AIMS AND SCOPE

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Electronic olfactometry. A new tool in analytical chemistry

Abstract

An overview about the electronic olfactometry is presented. The principles, sensor technologies and chemometric treatment of signals are briefly discussed. Applications of commercial instruments currently available in food technology, environmental analysis, the follow-up of biological processes and the future developments are reviewed.

Keywords: Electronic olfactometry, sensors, chemometric analysis.

C. García Pinto, Mª Esther Fernández Laespada, J. L. Pérez Pavón and B. Moreno Cordero*

Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Ciencias Químicas, Universidad de Salamanca, 37008 Salamanca (Spain).

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Introduction

Traditionally, the assessment and characterisation of smells has been carried out using sensory analysis with expert panels of previously trained human beings or by gas chromatography coupled with mass spectrometry. Such methods are costly and require relatively long analysis times and it has therefore become necessary to develop new non-destructive and almost automated techniques able to analyse smells in short times with sufficient precision and accuracy [1].

In this context, the past few years have seen an important development of a new technique called “electronic olfactometry” based on gas sensor technology that seems to cover the above requirements [2-4].

The term “electronic nose” is used to refer to a system that combines the response of a set of chemical sensors, with partial specificity for the measurement of volatiles, and techniques able to recognise patterns for data interpretation [5]. The absorption of volatiles on the surface of the sensor produces physical or electric changes in the sensor and these are recorded against time as an adsorption curve. The response generated by the sensors is basically a chemical fingerprint that allows identification of both individual compounds and of complex mixtures. The device imitates the human olfactory system and hence uses a limited number of sensors and a simulated “brain” [6].

In principle, the results obtained with a set of sensors allows the collection of qualitative and quantitative information about the composition of the gases in the headspace generated by a sample; the technique therefore has enormous potential in fields as diverse as the food industry, environmental analysis, medicine, microbiology, etc. The capacity of electronic olfactometry allows analysis of the set of volatiles present in a sample in almost real time and hence it is possible to identify any problem during the first steps of the production process with the consequent saving of time and money.

The ability of a set of sensors to differentiate odorous substances was demonstrated by Persaud and Dodd in the eighties at the University of Warwick (United Kingdom)
[7]. The first results of their work revealed that discrimination in an olfactory system could be achieved without highly specific receptors. Later, the electrochemist Philip Bartlett et al. designed and built a system comprising 12 SnO₂ sensors for the differentiation of aromas, reporting the applicability of the system in products such as coffee and beer [8]. At the end of the eighties, Jay Grate and his team at the Naval Investigation Laboratory of the United States constructed an electronic nose to detect chemical and toxic agents [9,10].

Currently, electronic olfactometry continues to be developed at both methodological and instrumental level. Investigations into this field cover areas as widely varying as sensor technology, electrochemistry, solid-state and polymer physics, among others, and many of the technological advances made have already been patented [11-15].

There are several instruments available commercially that are based on sensor systems and many of them are used at universities and research institutions. Interesting information concerning this can be found at the Internet site (http://nose.uta.edu/review/). This site contains the addresses of many different commercial companies and those of some of the research and development teams currently involved in different aspects of electronic olfactometry.

Recently, J.W. Gardner and P.N. Bartlett have published a book, with an ample reference section, that addresses the basic principles underlying electronic olfactometry and recent developments in this field as regards both technology and applications [16].

Sensor Technology

One of the main components in an electronic nose is the set of sensors; ideally, these should have a high degree of sensitivity and reliability. Most gas sensors are based on physical or chemical adsorption and desorption, optical desorption or chemical reactions that take place on the surface of the sensor or in the bulk of the material which the sensor is made of. These interactions cause changes in physical properties (conductivity, electric polarization, optical properties, magnetic and dielectric properties, etc) that can be detected [17].

Depending on the working temperature, currently available gas sensors can be divided into two main groups: sensors that function at high temperatures (175-450°C) and those that afford responses at room temperature (cold sensors).

Among the sensors that work at high temperatures, the most important are metal oxide semiconductors (MOS) [18-20] and metal oxide semiconductor field effect transistors (MOSFET) [21-23]. The former are based on the design known as Taguchi gas sensors, which detect analytes on a short ceramic tube onto which a fine layer of metal oxide has been deposited. Among the most important oxides are SnO₂, WO₃, and ZnO. The second type comprises a doped semiconductor and an insulating oxide (SnO₂) covered by a layer of catalytic metal. The type of metal used, the thickness of the layer and the operation temperature can be varied to obtain sensors with different degrees of selectivity. Of the sensors that operate at room temperature, conducting polymer sensors (CP) [24,25] are those most used. These devices are obtained from monomers by electrolymerization with different counterions [26,27]. Among the monomers most used are pyrrol, aniline, thiophene and indole. The sensitivity and selectivity of these sensors is achieved with the variation in functional groups, the structure of the polymer and using different ions. Sensors of the quartz microbalance type (QMB) [28-31], of the surface acoustic wave type (SAW) [32] and optical sensors [33,34] have also been used in electronic noses.

Metal oxide sensors are generally more robust (longer half-life time), less sensitive to temperature changes, and their times of response and baseline recovery are faster than sensors that work at room temperature. The selectivity and sensitivity of these sensors varies as a function of their technology. Cold sensors generally show better selectivity than hot ones. Sensitivity ranges between parts per million in the case of metal oxide sensors [41], quartz microcrystal sensors [17,31] and conducting polymer sensors [35], and parts per billion in surface acoustic wave sensors [36,37].

The open designs of most commercial devices offer complete flexibility in the selection of sensors both as regards type and number. Hybrid systems (Alpha MOS, EEV Chemical Sensor Systems, NST) are available on the market that use different sensor technologies in the same piece of apparatus (MOS, MOSFET, CP), allowing their use in many different applications.

Although sensor technology is perhaps the area in which most advances have been achieved, currently the main limitations of gas sensors are the dependence of the response on the humidity of the sample and their instability over time. Sample humidity strongly affects sensors that respond to polar molecules. Different methods have been proposed to palliate this problem, such as the use of a carrier gas with the same degree of relative humidity as the headspace generated by the sample or the recording of the signal of a sample of pure water and use of this as a blank for the samples [17,38].

The dynamic response of gas sensors with time may vary significantly owing to the effect of external parameters such as the relative humidity of the carrier gas, changes in temperature or drift in the sensors themselves. According to some authors, the variations in the response of these systems may reach values of up to 10% in periods of less than
three months [36,37,39]. It is therefore necessary to calibrate the sensor system; this should be based on the use of control or reference samples. Compensation of the drift of sensors with time is accomplished by applying different mathematical treatments to the signals before data analysis. Different calibration methods have been proposed, based on mathematical algorithms [40-43] and neural networks [37,43], with which it is possible to obtain reproducible responses over relatively long periods of time. Despite all this, however, replacement of complete sensor modules requires recalibration and “retraining” of the whole system [44].

Recently, Hewlett-Packard has commercialized a device—the “HP4440A” Chemical Sensor—that uses a mass detector to obtain information from the samples. The system employs an automatic headspace injection system to form the volatiles corresponding to a given sample. The molecules in the vapour phase thus generated are ionised and fragmented and the charged fragments are led to the mass sensor. Because the fragmentation and ionisation processes are highly reproducible, even the most complex samples afford reproducible responses of the detector. Among the advantages of this new system are the speed of response of the detector, and the possibility of performing measurements in the presence of species that saturate the gas sensor system and hence render it useless.

### Chemometric treatment of signals

The set of sensors of an electronic nose affords a large amount of information and generates a unique overall pattern of the volatiles of any given sample. Processing of the data generated by the system is an essential part of the concept of electronic olfactometry.

