0	89	2.7	87	1.5
Fenthion	$y = 3.32\ 10^{+4}x - 1.26\ 10^{+3}$	0.999	0.1	81	10.9	104	3.7
			0.5	89	7.7	----	----
			1.0	78	0.4	78	4.1

mg kg^{-1} for dichloran, azoxystrobin and fenthion and levels of 2.0 and 6.0 mg kg^{-1} for malathion.

The recoveries varied from 75 to 118% with RSD below 20% for all compounds. In case of inter-day precision, the recoveries varied from 78 to 104% while the RSD ranged from 1.5 to 13.1%. These results, which comply with SANCO [45] and ANVISA, are shown in Table III.

Overall, the results for intra-day and inter-day precision, linearity and accuracy suggest that this method is acceptable for the analysis of the selected pesticides in processed peach samples. Typical chromatograms of the spiked peach sample, extracted using the proposed QuEChERS method **(a)** and of the blank peach sample extract after the QuEChERS method **(b)** indicate the absence of interfering peaks at the same retention times as the analytes as well as good separation of the measured compounds, thus, indicating good selectivity (Fig. 1).

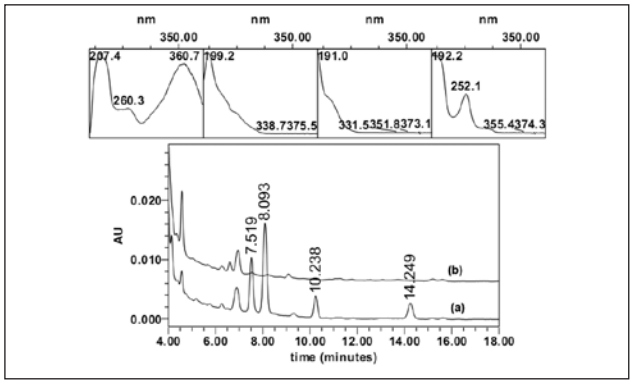


Figure 1. LC-DAD chromatograms and the identification spectra of the detected compounds of: (a) spiked peach sample; (b) blank peach sample extract after the QuEChERS method. The concentration of the pesticides dichloran (t_R 7.519 min), azoxystrobin (t_R 8.093 min) and fenthion (t_R 14.249 min) was 2.5 mg kg^{-1}, and 8.0 mg kg^{-1} for malathion (t_R 10.238 min), obtained at a wavelength of 230 nm

3.1.3 Matrix effect

Several factors affect the matrix effect, such as the chemical structure, concentration of analytes, time of interaction, the kind and concentration of the matrix [34].

In food samples, the recovery rate is not always satisfactory due to the fact that the matrix effects of the chromatographic analysis can lead to erroneous estimates of the levels of analytes [50,51]. The quantification of compounds can be affected by the presence of co-extracted matrix compounds. To compensate the matrix effect, the use of an analytical curve prepared in matrix extracts free of pesticides is recommended [52].

The existence of the matrix effect was evaluated by comparing the areas of pesticides in the blank matrix extract spiked after extraction (set 2) and the standard prepared in the solvent (set 1), according to Equation 2 [13].

$$ME(\%) = \frac{set\ 2}{set\ 1} \times 100 \quad (2)$$

The ME calculated in this manner may be referred to as an "absolute" matrix effect. This aspect of the matrix effect assessment is highly relevant for the development of selective chromatographic methods [13].

ME values of 100% indicate that there is no matrix effect; values above 100% indicate signal enrichment and must be determined carefully. When values are below 100%, there is signal suppression [53].

Despite the complexity of the matrix, a significant matrix effect was not observed, due to the chromatographic separation, free from interferences, at the same retention times as the analytes under investigation. The values for matrix effect observed were between 87-105% (Fig. 2)

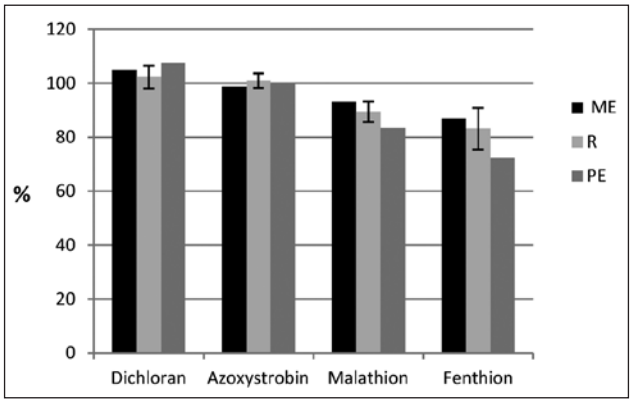


Figure 2. Relations between ME (%), R (%) and PE (%) for the analytes dichloran, azoxystrobin and fenthion at concentration levels of 0.5 mg kg^{-1} and for malathion at 4.0 mg kg^{-1}. Error bars indicate RSD values (%)

3.1.4 Process Efficiency

The process efficiency (PE) characterizes the overall performance of the proposed method and is defined by the comparison of the peak areas obtained in sets 1 and 3. It shows a matrix effect combined with the recovery of the analytes. The process efficiency was calculated according to Equation 3 [13].

$$PE(\%) = \frac{set3}{set1} \times 100 \text{ or } PE\ (\%) = \frac{ME \times R}{100} \quad (3)$$

PE (%) values around 100% generally indicate that both matrix effect and recovery are around 100%. Generally, signal enrichment caused by matrix effect causes an increase in process efficiency and signal suppression can cause low efficiency in the process, even with high recoveries [54].

In Fig. 2, the relations between ME (%), R (%) and PE (%) for the analytes dichloran, azoxystrobin and fenthion at concentration levels 0.5 mg kg^{-1} and for malathion, at 4.0 mg kg^{-1}, are shown. As can be seen, the process efficiency was higher than 70% for all compounds, indicating that the process was efficient with good recoveries and without any matrix effect.

Table IV. Comparison of the proposed method with published methods for the extraction and determination of pesticides in fresh peaches

Sample preparation	Solvent / volume	Determination	Recovery / Precision	LOQ (mg kg^{-1})	Reference
Solvent extraction	Ethyl acetate / 100 mL Methanol / 20 mL	LC-MS/MS	63-96% / 20%	0.005 – 0.8	55
Solvent extraction	Ethyl acetate / 250 ml Methanol / 10 mL	LC-APCI-MS	64-108% / < 14%	0.02	56
QuEChERS + d-SPE	Acetonitrile / 10 mL	GC-MS	85-101% / <5%	0.01-0.1	34
acetone and dichloromethane	Acetone / 30 mL light petroleum: dichloromethane (1:1, v/v) / 60 mL	GC-MS	73-145% / < 5%	0.02 – 0.2	57
PLE	Ethyl acetate / 22 mL	LC-IT-MS	58-97% / < 19%	0.025 – 0.25	25
QuEChERS	Acetonitrile / 10 mL	HPLC-DAD	75-118% / <20%	0.1 – 2.0	Proposed method

3.3. Comparison of the proposed method with previously published results

A comparison between the currently proposed method and previously published ones was made. It is important to point out that previous studies deal with the determination of pesticides in fresh fruit and not processed ones, as in the case of this study. Table IV illustrates some differences among the methods. It can be concluded that the proposed method has lower solvent and reagent consumption. In addition, although the achieved LOQs are similar or higher than the published ones, in this study the determinations were carried out in a HPLC-DAD, which is a detector common to most laboratories; thus, the cost is decreased by comparison with a mass spectrometer. Besides, the technique employed in this study is simpler and cheaper than a LC-MS or GC-MS.