In chemometric treatment of the signals produced by the response of the sensors, statistical packages—both commercial and custom-designed by the suppliers themselves of the devices—based on pattern recognition techniques are used. Among those most commonly employed are cluster analysis (CA), linear discriminant analysis (LDA), principal component analysis (PCA) and principal component regression (PCR).

The introduction of artificial neural networks (ANN) enables compilation of sufficiently robust classification models from the responses of the gas sensors of an electronic nose. A neural network can be defined as a set of very simple calculation units (neurons) joined by connections or “synapses” that start out from a database (the responses of the sensors) and convert it into a series of response values using a given transfer function among the neurons [45-48].

The task of a neural network can be divided into two parts: the training stage and the prediction stage. In the former, the assignation of weights to each connection is carried out in such a way that the prediction error will be as small as possible. Proposals have also been made of combinations of neural networks with some of the above-mentioned techniques (principal components as inputs of a neural network) as powerful tools to obtain models from the data afforded by the set of sensors.

When linear responses are to be analysed, all the above statistical methods, afford very good results. This is the case of the signals generated by the HP-4440 Chemical Sensor mass detector, whose statistical package does not include neural networks. However, owing to the fact that the responses of gas sensors are essentially non-linear, when they are used in electronic olfactometry neural networks afford better results [49], specially for calibration tasks.

In many cases, the combination of statistical treatments such as principal component analysis and neural network systems affords excellent results since PCA allows a reduction in the dimensions of the space, facilitating determination of the structure of the networks, eliminating the co-linearity of the experimental data and the random components of the signals measured, and degrading the model due to overtraining [50].

### Applications

The information, both qualitative and quantitative, about a sample derived from the sensors of an electronic nose means that a priori the technique has enormous potential in different research fields. The commercial instruments currently available find application in food and beverage quality control and environmental analysis, the follow-up of biological processes and even the diagnosis of different diseases.

#### Food Technology

Undoubtedly the field in which the greatest number of applications for electronic olfactometry can be found is food technology, both for the characterisation of different odours and for quality control during the production, storage and conservation of foods [44,51-58].

The capacity of electronic olfactometry in the determination of the sensory quality of meat products has been shown by comparing the technique with the above-mentioned expert human panels. Thus, correlations have been established between sensory analysis (panels) and the signals obtained using a set of MOS and MOSFET sensors that allow the prediction of the sensory attributes of meat products and the differentiation of pork samples as a function of the type of feed given to the animals [59]. Data treatment permitted the correct classification of 15 of the 17 samples analysed in the corresponding group. In this classification task the results concerning the discrimination among different samples of meat products as a function of the type and sex of
the animals and the origin of the products has been excellent [60-63].

The detection of bad smells and of products in poor condition is another of the possible applications of electronic olfactometry. In this sense, the literature refers to works aimed at the detection of meat contaminated with androsterone and skatole. Bourroulet et al. [64], using a system of 5 metal oxide sensors, established two groups of samples (with 84% success in classification) as a function of the androsterone content being above or below 1.7 mg g⁻¹. Using 12 conducting polymer sensors, Amor Frempong et al. [65] analysed samples with concentration limits of 4 mg g⁻¹ of androsterone and 1.6 mg g⁻¹ of skatole. They achieved classification success rates of 84 and 90% respectively and a correlation close to 0.8 with the responses of expert panel of 10 trained members.

The spoilage of meat products due to microbiological processes is another of the aspects addressed by electronic olfactometry. Winiust et al. [66], using neural networks for the treatment of the signals generated by an electronic nose, predicted the storage time of products such as minced pork and minced beef. The production of ethyl acetate, one of the major compounds to emerge during the first staged of the decomposition of meat, can be detected using a single MOS-type sensor [67]. The response of this type of sensor is linear between 1-200 ppm, permitting determination of the freshness of a given product.

Rossi et al. [68] were able to discriminate (100% in classification) between seven types of Micrococcus (4 aromatic and 3 pathogenic) found in fermented meat products on the basis of the volatiles generated. Arnold and Senter [69] have demonstrated the applicability of the signals produced by a set of sensors in the detection of compounds and the identification of the bacteria involved in spoilage processes.

Finally, proposals have been made to use systems based on gas sensors for the follow-up of food processing and the quality control of such products [23,52]. Application of principal component analysis to sensor responses and to the results of an expert sensory panel enabled Eklöv et al. to find relationships between the responses of a set of MOSFET sensors in the initial phase of the fermentation process and the final sensory quality of sausages, demonstrating that the methodology is rapid, simple and cheap when compared with sensory analysis or gas chromatography with mass spectrometry [23].

In the field of milk products, the profiles of the aromas obtained by electronic olfactometry have permitted the differentiation of milk types as a function of their treatment (pasteurisation, UHT, sterilization in the bottle) [70,71], and rapid and efficient discrimination among different classes of cheese [70,72,73]. Using a suitable chemometric treatment of the signals obtained it is possible to discriminate among cheese as a function of the maturation time [74-76].

A solid-phase microextraction procedure coupled with mass spectrometry and multivariate analysis of the signals obtained has been used to differentiate among milk samples with low fat contents and other similar samples subjected to processes of illumination, heating or microbiological contamination [77].

Electronic olfactometry has also been used to obtain the profiles of wine aromas to discriminate among different varieties and, eventually, their authentication [78]. Systems have been proposed for the differentiation not only of wines of different origins but also of wines from different harvests and even among vineyards from the same origin [79,80]. In the same context, methods have been developed for the analysis and quality control of different types of vinegar [81,82].

In the beer industry, electronic olfactometry has permitted the quantification of sensory characteristics at the pph level [35]. It has also been used for the analysis of grain, the characterisation and discrimination of malts and hops and for control of the stability of the final product [83,84].

Analysis of edible oils using chemometric treatment of the data obtained from a set of gas sensors allows the differentiation among different samples with a good correlation with the results obtained by panels of experts and by gas chromatography [85]. A procedure based on application of linear discriminant analysis has allowed the differentiation of virgin olive oils, non-virgin olive oils and seed oils with classification and prediction percentages in excess of 95% [38]. More complex chemometric treatments, such as artificial neural networks, permit the detection of fraudulent practice in these matrices, a very important aspect from the economic point of view [86]. Analysis of the volatiles produced in the processes of oxidation of oils by electronic olfactometry can be used in the assessment of oxidised samples [87].

Sensor systems have also been developed for the characterization of the aroma, and hence freshness, of fish [72,88] and the characterisation and discrimination of fruit and vegetables as a function of variety [89,90], the degree of maturity, and type of treatment and storage after harvesting [91-95]. Electronic olfactometry has also found application in the characterisation of and discrimination among different types of coffee [96-98], tea [99], chocolate [100], cereals [101-103], sugar [104-106], natural fruit juices [107] and aromatic spices [108], among others.

The containers in which products are stored may sometimes afford undesired sensory characteristics. In this sense, studies performed with electronic olfactometry permit
good differentiation of container materials that transmit bad smells from others that do not [109-111]. The process of manufacturing oak casks for storing wine may affect the composition and quality of the product. This process can be controlled using electronic olfactometry, with promising results [112,113].

**Environment**

The use of gas sensors, generally employing specific solid-state gas sensors, has for many years been a very useful tool in the characterisation of environmental pollutants. However, environmental monitoring requires simultaneous in situ measurement of a large number of different species. The results obtained with a set of non-specific sensors furnish qualitative and quantitative information about the composition of the headspace gases generated by a sample and hence the technique has enormous potential in environmental analysis [114].

Although application of electronic nose technology in environmental determinations has been limited, the smell profiles of different types of water have been used to establish the possibilities of this new technique in the field [115]. Thus, Stuetz et al. used a set of 12 conducting polymer sensors to detect pollutants in natural and treated drinking water [116]. Mathematical treatment of the signals obtained using multiple discriminant analysis permits accurate differentiation among unpolluted and polluted water samples and even the establishment of a certain correlation of the responses with the actual concentration of the organic pollutant. The detection levels lie between 1 mg L$^{-1}$ for phenol and 1 pg L$^{-1}$ for species such as geosmin, methylisoborneol and the measurements obtained are reproducible (RSD<4.5%).

The multivariate responses of the sensors of an electronic nose have been used in the characterisation of waste waters from different origins. Comparison with the results obtained by panels of experts has revealed good correlation when the waters are from the same manufacturing process [117]. In these cases, for example, relationships have been established between the responses and the biological oxygen demand (BOD) [118,119] and the concentration of hydrogen sulphide (H$_2$S) [120].

In agriculture, electronic olfactometry has been used to differentiate among residual smells of livestock as a function of the animals that produce them. The electronic nose is less sensitive than other techniques, such as sensory analysis or photoionization detectors. However, it has greater capacity for the discrimination of smells at high concentrations [121]. The differences in the chemical composition of the different residues were assed by GC-SM. Linear relationships were found between the mean responses of the sensors and the concentration of the smell (expressed as units of smell per m$^3$ of air) in air samples collected from fields treated with cattle manure [122].

Electronic olfactometry has also been used in the environmental field to detect bad smells [123], the evaluation of pollutants [124-126] and even in the quantification of some volatile organic compounds [31,127] in air and soil samples and, additionally, metal oxide sensor systems have been proposed for the selective detection of CO and NH$_3$ [128].

The European Space Agency incorporated a gas sensor system in a space mission and used it to detect changes in the air in closed systems, in particular in the MIR space station [129]. The results obtained revealed the ability of the system to detect changes in air quality of the station produced by the daily activities of the astronauts in real time. Particular contaminations of water (with ethyleneglycol) and of the environment (with ethanol and after a fortuitous fire) were clearly reflected in the results obtained.

**Follow-up of biological processes**

The control of on-line biological processes is a strongly developing research field [130]. The use of a set of gas sensors in combination with the appropriate chemometric techniques offers a faster and cheaper alternative method to traditional analyses using gas chromatography and mass spectrometry.

In fermentation processes, electronic olfactometry has proved to be suitable for detecting microbial contamination and assessing the yield of the process during the culture of microorganisms in the inoculation and production stages [131]. The use of artificial neural networks permits on-line determination of parameters such as glucose and ethanol in yeast fermentation processes [132-134]. Principal component analysis provides information about the development of fermentative [132,135] and pharmaceutical [136] processes.

The production of volatile compounds during the metabolism of fungi and bacteria can be used for the detection and identification of such microorganisms [137]. In this sense, Gibson et al. have used neural networks for the classification of 12 different bacteria and one pathogenic yeast with a success rate of 93% [138]. Gardner et al. studied Escherichia coli and Staphylococcus aureus and achieved success rates of 92% and 100%, respectively [139]. Keshari et al. analysed the headspace generated in the growth process of fungi and managed to differentiate six types, suggesting the potential usefulness of the technique for the detection, in the first stages, of the activity of these species [140].

By analysing the volatiles generated in the development of microbiological processes it is possible to classify different types of cereals as a function of their degree of humidity and to establish correlations between aroma and the number of colony-forming units of both fungi and bacteria [141,142] and to detect
the presence of acarids in wheat [143]. With electronic olfactometry it is possible to detect the deterioration of foods produced by microbial growth [144].

Other applications  
As well as the foregoing applications, electronic olfactometry has been used for the characterisation and quality control of perfumes. The use of both metal oxide sensors [145] and conducting polymer sensors [20] generate patterns of aromas that, with the appropriate chemometric treatment, allows the characterisation and classification of this type of sample.

The responses generated by the set of sensors of an electronic nose have been used in the analysis and discrimination of diesel fuels as a function of their origin. Chemometric treatment of these responses via PCA and LDA permits the correct classification of the samples as a function of their site of production [146]. An electronic nose has also been used for the detection of gases emitted in the leather industry [147], for the discrimination of tobacco samples with very similar aromas [148], and for quality control of paper pulp [149], among other applications.

One interesting possibility of electronic olfactometry is for the diagnosis of diseases or infection, in both animals and humans. Although applications in these fields are scarce, the Pacific NorthWest National Laboratory has carried research into the detection and identification of smells for medical purposes. In this sense, it has been proposed that an electronic nose could be used to detect infections in wounds [150] and diabetes [151]. The clinical results of the experiments carried out so far reveal that the method is suitable and painless.

Future Developments  
The important development of electronic olfactometry in recent years means that the future perspectives of the technique are very exciting. One of the broadest fields of research today is that of sensor technology [152-157]. Efforts are focused on improving reproducibility and sensitivity. Thus, the appearance on the market of systems with mass detectors opens new possibilities for the technique.

The miniaturisation of electronic olfactometry systems for “in situ” analysis of samples is another field of future developments of the technique. In this sense, Staples [158] and developed a device known as the zNose™, which is small and permits such analyses. The system is a combination of fast gas chromatography (FGC) and a surface acoustic wave sensor (SAW). It has been validated, among others, by the Environmental Protection Agency of the United States (EPA) and has been successfully applied to different environmental problems, drug detection, nerve gas, dioxins, etc.

The interesting perspectives of electronic olfactometry in the analysis of liquid samples has led some authors to propose the term “electronic tongue” for systems that apply similar concepts but for the analysis of liquid samples [159]. The measurement techniques that can be used include potentiometry [160-162], conductivity [162], spectrophotometry [163] and voltammetry [164,165], among others.

Recently, an electronic tongue system, called “hybrid” by the authors has been proposed; it consists of a combination of potentiometry, voltammetry and conductivity for the classification of six different types of milk, with good results [166]. The combination of an electronic nose and an electronic tongue could even improve the capacity of the system [167]. The use of other sensors that simulate the human senses (microphones for the ear, force sensor for touch, etc.) could be an important field of research in the future [168].

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References  


Influence of sample pre-treatment on the determination of As and Se in Antarctic krill by HG-ICP-AES

Abstract

Three digestion procedures for Antarctic krill samples were compared for the reliable determination of two environmentally significant trace elements. A dry ashing (DA) procedure with magnesium nitrate and two microwave (MW) acid assisted digestion methods (with HNO₃/H₂O₂ and HNO₃/HF) were tested for decomposing the certified reference material MURST-ISS-A2 (Antarctic krill) for the subsequent determination of As and Se by HG-ICP-AES. Microwave procedures, namely MW1 and MW2, were insufficient to digest As and Se in this marine organism. The recoveries achieved were: 37.3 ± 2.2% (MW1) and 30.9 ± 1.6% (MW2) for As; 43.2 ± 2.0% (MW1) and 41.6 ± 1.8% (MW2) for Se. On the other hand, the results obtained using the dry ashing method at 500°C were consistent with the reference values with recoveries around 100%. The optimum working conditions and the order of sample, NaBH₄, and KI mixing during the simultaneous hydride generation of As and Se were studied. Under optimized conditions detection limits of As and Se ranged between 22 and 50 ng·g⁻¹ for the three methods tested. In all cases precisions were better than 7%.

Keywords: Microwave digestion, dry ashing, Antarctic krill, arsenic, selenium, HG-ICP-AES detection.

J. Marrero¹, S. Farías² and P. Smichowski²

Comisión Nacional de Energía Atómica. Av. Libertador 8250, 1429-Buenos Aires (Argentina)
¹ Unidad de Actividad Geología, UPESN
² Unidad de Actividad Química, CAC

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Introduction

Environmental studies in Antarctic marine organisms are useful to evaluate pollution phenomena in the only continent still uncontaminated. Krill (Euphausia superba) is extremely abundant in the Southern Ocean and it is a prime food source for seals, penguins and whales, to such an extent that these animals probably would not survive in the absence of this crustacean [1].