3.4. Applicability of the method

The applicability of the method was verified by the analysis of three different brands of processed peaches.

In order to verify the possibility of pesticide transfer to the syrup during the conservation and storage of the product, an application using the matrix containing the syrup over the peaches was performed.

Considering the target pesticides no pesticides were detected in the samples, within the analyzed linear range (Fig. 3).

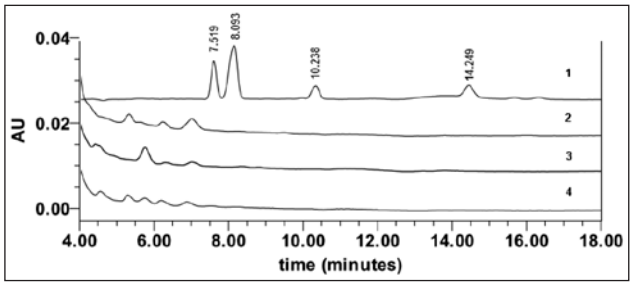


Figure 3. Chromatograms at 230 nm for standard solutions in acetonitrile (1) at concentration level 2.5 mg kg^{-1} for dichloran, azoxystrobin, fenthion and 8.0 mg kg^{-1}, for malathion. Chromatograms 2,3 and 4 represent processed peach samples for brands "A", "B" and "C", respectively.

4. Conclusions

This paper describes the development and validation of a multiresidue method for reliable and low-cost separation and simultaneous determination of four pesticides commonly used in peach, cultivation.

The results show that extraction with the modified QuEChERS method and determination by LC-DAD is effective, precise and accurate for the determination of azoxystrobin, dichloran, malathion and fenthion in processed peaches.

The modified QuEChERS method under analysis, developed for onion samples [31], was shown to be robust. When applied to processed peach samples, the method presented excellent results for all compounds. Moreover, the extraction procedure is extremely fast and does not require use of any purification step.

The short analytical run time of 18.0 min leads to effective cost and fast chromatographic procedure. Thus, the proposed method is rapid and selective with a simple sample preparation procedure that can be used for the convenient and effective determination of pesticide residues in processed peach samples.

To sum up, the method, which was successfully used for the determination of four pesticides in processed peach samples, proved to be very useful.

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Analytical Chemistry in Uruguay: A review (2000 – 2012)

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Abstract
A bibliographic review of the production of the Uruguayan scientific community in Analytical Chemistry and related fields, during the period 2000 – 2012 was performed.
The search was carried out using the TIMBO portal (www.timbo.org.uy) available in Uruguay as a universal access to online scientific literature and international technology patents banks. A total of 38 journals were selected taking into account the publications of the considered period in Analytical Chemistry and from this search a total of 90 publications were obtained. It was found that 51% of the papers published in this period exhibit cooperation with other countries, mainly with Spain and Brazil.
This review shows that Uruguayan scientific production in Analytical Chemistry has been increasing and also that there are contributions of Uruguayan researchers groups in high impact journals.

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Keywords: Uruguay, scientific production, Analytical Chemistry.

Introduction

Uruguay is a South American country situated between Argentina, Brazil and the Atlantic Ocean [1]. It is small if compared to its neighbors, with an area of 176.215 km^2, and a population of about 3.3 million inhabitants according to the latest census [2]. Exhibiting a high Human Development Index of 0.783 [1] its population is considered well educated and presents a culture strongly influenced by its European origins.

Higher education in Uruguay has for a long time been provided by the state-supported Universidad de la República (UdelaR), which was in fact the only university in the country until 1984, when the Catholic University (Universidad Católica del Uruguay, UCU) was founded, this being the first private university recognized in the country.

Currently, despite the existence of several private universities in the country, UdelaR, founded in 1849 and with about 80.000 students remains the most influential organization in terms of higher education and scientific research. Given that private universities are devoted mainly to the humanities, social sciences, engineering and informatics, UdelaR remains the only university in Uruguay where Chemistry is taught, a fact that is reflected in the high participation of its scientists in the total amount of published papers dealing with chemistry and related disciplines.

Other public institutions however do share the effort in chemistry-related research, among them the Laboratorio Tecnológico del Uruguay, LATU (National Technology Laboratory), the Instituto Nacional de Investigación Agropecuaria, INIA (National Institute of Agricultural Research), the Instituto Clemente Estable, and the Instituto Pasteur de Montevideo, the latter two dedicated to different aspects of biosciences.

Within the Universidad de la República, the School of Chemistry was officially created in 1929 but was not involved in scientific research for a long time. According to some authors, the modern concept of research in Chemistry was introduced into Uruguay by Giovanni Marini Bettolo, an Italian researcher who visited the School in 1948 and in subsequent years [3,4]. According to these authors, the first group of Uruguayan researchers in chemistry was formed from this contact. Under their influence, the next generations of Uruguayan researchers in chemistry were educated, including some distinguished names such as the late Tomás Hirschfeld, an outstanding scientist well known for his achievements in the fields of analytical infrared spectrometry [5,6].

The present work attempts to review the production of the Uruguayan scientific community in analytical chemistry and related fields, during the period 2000 – 2012.

Analysis of the references show two clearly different types of papers, i.e., those where the focus of the work is the development of analytical techniques / methods, and those where already established analytical methods are applied to obtain results which are used for a certain purpose.

Most of the papers can be classified as belonging to the second type, perhaps reflecting an early stage in the evolution of the analytical community.

Concerning the analytical techniques used, a significant number of papers deal with separation techniques such as gas and liquid chromatography, generally associated with selective detection techniques such as MS [7-37]. Many of these papers originate in research groups engaged in the determination of pesticides in food and other matrices.

Spectrochemical techniques are widely used, appearing frequently in the publications, with special emphasis on atomic spectrometry (AAS, ICP-OES, ICP-MS) [38-52] and near infrared (NIR) spectrometry [53-62], the latter applied to the analysis of different types of foods.