On the other hand, this marine organism, like many others, can be seriously endangered by the presence of pollutants. This also can be the case for some potentially toxic chemical elements, although they occur naturally in seawater at very low concentrations. As and Se are acknowledged to behave as essential elements at ultratrace levels which can exert toxic effects depending on the concentration and the chemical form in which they are present. Selenium is an element with a very narrow allowance range in organisms.

The determination of trace metals in marine matrices is subject to a number of errors of various nature for the vast majority of samples. Analysis requires adequate digestion to be reliable. Pre-treatment of samples is a critical step and for most techniques it is necessary to oxidize completely the organic matrix to obtain accurate and reproducible results. Marine animals are known to contain As mostly in the form of arsenobetaine (AsBet) as it
is the end product of arsenic metabolism [2]. Arsenocholine (AsChol), the tetrathiomethylarsinous ion and tri methylarsine oxide are also found in marine animals but they generally occur only as minor components [3]. Arsenosugars and arseneolipids also exist [4]. In the case of selenium, selenomethionine (SeMet) and selenocysteine (SeCys) are the predominant species.

In order to quantify the expected levels of As and Se in marine samples by inductively coupled plasma atomic emission spectrometry (ICP-AES), it is necessary to increase the detection power by the conversion of these elements into their hydrides. The hydride generation (HG) method provides excellent detection limits for As and Se compounds, but cannot detect AsBet, AsChol, SeMet and SeCys. These organic compounds do not yield volatile hydrides and can be converted into hydrides only after their molecules have decomposed. From this point of view, it is mandatory to convert all the organic species present in the sample to species capable of generating their respective hydrides. In this context, it is evident that marine organisms, like krill, require a more complete decomposition procedure to ensure efficient mobilisation of As and Se into solution and it is of great importance to choose an appropriate mineralisation procedure for the analysis of these elements in biological matrices.

The necessity to oxidise completely the organic matrix of biological samples to produce accurate and reproducible results is widely recognised [5]. Different digestion approaches have been reported for decomposing organic matter, including dry ashing [6, 7], wet ashing with different mixtures of reagents [8, 9], microwave acid assisted digestion [10] and UV radiation for easy-to-digest samples [11].

Wet digestion using different acid decomposing mixtures of mineral acids (HNO₃, H₂SO₄, HClO₃) and oxidants (generally hydrogen peroxide) in open [8] and closed devices [9] was widely used to destroy organic matrices. Neve et al. [12] recommended the use of perchloric and sulfuric acids to attack biological materials as they led to complete digestion of the samples for the determination of the total Se content. The attack with the mixture HNO₃·HClO₃·H₂SO₄ was employed to digest samples of marine origin and reference materials for As determination [13].

Microwave dissolution has been widely used for digestion of almost all matrices, including biological [11, 14], geological [15, 16], botanical [17, 18] and marine tissues [19, 20] samples. Microwave digestion procedures have replaced many acid decomposition and dry ashing methodologies because digestion time can be reduced. In addition, the reduced volume of acids required contribute to minimise the overall contamination. In spite of all the recognised advantages, Damkröger and co-workers reported that the microwave system was insufficient to mineralise As in mussel tissues and cod muscle samples [21].

It is known that many of the reported direct HG-AAS or HG-ICP-AES methods that employ wet digestion procedures of marine samples may involve an underestimation of the total As and Se content. Better recoveries were obtained after a dry ashing sample pre-treatment. Dry ashing (DA) was used for decomposing organic matrices in different kind of samples [22, 23]. DA in open systems is easy to use and offers the possibility to handle higher amounts of sample in comparison with wet digestion methods. Mg(NO₃)₂ or MgO + Mg(NO₃)₂ are required to prevent volatilisation of elements like As and Se. DA in the presence of Mg(NO₃)₂ was employed for the pre-treatment of selenium containing samples of fish [24], mussels [25] and oyster [24, 25] in HG-AAS studies. Brumbaugh and Walther improved Se recovery from tissues with a modified sample decomposing method using dry ashing in presence of MgSO₄·HCl·HNO₃ [26].

In this study, different digestion procedures were evaluated with the aim of establishing a suitable method that ensures the efficient mineralisation of krill samples for the subsequent determination of As and Se by HG-ICP-AES. Problems concerning the simultaneous hydride generation of both elements in a continuous mode will also be discussed.

Experimental

Apparatus

Determinations were performed with a Perkin-Elmer (Norwalk, CT, USA) ICP 400 sequential inductively coupled Ar plasma atomic emission spectrometer. The instrument was equipped with a pneumatic cross-flow nebuliser and a disposable quartz torch. The operating conditions are shown in Table 1. The continuous manifold used to generate the hydride was based on the use of an eight channel peristaltic pump (Gilson Minipuls 3, Villiers Le Bel, France), a mixing and reaction coil (250 μL Teflon tubing and a U tube liquid separator. The cylindrical glass cell (id., 2.5 cm) of the gas-liquid separator provided efficient separation of the hydrides and H₂ from the liquids, and mixing of the gaseous products with the carrier gas. The hydrides generated were swept out by Ar connected directly to the inlet tube of the plasma torch. The spray chamber was disconnected and replaced with glass tubing to connect the phase separator with the torch. The transfer line was short enough to avoid transport losses. A schematic diagram of the coupling used for the simultaneous determination of As and Se is illustrated in Figure 1 where the order of reagents mixing is noted.

As and Se were also determined by electrothermal atomic absorption spectrometry (ETAAS) using a Perkin-Elmer (Norwalk, CT, USA) 5100 instrument equipped with Zeeman background correction, a THGA graphite furnace and an AS-71 autosampler. The analytes were atomized from the surface of pyrolytic graphite coated tubes with inserted py-
Table 1. Optimised working conditions for the HG-ICP-AES coupling

<table>
<thead>
<tr>
<th>Plasma</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward rf power</td>
<td>1.1 kW</td>
</tr>
<tr>
<td>Frequency of rf generator</td>
<td>40 MHz</td>
</tr>
<tr>
<td>Coolant gas flow rate</td>
<td>15 L min⁻¹</td>
</tr>
<tr>
<td>Auxiliary gas flow rate</td>
<td>2 L min⁻¹</td>
</tr>
<tr>
<td>Sample flow rate</td>
<td>0.75 L min⁻¹</td>
</tr>
<tr>
<td>Wavelengths</td>
<td>As(I): 193.7 nm; Se(I): 196.0 nm</td>
</tr>
<tr>
<td>Viewing height above load coil</td>
<td>15 mm</td>
</tr>
<tr>
<td>Integration time</td>
<td>20 s</td>
</tr>
</tbody>
</table>

Hydride generation

<table>
<thead>
<tr>
<th>Samples and reagents flow rate</th>
<th>1.5 mL min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample acidity</td>
<td>5 M HCl</td>
</tr>
<tr>
<td>NaBH₃ concentration</td>
<td>0.8 % (w/v)</td>
</tr>
<tr>
<td>KI concentration</td>
<td>6%</td>
</tr>
<tr>
<td>Coil volume</td>
<td>250 μL</td>
</tr>
<tr>
<td>Tube size for reagents</td>
<td>1.1 mm (i.d.)</td>
</tr>
</tbody>
</table>

Welding argon from Indura (Buenos Aires, Argentina) was found to be sufficiently pure for As and Se determination. All reagents were of analytical reagent grade unless otherwise mentioned. Deionised distilled water (DDW) was produced by a commercial mixed-bed ion-exchange system Barnstead (Dubuque, IA, USA) fed with distilled water. Commercially available 1000 mg L⁻¹ As and Se standard solutions (Merck, Darmstadt, Germany) were used. Dilute working solutions were prepared daily by serial dilutions of these stock solutions. Sodium tetrahydroborate (III) solution, 3% (w/v) was prepared by dissolving NaBH₃ powder (Baker, Phillipsburg, NJ, USA) in deionized water, stabilising in 1% (w/v) NaOH (Merck) and filtering through Whatman N 42 filter paper to eliminate turbidity. The solution was stored in a polyethylene flask at 4 °C. Diluted working solutions were prepared before use.