Flow analysis and automation is a consolidated research line in Uruguay, and papers on these subjects appear frequently. Several techniques such as flow injection analysis (FIA) [63-70], multicommutated flow analysis (MCFA) [71-76] and sequential injection analysis (SIA) [77-80] have been used for the determination of organic and inorganic analytes. Several papers dealing with the flow analysis of lubricating oils also show a specific application of these techniques [63, 67, 68, 69, 71].

Lately, new research lines have evolved, focusing on the development of different types of biosensors based on the use of nanoparticles and electrochemical detection [81-85].

Some interesting experiments for teaching analytical chemistry in university laboratories were published in educational journals [86-88].

Other techniques involve the use of immunoassays, spectrofluorimetry, NMR and chemometrics among other miscellaneous topics [89-97].

Methodology

The search was performed using the TIMBO portal (http://www.timbo.org.uy/) available in Uruguay as a universal access to online scientific literature and international technology patents banks. Scopus was the main bibliographic database consulted, along with Scirus and Google Scholar.

A total of 38 journals were selected taking into account the publications of about the last ten years in Analytical Chemistry.

The selected journals were: Journal of Agricultural and Food Chemistry, Talanta, Meat Science, Analytical and Bioanalytical Chemistry, Biosensors and Bioelectronics, Journal of Chromatography A, Agricultura Técnica, Journal of Chemical Education, Environmental Geochemistry and Health, Journal of Automated Methods and Management in Chemistry, Atomic Spectroscopy, Journal of Pharmaceutical and Biomedical Analysis, Journal of Food Composition and Analysis, Analytical Methods, Journal of Near Infrared Spectroscopy, Journal of Environmental Science and Health - Part B, Avances en Ciencias e Ingeniería, Journal of Chromatography B, Food Chemistry, Analytica Chimica Acta, European Food Research and Technology, Analytical Chemistry, Applied Spectroscopy Reviews, Archives of Environmental Contamination and Toxicology, Atmospheric Environment, Bulletin of Environmental Contamination and Toxicology, Environmental Geology, International Journal of Environmental Research and Public Health, International Journal of Analytical Chemistry, Journal of Chromatographic Science, Journal of Electroanalytical Chemistry, Journal of Food Engineering, Livestock Production Science, Plant Physiology and Biochemistry, Soil &Tillage Research, Trends in Analytical Chemistry, Spectrochimica Acta B and Journal of the Science of Food and Agriculture.

From this search a total of 90 publications were obtained.

Results and Discussion

The total population in Uruguay is 3.3 million inhabitants according to the latest census [2] and the number of researchers per million inhabitants increased from 277.7 in 2000 to 346 in 2008 [98], showing an important development of scientific research in the last decade. This is reflected in the increase of publications per year in the area of Analytical Chemistry, as shown in Figure 1, however it is still a small discipline in relation to the country's scientific community.

It is noteworthy that 51% of the papers published in the period considered correspond to work carried out in cooperation with other countries, mainly with Spain and Brazil. Distribution of the collaboration with other countries is shown in Figure 2.

The search employing the TIMBO portal (www.timbo.org.uy) showed that the 78% of the publications were from Universidad de la República (UdelaR), which means that at least one of the authors belongs to the public university.

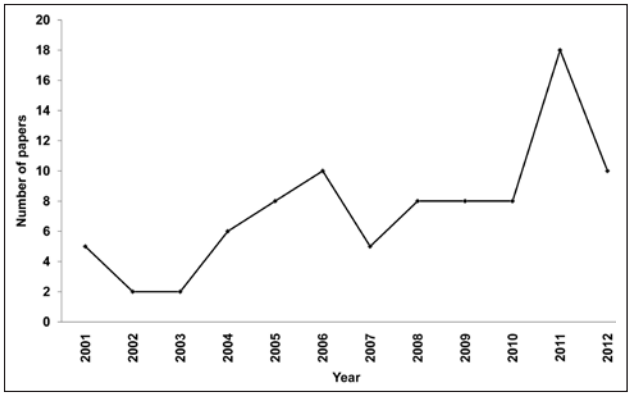

Figure 1. Evolution of the number of papers published in the last decade

Two of the most productive groups in terms of publications in Analytical Chemistry in the last five years are those devoted to the investigation of organic contaminants at trace levels in food, and to flavour analysis and enology.

Hence the significant number of publications in journals such as the Journal of Agricultural and Food Chemistry, Journal of the Science of Food and Agriculture and Food Chemistry, among others.

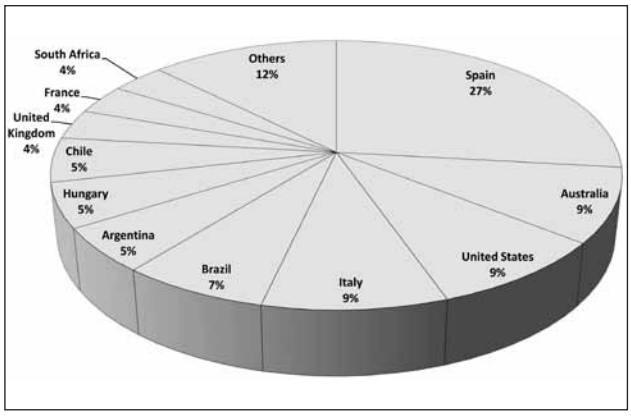

Figure 2. Cooperation with other countries in terms of publications in the last decade

Publications produced by groups dedicated to analytical automation and development of flow analysis systems can be found mainly in Talanta and Journal of Automated Methods and Management in Chemistry and, to a lesser extent, in the Journal of Pharmaceutical and Biomedical Analysis, and Analytical Methods, among others. Other important research areas in Uruguay are related to environmental health, toxicology and electroanalytical chemistry.

The distribution of the 90 selected publications is summarized in Table I. This distribution also shows an interesting number of publications in Meat Science, a fact that is in good agreement with the application of Analytical Chemistry in topics related with one of the main productive activities of the country. The same observation can be made regarding the publications in Agricultura Técnica.

While the number of publications is not high in the last decade, the highest percentage of them were published in journals whose ISI impact factor goes from 2.275 to 5.602. This shows that high impact journals in Analytical Chemistry include the contribution of Uruguayan researchers.