Ultrapure hydrochloric acid (Ultrex II, Baker) was employed to generate the hydrides. Analytical reagent nitric acid (Merck, 70%) was used after additional purification by sub-boiling distillation in a quartz still. Mg(NO₃)₂ + 6 H₂O (Merck) was used as ashing aid.

Samples

A certified reference material (CRM), Antarctic krill (MURST-ISS-A2) was used in this comparative study. The certified concentrations of As and Se are: 5.03±0.42 and 7.37±0.91 μg g⁻¹ respectively.

Sample digestion procedures

All the experiments were performed by triplicate. In all cases, a set of digestion blanks were prepared together with each digestion procedure tested. In order to decrease digestion blanks, purified nitric acid was used. The procedures followed to digest krill samples are detailed as follows:

Microwave digestion procedure Nº 1 (MW 1)

0.5 g of freeze-dried krill samples were transferred into Teflon vessels and 5 mL of purified concentrated HNO₃ (Merck, 70%) and 1.0 mL of HF (Merck, 40%) were added. The average MW power applied during the digestion steps varied from 300 to 600 W and the complete mineralization cycle was less than 30 min. The operating conditions for the MW digestion of Antarctic krill are detailed in Table 2. An optimised selection of the power and time in the MW digestion is important to achieve a complete dissolution of the samples. After cooling, the vessels were opened and placed on a hot plate and they were evaporated to dryness. Then, the residue was dissolved by adding drop by drop 5 mL of (1+1) HCl solution. The resulting solution was placed in a boiling water bath (loosely covered with a watch glass) and heated at 95 °C for about 15 min. The digest was allowed to cool and then transferred into a 25 mL volumetric flask. A 8 mL of concentrated HCl were added and the solution was diluted to volume with DDW.

Microwave digestion procedure Nº 2 (MW 2)

0.5 g of freeze-dried krill samples were transferred into Teflon vessels. 5 mL of purified concentrated HNO₃ (Merck, 70%) and 1.5 mL of H₂O₂ (Merck, 30%) were added and the solution was diluted to volume with DDW.

Table 2. Microwave acid digestion program (MW1) to mineralise krill samples

<table>
<thead>
<tr>
<th>Sample weight</th>
<th>0.50 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagents</td>
<td>5 mL HNO₃ + 0.5 mL HF</td>
</tr>
<tr>
<td>Final volume</td>
<td>25 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microwave program:</th>
<th>applied power (W)</th>
<th>time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>5</td>
</tr>
<tr>
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<td>1</td>
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<td></td>
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<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3. Microwave acid digestion program (MW2) to mineralise krill samples

<table>
<thead>
<tr>
<th>Sample weight</th>
<th>0.50 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagents</td>
<td>5 mL HNO₃, 1.5 mL H₂O₂</td>
</tr>
<tr>
<td>Final volume</td>
<td>25 mL</td>
</tr>
</tbody>
</table>

Microwave program:
- Applied power (W)
  - 250: 1
  - 50: 1
  - 250: 5
  - 400: 5
  - 600: 5

were added. The heating program shown in Table 3 was run to completion. The complete mineralisation cycle lasted 17 min. After cooling, the vessels were opened and placed on a hot plate and they were evaporated to dryness. The residue was then dissolved by adding drop by drop 5 mL of (1+1) HCl solution. The resulting solution was placed in a boiling water bath (loosely covered with a watch glass) and heated at 95 °C for about 15 min. The digest was allowed to cool and transferred into a 25 mL volumetric flask. A 8 mL of concentrated HCl were added and the solution was diluted to volume with DDW. The whole sample pre-treatment process lasted about 30 hours.

**Calibration**
Calibration curves were obtained with standards prepared in 5 M HCl acid. In spite of the complexity of the matrix investigated, no standard addition calibration was necessary.

**Results and discussion**

The simultaneous determination of As and Se at trace levels in marine samples is subject to errors during sample preparation, pre-reduction of the analytes to the required oxidation states and hydride generation. In this context, and in order to prevent these errors, careful optimisation of experimental parameters and hydride generation conditions are essential for obtaining reliable results.

**Optimisation of hydride generation conditions**

Owing to the high oxidation potential of acid mixtures, most part of the arsenic and selenium species are converted into arsenate and selenate respectively. A pre-reduction step for converting seleno species into Se(IV) is necessary in all HG procedures because only Se(IV) is capable to generate Se hydride. It is recommended to perform this reduction step at high temperature (90-100 °C) since this redox equilibrium is strongly temperature dependent [27]. To convert Se(VI) to Se(IV) for maximum efficiency in the generation of SeH₄, the digested samples were heated at 95 °C for 15 min. in (1+1) HCl solution. However under these conditions, arsenic remains as As(V) and a pre-reduction step is mandatory to convert As(V) to As(III). As(V) can be reduced with potassium iodide, thiourea, L-cysteine or mixtures of hydrogen sulfide-thiosulfate and iodide-ascorbic acid. KI is the most often reducing agent employed but in the case of the simultaneous determination of As and Se, Se will be reduced to its elemental form and the hydride will not be formed. Nygaard and Lowry [28] reported that in a continuous mode system, the addition of KI to the sample after NaBH₄ addition allows Se to generate the hydride before the reduction to elemental Se can occur. The effectiveness of this approach was also observed in our study. Differences in the kinetics of As and Se hydride evolution may be responsible for this behaviour. The generation of SeH₄ is very fast and it was estimated to take place in less than 10 ms [29]. In the case of As, kinetics studies of arsine generation have shown that the hydride is produced from As(III) species in about 100 ms [30]. The concentration of iodine was optimised and a maximum yield of arsine was obtained with a KI concentration of 6%. The order of reagents mixing during hydride generation is shown in detail in Figure 1.

The effect of tetrahydroborate(III) concentration on As and Se response was also examined. The optimal As and Se signals were achieved with 1% (w/v) NaBH₄. In spite of this, the higher production of H₂ observed at this concentration, led to a violent reaction (eventually the plasma extinguished) and made necessary to reduce the NaBH₄ concentration to 0.8%. This concentration was used in further experiments.

Under the conditions reported in Table 1 and the order of reagents mixing during the hydride generation
process, As and Se were generated with maximum hydride evolution.

Comparison of sample digestion procedures

All digests were prepared using optimised microwave conditions (Tables 2 and 3). Preliminary studies demonstrated that a maximum power last stage was beneficial for better digestion. It is attributed to the fact that the rate of the digestion reaction of acid decomposition and oxidation power of the acids are increased with higher powers. The addition of hydrogen peroxide to the nitric acid was necessary to increase the oxidation efficiency of fat tissues. Addition of HF was tested to remove siliceous components that are present in the caparace (shell) of krill. The cool steps in the MW program were included to ensure that the reaction was brought under control.

Sample preparation is without any doubts a critical stage in As and Se determination in marine samples like krill. The effectiveness of the three digestion procedures was compared in terms of As and Se recoveries. The resulting recoveries of As and Se from MW 1, MW 2, and DA correspond to mean values of three sets of replicates, each set consisting of five measurements.

Microwave digestion procedures (MW1 and MW2)

When the MW 1 digestion procedure was tested to digest krill samples recoveries of only 37.3±2.2 and 43.2±2.0% were obtained for As and Se respectively. The recoveries achieved after the application of the MW2 digestion procedure were: 30.9 ± 1.6 and 41.6 ± 1.8% for As and Se respectively.

The over-all picture of both MW procedures shows that organic As and Se compounds were not quantitatively decomposed by the acid mixtures to inorganic species. This fact confirms that some organoarsenic and organoselenium species are extremely resistant to wet chemical oxidation and persist in the final digest, thus producing lower results in HG procedures. Damkröger and coworkers [21] also found an incomplete mineralisation of organic As species when a high-pressure ashing system was employed to attack marine tissues. The same difficulty in mineralising organic Se species was observed by Nève et al. [12].

The quick heating of the MW system may be responsible for the low recoveries obtained. It is quite evident that the MW treatment can not decompose the organic molecules.

Dry ashing digestion procedure (DA)

Another issue that arose during this study was to optimise the temperature in the dry ashing method. In order to remark the effect and the importance of temperature on As and Se recoveries, the dry ashing procedure was performed at 250 and 500 °C. The results achieved with HG-ICP-AES detection are decisively influenced by the temperature. Almost quantitative recoveries were obtained at 500 °C. The recoveries obtained when the DA procedure was tested at 250 °C were: 63.5 ± 4.3% for As and 54.0 ± 3.2 for Se. In contrast, the recoveries obtained using the same procedure at 500 °C were: 101 ± 5.7 and 98.3 ± 5.3% for As and Se respectively. A higher temperature must be employed to destroy completely the stable organic molecules contained in the samples. Our results are in accordance with those reported by Fecher and Ruhmk [31] who also observed a drastic increase in the recovery of As when a certified reference material (BCR
was subjected to high pressure ashing (HPA) treatment in a quartz vessel at different temperatures.

**Analysis of krill samples by ETAAS**

In order to verify that As and Se were not partially volatilized when samples were evaporated to dryness after microwave treatments (MW1 and MW2), krill samples were also analysed by ETAAS. Utilisation of the stabilised temperature platform furnace (STPF) and Zeeman effect background correction was necessary to eliminate spectral interferences caused by atoms having absorbing lines within the As and Se spectral bandpass. A mixture of 5 μg Pd + 3 g Mg(NO₃)₂ was used as matrix modifier. The recoveries obtained were: 95.9 ± 3.9 and 103 ± 3.2% for As and Se, respectively (after MW1 treatment) and 98.0 ± 3.3 and 102 ± 3.9% for As and Se, respectively (after MW2 treatment). These results confirm that both elements remained in solution after the evaporation to dryness.

**Quality parameters**

The three sample pre-treatment were also compared in terms of their analytical performance. The quality parameters were determined under the optimised operating conditions established in the foregoing investigation.

The detection limits were calculated following the IUPAC rules [32] on the basis of the 3 criterion for ten replicate measurements of blank solutions that were subject to the same treatment that the samples. The detection limits averaged between 22 and 50 ng g⁻¹ (Table 4) depending on the sample pre-treatment tested. Precision was evaluated for the three methods studied and the RSD values obtained were in the range 3.6-6.8% for ten replicate measurements of MURST-ISS-A2 solutions. The detection limits (LD) and relative standard deviation (% RSD) were in general satisfactory if the complexity of the matrix analysed is taken into account.

No significant differences are evident for the three digestion approaches tested and in all cases are limited by the purity of the reagents rather than by the sensitivity of the coupling used.

**Conclusions**

The comparison of three digestion pre-treatment of krill samples demonstrated that to obtain satisfactory recoveries of As and Se in marine organisms, drastic digestion conditions are required. Even when the dry ashing procedure is extremely time consuming, it is the best alternative when HG-ICP-AES is used for the detection as a consequence of the resistance of some organic compounds to generate volatile hydrides.

The results obtained evidenced that when dry ashing was used as sample pre-treatment, a complete destruction of organic matter was achieved without loss of As and Se through volatilisation. Another advantage to highlight is that a large amount of sample can be processed. This fact is of great importance when very low concentrations of analytes are expected in the samples.

**Acknowledgements**

The authors gratefully thank Dr. Sergio Caroli for supplying the krill samples and Lic. Liliana Valiente for the ETAAS measurements. Dr. Scott Willie and Lee L. Yu are also acknowledged for their kind cooperation. Financial support was provided by the Agencia Nacional de Promoción Científica y Tecnológica through Project PICT-N° 06-00000-00354. This work is part of CNEA-CAC-UAQ projects 97-Q-02-03 and 97-Q-02-05.

**References**


**Table 4** Detection limits (3σ criterion) and precision (% RSD) achievable for Antarctic krill using three digestion procedures

<table>
<thead>
<tr>
<th>Element</th>
<th>Detection limit (ng g⁻¹)</th>
<th>Precision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MW1</td>
<td>MW2</td>
</tr>
<tr>
<td>As</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>Se</td>
<td>50</td>
<td>35</td>
</tr>
</tbody>
</table>

(n=10)
Practical aspects of separation of selenite, selenate, selenocysteine and selenomethionine in urine with anionic exchange liquid chromatography

Abstract

We study the behaviour of selenite, selenate, selenomethionine (SeMet) and selenocysteine (SeCys) in human urine in an anionic-exchange polymer-based chromatographic column. The coupled technique liquid chromatography-UV irradiation-hydride generation-quartz cell atomic absorption spectrometry (LC-UV-HG-QCALS) is used for the study. Several clean-up cartridges are assayed and C18 gives the best results. Adsorption of SeMet and SeCys on the column is observed and studied in detail. Under the optimised working conditions detection limits and precision are established and analysis of several samples have been carried out. The detection limits (expressed as Se) are 42 µg l⁻¹ for selenite, 51 µg l⁻¹ for selenate and 12 µg l⁻¹ for SeMet. Detection limit of SeCys could not be established, since the adsorption of this compound did not allow its quantification. Precision at concentrations 10 times those of the detection limits is lower than 6.5 % R.S.D. for all the Se compounds. Only SeMet was detected in one of the donors' samples.

Keywords: Selenite; selenate; selenomethionine; selenocysteine; selenium speciation; urine. Liquid chromatography-UV photoreaction-hydride generation-atomic absorption spectrometry.

M. Vilanó, R. Rubio* and G. Rauret

Departament de Química Analítica, Universitat de Barcelona. Martí i Franquès, 1-11. 08028 Barcelona (Spain).

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Introduction

Selenium is an essential element in human diet, but it can be toxic at high concentration [1]. Se intake is usually provided by a normal diet, and it is mainly excreted in urine [2]. For this reason, a relationship between the concentration of Se in urine and Se intake has been established [3]. Se intake may thus be monitored by analysing urine. Se concentration of 100 µg l⁻¹ in urine is an indicative of organisms toxicity [3, 4], but significantly higher concentrations have been reported [3]. The toxicity of Se depends on its chemical form. The relative toxicity of Se compounds has been established as selenite > selenate > selenomethionine (SeMet) > (selenocysteine (SeCys) = (selenoethionine (SeEt)) [5], whereas trimethylselenonium ion (TMSe⁺) is not toxic [3, 6].

All these Se compounds can be found in urine, as well as other unidentified species [7, 8]. Nevertheless, there is some controversy on the presence of such compounds in urine. Thus, Yang et al. [7] report the presence of TMSe⁺, selenite and an unidentified compound in urine, whereas González LaFuente et al. [8] found inorganic Se and two unidentified species; Muñoz Olivas et al. [9] report the presence of SeCys in several urine samples.

Se speciation in urine is performed by coupling liquid chromatography with a selective, sensitive detection technique, such as induc-
tively coupled plasma-mass spectrometry (ICP/MS) [7-11], hydride generation-quartz cell-atomic absorption spectrometry (HG-QCASA) [8,12], or hydride generation-inductively coupled plasma-optical emission spectrometry (HG-ICP/OES) [8].

Recently, the coupled technique liquid chromatography (LC)-UV irradiation-HG-QCASA was optimised and established for selenite, selenate, SeCys and SeMet determination in water [13]. The present paper reports the attempt to adapt and apply this method for selenium speciation in urine, testing several species that could be of interest for assessing toxicity. The chromatographic process was studied in detail, since selenoamino acids in urine samples showed strong adsorption on the polymer-based anion-exchange chromatographic column after few injections, and this effect was more pronounced for selenocystine. Several attempts were carried out in order to overcome these analytical difficulties. Urine samples from several donors were analysed in the study.