Table I. Publications in the selected journals

Journal	Percentage of publications
Journal of Agricultural and Food Chemistry	14
Talanta	10
Meat Science	6
Analytical and Bioanalytical Chemistry	6
Biosensors and Bioelectronics	4
Journal of Chromatography A	4
Journal of Automated Methods and Management in Chemistry	4
Agricultura Técnica	3
Journal of Chemical Education	3
Environmental Geochemistry and Health	3
Atomic Spectroscopy	3
Journal of Pharmaceuticals and Biomedical Analysis	3
Journal of Food Composition and Analysis	3
Analytical Methods	2
Journal of Near Infrared Spectroscopy	2
Journal of Environmental Science and Health - Part B	2
Avances en Ciencias e Ingeniería	2
Analytica Chimica Acta	1
European Food Research and Technology	1

Journal	Percentage of publications
Journal of Chromatography B	1
Analytical Chemistry	1
Food Chemistry	1
Applied Spectroscopy reviews	1
Archives Environ. Contam. Toxicol	1
Atmospheric Environment	1
Bulletin of Environmental Contamination toxicology	1
Environmental Geology	1
International Journal of Environmental Research and Public Health	1
International Journal of Analytical Chemistry	1
Journal Chromatographic Science	1
Journal of Electroanalytical Chemistry	1
Journal of Food Engineering	1
Livestock Production Science	1
Plant Physiology and Biochemistry	1
Soil & Tillage Research	1
TrAC Trends in Analytical Chemistry	1
Spectrochimica Acta B	1
Journal of the Science of Food and Agriculture	1

Conclusions

This review shows that while scientific production in Analytical Chemistry in Uruguay is still small when compared to other countries in the region, it has been increasing in the last 10 years. Also it can be concluded that many contributions of Uruguayan research groups are published in high impact journals.

Considering that research and development in Analytical Chemistry relies heavily on the investment in modern instrumentation, it can be hoped that the creation in 2005 of the National Agency for Research and Innovation (ANII) will have a significant impact reflected in a sustained growth and improvement in scientific production in this area.

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Grass as a biomonitor: impact of copper suplementation in the environment

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Abstract

In this work copper, molybdenum and sulfur determinations in grass, used as biomonitor of the soil, were performed during the period 2008-2009 and compared with the levels obtained in 2000-2001 in the same locations with the aim of observing environmental changes after the implementation of a program recommending oral and injected copper supplements in cows and after the use of fertilizers containing molybdenum and sulfur elements that can affect copper bioavailability. Several correlations with meteorological factors were also studied. The results showed that the copper level increased in grass but the concentrations remained below the recommended values. The mean copper concentrations in spring, summer, autumn and winter were 2.44, 3.12, 4.29 and 5.21 mg kg^{-1} DM, respectively, and for molybdenum were 0.20, 0.16, 0.22 and 0.17 mg kg^{-1} DM, respectively (DM = dry matter). In many samples, the molybdenum concentration was above the recommended range and consequently a secondary deficiency can be observed. In addition, a seasonal variation in grass, an inverse correlation between copper and molybdenum concentrations and a direct correlation between copper levels and the relative humidity were observed permitting visualization of the most critical periods of hypocuprosis.

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1. Introduction

The health of living organisms depends, among other factors, on their ability to obtain from the external chemical environment essential trace elements [1].

In Uruguay, where almost 90% of its land is devoted to raising livestock with extensive breeding, an important health problem related with copper deficiency was reported at the end of the twentieth century, in Salto Department [2] where a nosologic entity, named "growth syndrome", was detected in bovine and ovine herds showing anemia, low weight, bone deformities, depigmentation, persistent diarrhea, and low milk production, among others. Similar observations were performed in the nearby regions from Brazil and Argentina [3] [4].

Taking into account these observations, an interdisciplinary group was formed at the end of the nineties with the aim of studying, in Salto Department of Northern Uruguay (Fig. 1) [5], the copper lacking cattle, the causes of this deficiency and alleviation with copper complexes as supplements [6-8].

As a part of these studies, Cu, Mo and S levels in grass were determined in different locations of the milk, in production area Salto Department, particularly in Itapebí, San Antonio, EEFAS and Las Margaritas, in the period 2000-2001. The obtained results showed that copper content in grass was below adequate levels (10 mg kg^{-1}) most of the year [7]. Although there is no complete agreement with the copper requirements for cows, 10 mg kg^{-1} of dry matter (DM) is usually considered adequate or recommended [9]. Besides, as a conclusion of this previous study, a positive correlation between copper content in grass and serum copper level in cows was established [7], showing

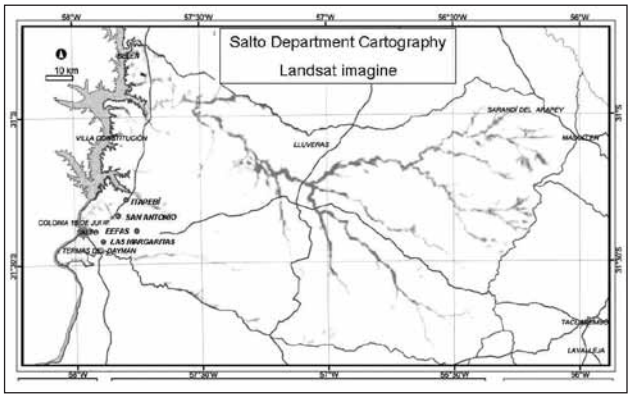

Figure 1. Salto Department cartography showing the sampling locations and their coordinates (Dirección General de Recursos Naturales Renovables Ministerio de Ganadería Agricultura y Pesca Renovables, 2010).

that the evaluation of copper levels in grass is a good tool to understand the copper status in cows.

On the other hand, it is well known that copper deficiency in cows can occur because of low copper content in grass (primary deficiency) but also due to other factors that impact copper bioavailability (secondary deficiency) [10]. Molybdenum and sulfur are the two most important factors that reduce copper absorption. Molybdenum can form copper compounds like $CuMoO_4$ and $CuMoS_4$, of low bioavailability, the latter with S^{2-} produced in the rumen from fresh grass [1][11]. For this reason it is important to evaluate Mo and S concentrations in grass.

Due to these observations, veterinarians of the region began to recommend oral copper supplements to alleviate the "growth syndrome" [12-13]. Oral supplementation with copper salts sometimes appears to be unsatisfactory for ruminants [14]. Especially, it is well known that only low amount of copper (near 5%) are absorbed in cows from feed and consequently most is excreted, principally through the feces, and remains in the environment [15].

Another important aspect about copper and environment is the use of fertilizers containing trace elements that could interfere with copper absorption. One of them is molybdenum, which can affect copper bioavailability.

In this work new Cu, Mo and S seasonal measurements in grass were performed during the period 2008-2009 with the aim of evaluating the bioavailable mineral levels that can be absorbed by grass after changes in the animal supplementation program. Then, a comparison with the results obtained in the period 2000-2001 was performed. Also, several correlations with meteorological factors were studied to understand the results observed in mineral content and consequently to advise the producers affected by the "growth syndrome".

2. Experimental

2.1. Materials

All reagents were of analytical grade. Purified water (ASTM Type I) was obtained from a Millipore (São Paulo, Brazil) Simplicity 185 purifier fed with glass-distilled water.

All bottles for storing samples and standard solutions were immersed in 10% (v/v) nitric acid for 24 h, rinsed with ultrapure water and dried before being used.

2.2. Grass samples

A total number of 35 samples of natural grass were obtained from four different locations named Itapebí, San Antonio, EEFAS and Las Margaritas (see Fig. 1). The minimum number of samples was 5 for each location with a minimum distance of 1 meter between each one. They were collected by the hand clipping method based on animal selectivity. The samples were collected in the period July 2008 – August 2009, washed with purified water and dried at 50 ºC until constant weight.

2.3. Analytical methods

Grass samples were prepared as follows: 10 g of dried grass was accurately weighed and ashed at 500 °C in an Atec furnace (Montevideo, Uruguay), model HFA10, until constant weight. Inorganic ashes were dissolved with 50 mL of 50 % v/v aqueous HCl solution according to AOAC International method 975.03 [16]. This sample treatment was run in duplicate. The solutions obtained were used for the analytical determination of Cu and Mo.

According to the preliminary information, Cu level in grass is usually in the order of mg kg^{-1}. For this reason the flame atomic absorption spectrometry (FAAS) technique is adequate, while for Mo determination (in the order of $\mu g\ kg^{-1}$) electrothermal atomic absorption spectrometry (ETAAS) was selected [17].

S levels in grass samples were also determined. For this purpose, 1 g of accurately weighed dried sample was mixed with a $Mg(NO_3)_2$ solution and heated in a furnace until samples were completely oxidized as described in AOAC International, method 923.01 [18].

Sulfur was determined as sulfate ion (SO_4^{2-}) in the final solution by a turbidimetric method using an Oakton TN-100 instrument according to the standard method 4500E-APHA [19].

2.3.1. Copper determination

The Cu determinations were carried out using the FAAS technique after sample preparation according to the AOAC International, method 975.03.

A Perkin Elmer (Norwalk, CT, USA) model 5000 flame atomic absorption spectrometer, with an air-acetylene flame and a copper hollow cathode lamp (Photron, Narre Warren, Australia) was used for Cu determination. The selected analytical wavelength was 324.8 nm.

The Cu stock solution (1000 mg L^{-1}) was prepared by dissolving electrolytic wire (purity > 99.7 %) in HNO_3 1 % (v/v).

Aqueous standards in the range of 1-5 mg L^{-1} were freshly prepared by appropriate dilution of the stock solution in purified water.

2.3.2. Molybdenum determination

The Mo determinations were carried out using the ETAAS technique after sample preparation according to the AOAC International, method 975.03.

These determinations were performed with a Varian AA-240 atomic absorption spectrometer with a deuterium background correction, a GTA-120 electrothermal atomizer and a PSD-120 autosampler (Victoria, Australia). Wall atomization with pyrolytic coated partitioned graphite tubes was used. The Mo hollow-cathode lamp (Photron, Narre Warren, Australia) was run under the conditions recommended by the manufacturer. The selected analytical wavelength was 313.3 nm. The graphite furnace temperature program for this analyte was 1000 ºC for pyrolysis and 2800 ºC for

atomization. All measurements were performed using integrated absorbance (peak area).

A 1000 mg L^{-1} stock standard solution of Mo was prepared from ammonium molybdate tetrahydrate (J.T. Baker, NJ, USA) in NH_4OH 1% (v/v). Working standards in the range of 4-80 μg L^{-1} were freshly prepared by appropriate dilution of the stock solution in 0.1 % (v/v) HNO_3.

2.3.3. Sulfur determination

Turbidimetric determinations where performed by an external laboratory. This laboratory was selected because it meets the quality standards to provide reliable results. The determinations were performed as was explained in section 2.3 with no modifications to the standard method referenced.

2.4. Meteorological factors

Temperature, relative humidity and rainfall data were obtained from INIA (Instituto Nacional de Investigación Agropecuaria, Salto, Uruguay), measured in the studied region [20].

3. Results and discussion

3.1. Copper and molybdenum determinations

Regarding the Cu determinations we investigated the possible existence of interferences. It was found that the slopes did not show significant differences. Consequently the measurements were carried out by direct calibration since no evidence of matrix interferences was found. Reagent blanks were also run.

The mean figures of merit were detection limit 10 μg L^{-1} (LD, 3s) in solutions, corresponding to 0.050 mg kg^{-1} in grass samples, linear range up to 5 mg L^{-1} and the day - to - day analytical precision, expressed as RSD (%), was better than 5%.

In order to evaluate the sample preparation procedure a spike/recovery approach was carried out. For the spiked samples analyzed, recoveries were in the range 95%-105%, showing no losses of the analyte during the procedure.

The same study was performed for Mo analytical determinations, after investigating the possible existence of multiplicative interferences by comparing the slope of the calibration curve with that of the standard additions curve by means of statistical hypothesis testing. It was found that the samples showed significant differences in the slopes. This suggests the existence of matrix interferences.

For this reason standard additions on samples were carried out. Reagent blanks were also run.

The mean figures of merit were detection limit 4.3 μg L^{-1} (LD, 3s) in solution, corresponding to 0.022 mg kg^{-1} in grass samples, linear range up to 80 μg L^{-1} and the day - to - day analytical precision, expressed as RSD (%), was better than 10 %.

The method was verified for grass samples by a spike/recovery approach. For the spiked samples analyzed, recoveries were in the range 90%-110%.

3.2. Cu, Mo and S levels in the period 2008-2009

Seasonal variation of mean Cu and Mo levels considering all grass samples from Itapebí, San Antonio, EEFAS and Las Margaritas locations are shown in Figure 2.

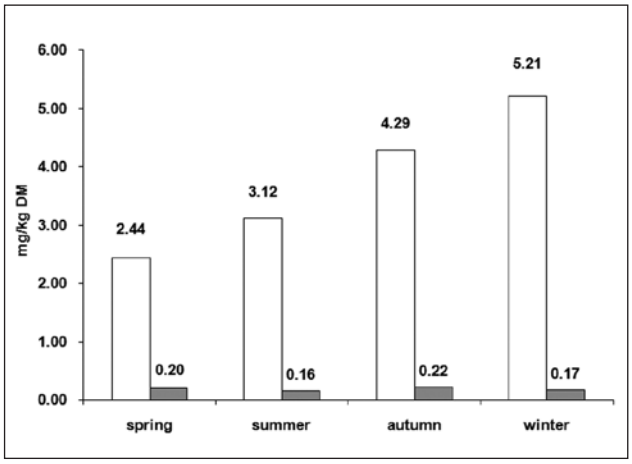

Figure 2. Seasonal variation of mean Cu (white bars) and Mo (gray bars) levels in grass samples from Itapebí, San Antonio, EEFAS and Las Margaritas locations in the period 2008-2009.

As Figure 2 shows, all the samples were well into the recommended level (10 mg kg^{-1} of dry matter (DM)). A seasonal fluctuation of Cu level in grass was observed, the lower values occurring in spring and summer, seasons with low humidity and high temperatures [20]. The correlation between humidity and copper levels in grass will be presented in section 3.4.