**Experimental**

**Apparatus**

All the measurements were performed using the LC-UV-HG-QCASA system [13].

A Perkin Elmer 250 LC binary pump (CT, USA) and a Rheodyne 7125 injector (Cotati, CA, USA) with a 100 µL loop were used. The stationary phase was a polysiloxane divinylbenzene-based anion exchange column Hamilton PRP X100 (Reno, NV, USA) with trimethylammonium exchanger, 10-µm particle size (250 mm x 4.1 mm). The chromatographic system was also equipped with a Hamilton PRP X100 guard column.

The photoreaction system [13] combines a water-cooled high-pressure Hg vapour lamp Heraeus TQ 150 (Hanau, Germany) with PTFE tubing (I.D. 0.55 mm) from Cole Parmer (Vernon Hills, IL, USA).

Hydride generation unit: a Gilson Minipuls peristaltic pump (Middleton, WI, USA), a reaction coil (L: 75 cm; I.D.: 1 mm) and a gas-liquid separator.

A Perkin Elmer 1100B atomic absorption spectrometer (CT, USA) equipped with a Se EDL lamp operating at 4.5 W and a deuterium lamp for background correction, was used as detector. Atomisation was performed in a Perkin Elmer AS-90/91 electric heating system equipped with a quartz cell (cell temperature: 900 °C). Measurement conditions: Wavelength: 196.0 nm; slitwidth: 2 nm.

Data acquisition and processing of the signal from the spectrometer were performed by microcomputer [14].

**Reagents, standards and samples**

All solutions were prepared with doubly-deionized water (USF purelab plus, Ransbach Baumbach, Germany) with 18.2 MΩ cm resistivity.

1000 µg L⁻¹ Se stock solutions were prepared from the following reagents: selenite, Na₂SeO₃ 99% (Aldrich, Milwaukie, WI, USA), selenate, Na₂SeO₄ 99% (Aldrich), Seleno-DL-cystine (SeCys) (Sigma, St. Louis, MO, USA) and seleno-DL-methionine (SeMet) (Sigma). SeCys and SeMet were weighed under nitrogen to prevent sample degradation, as recommended by the manufacturer, and dissolved in 0.5% (v/v) hydrochloric acid (Merek 37% "pro analysi", Darmstadt, Germany).

NaBH₄ 1% (w/v) was prepared daily from NaBH₄ 97% "parum" (Fluka, Buchs, Switzerland) in NaOH 0.5%; the resulting, slightly turbid mixture was filtered through a 0.45 µm Nylon membrane.

Phosphate buffer solutions were prepared by dissolution of Na₃PO₄·2H₂O 99% and Na₂HPO₄·2H₂O 99% (Carlo Erba, Milan, Italy) in water. These solutions were filtered through a 0.22-µm Nylon membrane before use.

Nitric acid, HNO₃ 70% "Baker Instr. Analyzed" was purchased from J. T. Baker (Deventer, The Netherlands).

Methanol 99.8% "Lichrosolv gradient grade" was purchased from Merck.

24-hour samples were collected daily [5,13] from healthy donors and kept at 4 °C for 12 h before analysis. The samples were then filtered, first through a 0.45 µm, and then through a 0.22 µm nylon membrane, to avoid overloading of the 0.22 µm membrane.

C₁₈ snap-carpillaries (600 mg) Lida (Kenosha, WI, USA) were used for cleaning up urine.

**Procedure for C₁₈ clean up cartridges conditioning and clean up process**

5 mL MeOH and 5 mL water were pumped consecutively through the C₁₈ cartridge at 1 mL min⁻¹ in order to condition it. Then, 10 mL urine was passed through the cartridge at 1 mL min⁻¹. The first 2 mL (mainly containing conditioning solutions) were discarded. A new clean up cartridge was used for every urine aliquot.

**Procedure for washing of the chromatographic column**

Water (60-70 mL), then 0.01 mol L⁻¹ HNO₃ (60-70 mL), and finally water (60-70 mL) were pumped at 1 mL min⁻¹ through the column. After this, the column was conditioned with 60-70 mL mobile phase at 1 mL min⁻¹.

**Procedure for quantification of selenite, selenate and SeMet**

The coupled system LC-UV-HG-QCASA was used. 10 µL of the cleaned-up sample was injected into the LC system, and the Se compounds were eluted with the 40 mmol L⁻¹ phosphate buffer at pH 7.0 at 1 mL min⁻¹ as mobile phase. The eluate was first UV-irradiated on-line for 60 s in the photoreactor, and then introduced into the hydride generation system, through which HCl 32% at 1 mL min⁻¹ and NaBH₄ 1% at 1 mL min⁻¹ were pumped. After reduction in the coil, the hydrogen selenide generated was carried by an argon flow into the heated quartz cell. Standard addition to the urine sample before clean up was performed for quantification of the Se compounds. Either peak area or peak height (after applying the Savitzky-Golay filtering method [16]) measurements were used for quantification.

The column must be washed with 0.01 mol L⁻¹ HNO₃ after 10 sample injections, according to the procedure described above.
Results and discussion

Aliquots of an aqueous standard mixture containing selenite, selenate, SeCys and SeMet, and filtered urine spiked with the same species were analysed and the results were compared. The final concentrations in the aliquots were 25 μg L⁻¹ Se as selenite, 500 μg L⁻¹ Se as selenate, 40 μg L⁻¹ Se as SeCys and 120 μg L⁻¹ Se as SeMet, which corresponded to approximately ten times the concentration of the detection limits for such species in water [13]. A non-spiked urine sample was also measured as a blank. Figure 1 shows the influence of the urine matrix on the analytical response. It is particularly remarkable the split in two peaks for selenite and SeMet. Whereas the analytical responses of the four Se compounds versus concentrations were linear for aqueous aliquots, the absorbance signals for SeCys and SeMet decreased with the number of urine aliquots injected. This behaviour can be seen in Figure 2, which shows the chromatograms of aqueous standards obtained when the chromatographic column was previously exposed to several injections of urine. The pattern of those sequential chromatograms could indicate that the selenoamino acids were adsorbed on the column. In order to avoid this matrix effect, dilution was tested. As a result, a decrease in the absorbance signals of SeCys and SeMet was still observed when diluting urine 1:1 and 1:3 (either with water or with mobile phase), and the background signal was high.

Several clean up cartridges (neutral, basic and acidic alumina, SAX anion exchange and C18) were tested. Background signal was significantly lowered after cleaning up the samples. The recovery of Se species calculated after clean up is shown in Table 1. We chose C18 (600 mg cartridge), since no losses of selenite, SeCys or SeMet was observed in the clean up process, although selenite was the only compound significantly retained by the cartridge.

Clean up did not prevent SeMet or SeCys adsorption in the column. Figure 3 shows the variation of the peak area of SeCys and SeMet as a function of the number of injections. Forty consecutive urine injections were performed. A significant decrease in SeCys peak area was observed after the second injection. No decrease in signal was observed for SeMet after 25 consecutive injections, and then major loss of signal was observed. The adsorption of both SeMet and SeCys on the column could be explained by the nonlinear response of their signal after 40 spiked urine injections versus concentration. The behaviour of SeCys and SeMet before and after urine injections in the column is shown in Figure 4. By considering the differences between the respon-

![Figure 1. Chromatograms from spiked water (a) and spiked urine (b). The system LC-UV-HG-QCAAS was used in the measurements. 1=SeCys (40 μg L⁻¹ Se), 2=selenite (25 μg L⁻¹ Se), 3=SeMet (120 μg L⁻¹ Se), 4=selenate (500 μg L⁻¹ Se).]
Figure 2. Chromatograms corresponding to aqueous standards containing the four selenium compounds after 25 urine sample injections, (a) after 35 urine injections (b) and after 50 injections of urine (c) in the system LC-UV-HG-QCAAS. 1 = SeCys (40 μg L⁻¹ Se), 2 = selenate (25 μg L⁻¹ Se), 3 = SeMet (120 μg L⁻¹ Se), 4 = selenite (500 μg L⁻¹ Se).