These results permitted better understanding of the "growth syndrome" in several cows, the nosological entity still reported in this region.

The recommended molybdenum levels in grass are under discussion and are not well established, oscillating between 0.03 and 0.15 mg kg^{-1} DM [21-22].

The obtained results in the region were in the range of 0.063-0.480 mg kg^{-1} DM. Fifteen samples were in the recommended range and twenty samples above this range. Nowadays, some producers use several fertilizers containing Mo, such as $Na_2MoO_4 \cdot 2H_2O$, MoO_3, or $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$, as a nutrient that can be applied to soil or foliage. Usually, Mo concentration in these products is in the range 0.0008-0.025% and they can also contain other trace elements, including Cu.

This Mo supplementation might be the cause of the number of samples out of the recommended range.

The S levels were, in this period, in the 0.1-0.3 % DM range, below the recommended values (0.3-0.5 % DM) [23-24]. This behavior discards the possibility of S interference in Cu absorption [25]. In spite of the fact that S deficiency is a growth limiting factor that should be treated, it is likely that correcting any sulfur deficit would decrease the Cu status.

3.3. Correlations

3.3.1. Cu and Mo monitoring during the periods 2000-2001 and 2008-2009

To evaluate changes in Cu, Mo and S levels in grass after veterinarian prescription of supplementing the animals, Las Margaritas and San Antonio locations were selected for monitoring Cu and Mo levels based on our previous research. In the other locations (Itapebí and EEFAS) copper and molybdenum seasonal variation was not studied in the period 2000-2001.

Figures 3 A and 3 B show the comparative seasonal copper levels in grass during the period 2000-2001 (previously reported) [7] and 2008-2009, in Margaritas and San Antonio locations, respectively.

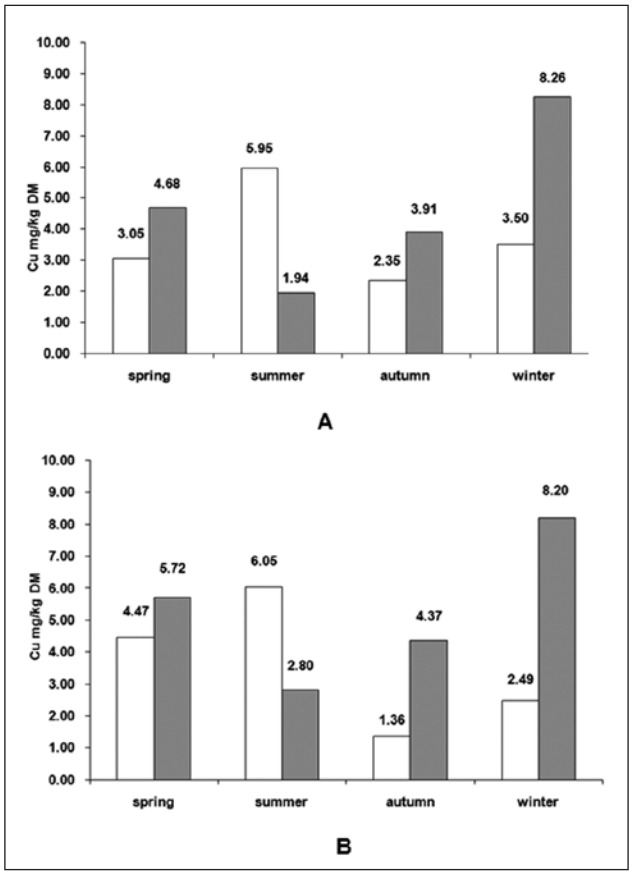

Figure 3. A) Cu levels in the periods 2000-2001 (white bars) and 2008-2009 (gray bars) in Las Margaritas; B) Cu levels in the periods 2000-2001 (white bars) and 2008-2009 (gray bars) in San Antonio.

As shown in Figures 3 A and 3 B copper increase was observed in the period 2008-2009 compared to that observed in the period 2000-2001, except in summer. This general behavior might be due to the copper supplementation of animals in the studied locations. This is in agreement with the fact that only a low copper percentage is absorbed in cows from feed as explained in section 1 [15].

The copper level decrease observed in summer could be related with the significant drought during this season in the period 2008-2009 that decreased the concentration of soluble Cu species and consequently the absorption [20]. Apart from this factor, the stage of development of plants could also affect the root uptake and hence the mineral content of grass in this season [1].

On the other hand, Figs. 4 A and 4 B show the comparative seasonal Mo levels in both periods, in Las Margaritas and San Antonio locations respectively.

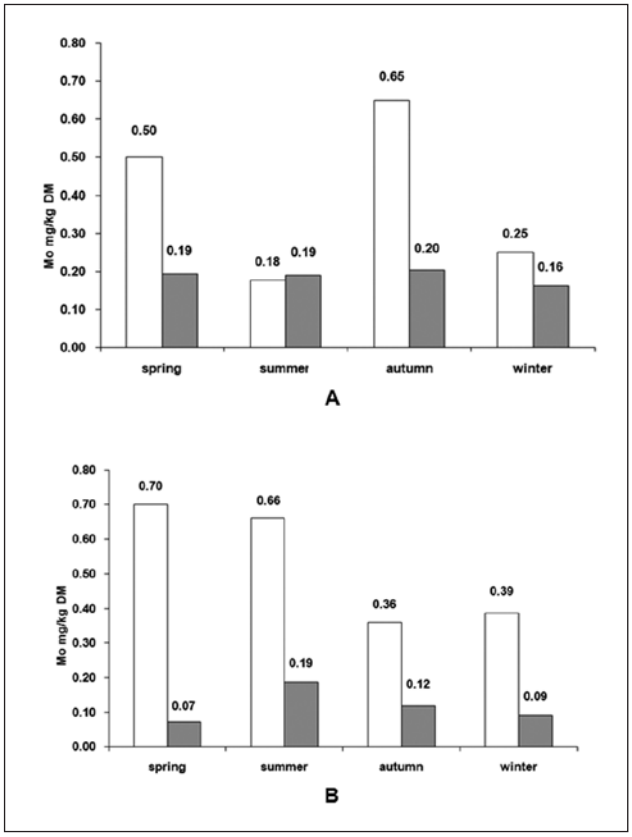

Figure 4. A) Mo levels in the periods 2000-2001 (white bars) and 2008-2009 (gray bars) in Las Margaritas; B) Mo levels in the periods 2000-2001(white bars) and 2008-2009 (gray bars) in San Antonio.

As figures 4 A and 4 B show, there was an important and unexpected Mo decrease in grass during the period 2008-2009 in almost all the seasons (except in summer in Las Margaritas location) even falling below the recommended limit for grass, in some cases.

This behavior could respond to the general soil impoverishment or to the formation in the soil of not bioavailable species that grass could not absorb. If we consider that some producers often used fertilizers with products containing molybdenum species, the second hypothesis would be the most appropriate.

3.3.2. Mo *vs* Cu levels

A Mo *vs* Cu correlation was analyzed using the statistic

table from Pearson et al. for a unilateral test with two degrees of freedom (N-2). The correlation coefficient (R) obtained for the analytical data was compared with the critical values of the Pearson table with a probability of 5% [26].

In Figs. 5A and 5B, the Mo concentrations *vs* Cu concentrations in Las Margaritas and San Antonio locations were plotted.

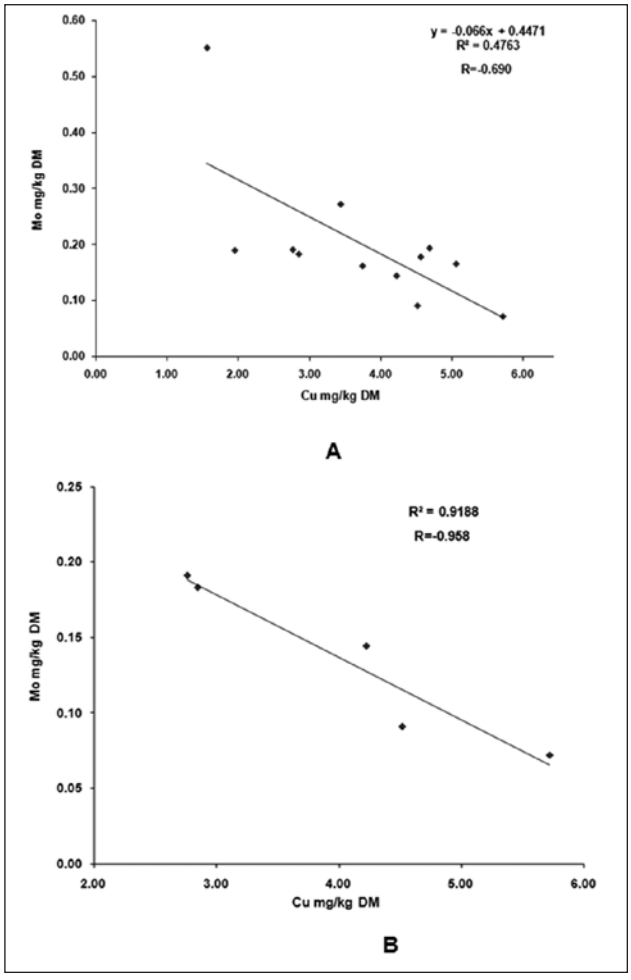

Figure 5. A) Correlation between Cu and Mo concentrations in grass obtained from Las Margaritas and San Antonio locations; B) Correlation between Cu and Mo concentrations only for San Antonio.

As expected taking into account previous reports [1] [23], Fig. 5 shows an inverse correlation between both metals (R = -0.690, critical absolute value: 0.576) when San Antonio and Las Margaritas data are plotted together. A better correlation was obtained for individual data from San Antonio (R = -0.958, critical absolute value: 0.878) (see Fig. 5 B) [26].

3.4. Cu and Mo levels vs meteorological factors

As explained in 3.3.2. the correlation was analyzed using the statistical table from Pearson *et al.* [26].

The correlations of Cu and Mo concentration with meteorological factors like temperature, relative humidity and rainfall data were performed [20].

Fig. 6 shows Cu levels from Las Margaritas and San Antonio locations *vs* mean daily relative humidity (%).

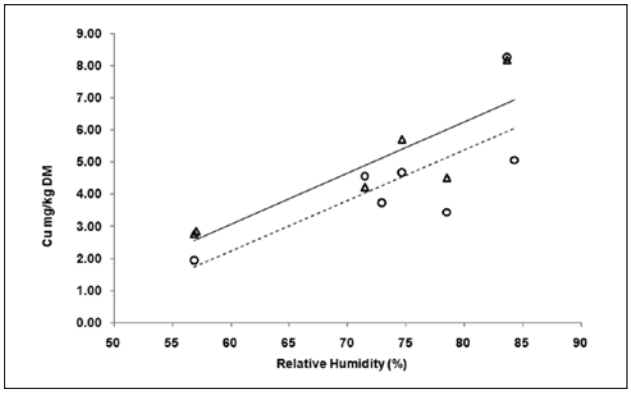

Figure 6. Cu levels from Las Margaritas (o) and San Antonio locations (Δ) vs mean daily relative humidity (%).

As Fig. 6 shows, a good positive correlation was observed in both locations, obtaining R = 0.752 and critical value 0.754 in Las Margaritas, and R = 0.878 and critical value 0.811 in San Antonio [26].

This result is in accordance with the fact that the increase of the environmental humidity could produce an increase of soluble Cu compounds and consequently higher Cu levels in grass.

No correlation was found with Mo levels and relative humidity or with Cu and Mo vs other meteorological factors.

4. Conclusions

The study of Cu levels in grass permitted better understanding of the "growth syndrome" in cows, nosological entity still reported in the region of Salto Department. This is a very important problem not only in Uruguay but also in other countries in the region devoted to livestock production, like Argentine and Brazil.

The monitoring of mineral levels in the periods 2000-2001 and 2008-2009 produced interesting data and show that supplementation with Cu compounds, promoted by veterinarians of the region in the first period, ten years ago, increased the Cu level in grass. However, the concentrations remained below the recommended values. Besides, the Mo levels decreased with time but several samples remained above the recommended range and consequently they can affect copper bioavailability, producing a secondary deficiency. For this reason it is important to control the addition of fertilizers containing Mo compounds. The low S levels obtained in grass samples discard the possibility of interference in copper absorption.

In addition, the seasonal mineral variation in grass, the inverse correlation between Cu and Mo concentrations and the direct correlation between Cu levels and the relative

humidity permitted visualization of the most critical periods for animal health.

Taking this work as a base, our future aim is to investigate other analytes such as Zn, Mg and Se and to perform similar studies in other regions and for a longer period.

Acknowledgement

The authors would like to thank PEDECIBA (Program for the Development of Basic Science) for financial support, the Uruguayan Agency ANII (Agencia Nacional de Investigación e Innovación) for the scholarship for Verónica Bruné and the Project "Enlaces" from the European Union.

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Point of View

Analytical chemistry as a tool in the drug abuse prevention in corporate programs: legal frameworks and prospects of brazilian scenario

With the expansion of oil exploration in Brazilian coast by foreign companies, control programs of psychoactive substance used by employees at risk activity were incorporated into the scenario of this type of activity.

In the scenario developed in the early 90s, the staff was subjected to random drug tests, motivated, pre-functional, pre-admission and post-accident. Given the lack of specific legislation, much controversy has been generated on the basis of analytical methodology available and applied tests.

Imunnoassays tests, generically called "rapid tests", were applied in profusion, and depending on their analytical limitation many false-positive results were reported by the occurrence of cross-reactivity with other analytes. A classical example of this type of occurrence is the unwanted cross-reaction of local analgesic alkaloids within cocaine positive results. In addition, false-negative results may also occur.

Mass spectrometry coupled to a separation methodology (generally chromatography) contained in international law (Department of Transportation North American, in eg.) is the unique choice as a standard for the identification of excretion metabolites of psychoactive substances in biological matrices.

The gap on Brazilian legal guidelines for drug testing employees generated a lot of controversy for two decades regarding the methodology of choice for employment in this exam, causing revulsion by the employer and legal burden generating due to false-positive unfairly imputed to the employee tested.

The legal framework that guided the implementation of programs of this nature, was the publication of RBAC 120 and IS120-002A, both authored by ANAC (National Agency of Civil Aviation) in the years 2011 and 2012, respectively. For the first time in Brazil, an official organ legislated on the subject, establishing finally the analytical method of choice for drug testing as well as defining cutoff values for the group of substances monitored and prohibited for employees engaged in risky activity.

In this scenario, transformed from 2011 as a result of this legal framework, the testing laboratories become fundamental tools to support these programs. Investment in accreditations such as ISO 17025, is paramount given the complexity of the test, the reliability of results and ensuring the chain of custody of the sample are critical to the efficiency and credibility of the program.

As occurred in other countries, and with the tools provided by the Analytical Chemistry, Brazil is rapidly moving to create a work environment free of drugs, increasing the safety of business and contributing to society, since the employee is forced to choose between his job and drug addiction.

Prof. Dr. Fábio de O. Martinez Alonso
Technical Director
CONTRAPROVA - Química e Toxicologia Forenses

Speciation analysis based on generation of substituted hydrides - is the analytical community familiar with its benefits?

Speciation analysis undoubtedly represents one of the current trends in analytical chemistry. The demand for identification and quantification of the most important forms of "important" elements at lower and lower concentrations (or masses) is increasing to keep up with requirements of various scientific disciplines such as toxicology, pharmacy, medicine, clinical chemistry and biology.

On-line coupling of liquid chromatography (LC) to inductively coupled plasma mass spectrometry represents the most often employed, classical, approach to the speciation analysis. The main advantage of the classical approach is that it is rather universal - applicable to a broad range of elements and their species in a broad range of matrix types. However, as everything in the world, it has its limitations. First, limits of detection are negatively influenced by three factors inherent to the approach: low sample volume, relatively broad peaks produced by liquid chromatography columns and low efficiency of analyte transport from column to the plasma torch. Second, and perhaps even more serious, limitation is a risk of changing the sample speciation during sample preparation/LC separation process. Third, the analysis is not feasible for samples which cannot be introduced to the LC column.

An alternative approach to speciation analysis is useful for determination of species which can be converted by hydride generation to differently substituted hydrides. This approach, tentatively termed "generation of substituted hydrides" employs this scheme: hydride generation followed by cryotrapping and subsequent hydride separation in a gas chromatographic way and, finally, hydride atomization and detection by an atomic spectrometer. The field of application is rather limited - to the speciation analysis of ionic alkyl substituted compounds of several hydride forming elements. This is virtually the only disadvantage of generation of substituted hydrides. All its other features are highly positive: (i) minimum sample pretreatment is required - the risk of changing the speciation during the analysis is thus minimized; (ii) analysis of complex biological matrices or even slurries which cannot be separated on LC columns can be performed; (iii) much sharper peaks than with LC; (iv) high sample volumes can be used. Resulting from points (iii) and (iv), generation of substituted hydrides can yield extremely high sensitivity. Consequently, detection limits lower than with the classical approach can be achieved even when using the relatively simple and cheap, both in investment and running costs, atomic absorption detector. Substantially lower detection limits can be achieved when replacing atomic absorption by atomic fluorescence detector (which is also simple and cheap) or by inductively coupled plasma mass spectrometer.

In conclusion, the generation of substituted hydrides renders possible to perform speciation analysis of ionic alkyl substituted compounds of several hydride forming elements in laboratories possessing only the simple equipment and for low price. Besides, it can handle sample matrixes which cannot be separated on LC columns.

Jiří Dědina
Institute of Analytical Chemistry of the AS CR, v. v. i.
Czech Republic

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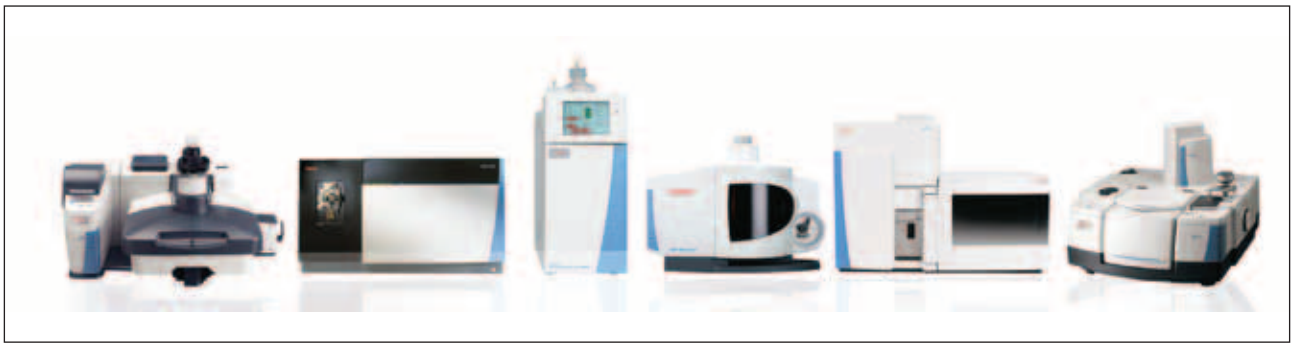

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6. Regitz, M. in *Multiple Bonds and Low Coordination in Phosphorus*

Chemistry; Regitz, M.; Sherer, O.J., eds.; George Thieme Verlag: Stuttgart, 1990, Chap. 2.

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7. Bash, T.; Dot, K.; Petersen, G.; Orbfield, B.; Cooper, D.; Sabbag, L.; Fce. *Le Kokai Tokkyo Koho 54 95,489* 1975. *(CA 233:P179341v)*
8. McDonald, L.B.; *US pat. 9,236,006* 1987. *(CA 233:P49542y)*

- ***Software:***

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

- ***Theses:***

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

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

11. Ferreira, A. B.; Brito, S. L.; *Resumos da 20ª Reunião Anual da Sociedade Brasileira de Química,* Pocos de Caldas, Brazil, 1998.

- ***Internet pages:***

12. http://www.sbq.org.br/jbcs, accessed June 2001.

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