Table 1. Recoveries of Se compounds in a spiked urine aliquot obtained by using several clean-up cartridges. Standard addition was used for quantification. Se compounds concentration were 25 μg L⁻¹ Se as selenite, 500 μg L⁻¹ Se as selenate, 40 μg L⁻¹ Se as SeCys and 120 μg L⁻¹ Se as SeMet.

<table>
<thead>
<tr>
<th>Clean up cartridge</th>
<th>SeLente</th>
<th>Selenate</th>
<th>SeCys</th>
<th>SeMet</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 mg C18</td>
<td>8 ± 0.5</td>
<td>93 ± 15</td>
<td>85 ± 7</td>
<td>72 ± 11</td>
</tr>
<tr>
<td>600 mg C18</td>
<td>6 ± 0.4</td>
<td>99 ± 4</td>
<td>97 ± 3</td>
<td>105 ± 1</td>
</tr>
<tr>
<td>SAX</td>
<td>27 ± 6</td>
<td>103 ± 5</td>
<td>46 ± 15</td>
<td>n.l.</td>
</tr>
<tr>
<td>Neutral Alumina</td>
<td>6 ± 1</td>
<td>94 ± 4</td>
<td>11 ± 6</td>
<td>n.l.</td>
</tr>
<tr>
<td>Basic Alumina</td>
<td>9 ± 2</td>
<td>128 ± 12</td>
<td>19 ± 6</td>
<td>n.l.</td>
</tr>
<tr>
<td>Acidic Alumina</td>
<td>5 ± 2</td>
<td>77 ± 8</td>
<td>20 ± 6</td>
<td>n.l.</td>
</tr>
</tbody>
</table>

n.l. = no linear response was obtained in the quantification.

Figure 3. Chromatographic responses (peak area) during the consecutive injection of spiked urine. Measurements were performed using LC-UV-HG-QCAAS. Selenium concentrations in the urine aliquot (expressed as Se) were 120 μg L⁻¹ as SeMet and 40 μg L⁻¹ as SeCys.
Figure 4. Behaviour of selenoamino acids before and after 40 spiked urine aliquot injections. a) SeCys, b) SeMet.

Figure 5. Retention times (a) and resolution between SeMet and selenate (calculated from SeMet and selenate retention times and base width values), (b) after consecutive injections of spiked urine. The system LC-UV-HG-QCAAS was used in the measurements.

The resolution between SeMet and selenate was also affected. Thus, the retention times of SeMet and selenate decreased with the number of consecutive urine injections (Figure 5a), leading to loss of resolution between these two peaks (Figure 5b). Resolution reached a constant value (approximately 0.95) after fifteen injections of urine.

Several washing solutions were tested to restore the original performance of the stationary phase. Washing solutions containing organic solvents, such as methanol or acetonitrile, did not improve the performance of the column [17]. However, nitric acid solutions were efficient. Several assays were carried out in order to optimise the concentration of the HNO₃ solution used for column washing. For this, about 25 injections of urine were performed to provoke selenoamino acids, curves derived from both Langmuir and Freundlich isotherms were fitted. The linear correlation observed (R² greater than 0.99 in all cases) confirms the hypothesis of adsorption of SeCys and SeMet on the column.
Table 2. Quality parameters. The system LC-UV-HG-QCAAS was used in all the measurements.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Selenite</th>
<th>Selenate</th>
<th>SeMet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (µg L⁻¹ Se)</td>
<td>1800</td>
<td>2000</td>
<td>1500</td>
</tr>
<tr>
<td>Detection limit (µg L⁻¹ Se)</td>
<td>42</td>
<td>51</td>
<td>12</td>
</tr>
<tr>
<td>Repeatability (R.S.D.)</td>
<td>3.7</td>
<td>4.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

¹ Calculated using peak areas.
² Calculated as 3σ of the background signal.
³ Repeatability expressed as R.S.D. of twenty peak area measurements, in the same working session, of a urine sample spiked with SeMet and selenate at concentrations ten times those of the detection limits.

Figure 6. Chromatogram obtained from a filtered, cleaned up urine sample from a healthy male donor. The system LC-UV-HG-QCAAS was used in the measurement. 1. SeMet.

adsorption and the loss of resolution between SeMet and selenate. After washing the chromatographic column with HNO₃ solutions ranging from 0.01 mol L⁻¹ to 0.1 mol L⁻¹, the original linear response of selenoamino acids aqueous standards at low concentrations was totally recovered. Nitric acid concentrations of less than 0.01 mol L⁻¹ were inefficient. Consequently, 0.01 mol L⁻¹ HNO₃ was adopted (the working pH range of the column is 1-13, according to the manufacturer).

According to this study, an analytical procedure such as that described in the Experimental section could be proposed for the determination of selenite, selenate and SeMet in urine. The quality parameters were evaluated and repeatability, detection limits (3σ) and linear ranges are summarised in Table 2.

2. Application to the analysis of urine from several donors

Urine form five donors was analysed in order to test the applicability of the proposed procedure. To do this, urine from both men (3) and women (2) was measured, all of them healthy people, with age ranges of 24-55 for men and 22-53 for women. All the samples were measured in triplicate and in two working sessions, in order to establish the precision of the analysis. However, only in one sample could SeMet be quantified (male donor: 23 ± 2 µg L⁻¹ Se as SeMet). Nevertheless, repeatability, linearity and sensitivity were similar in all cases whatever the origin of the sample, and also similar to the values reported in Table 2.

Conclusions

The complex composition of human urine makes the speciation analysis of Se difficult. With the chromatographic system used, adsorption of SeCys and SeMet on the column was observed when urine aliquots were analysed. This adsorption, as well as the resolution power of the column was deeply studied. The use of C18 clean up cartridges significantly reduced the background signal. 0.01 mol L⁻¹ HNO₃ was revealed as an efficient washing system for restoring the analytical performance of the polymer-based column.

Consequently, the proposed method may be used for Se speciation in urine.

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Free and immobilized yeast for chromium trace preconcentration and wastewater clean up (remediation)

T. Pérez-Corona, Y. Madrid-Alharrán and C. Cámara*

Departamento de Química Analítica, Facultad de Químicas, Universidad Complutense de Madrid, Ciudad Universitaria s/n, 28040 (Madrid).

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Abstract

Inexpensive baker’s yeast characterized as *Saccharomyces cerevisiae* was used to selectively accumulate and preconcentrate Cr(III) from aqueous matrices. The substrate was used in batch and continuous operation, immobilized in the latter case on alginate. Several parameters affecting the degree of bioaccumulation of Cr(III) and Cr(VI) were evaluated: pH, temperature, incubation time, amount of biomass, etc. In the presence of yeast, aqueous Cr(VI) remains unaltered and in solution, while Cr(III) is quantitatively bound to the yeast at pH 13. The experimental data were modelled using sorption isotherms and could be fitted to Langmuir-type expressions. By using the adsorbent *S. cerevisiae* immobilized on alginate, an adsorption-elution method was developed for Cr(III) preconcentration based on retention on a column of immobilized yeast and further elution with 3 mL of 0.3 mol L⁻¹ HNO₃. The system developed was applied for on-line remediation of wastewater samples and trace chromium preconcentration in a certified river water sample.

Keywords: Yeast, chromium, immobilization, ETAAS.

Introduction

The biosorption of metals by biological substrates such as algae, fungi, yeast, bacteria, plant-derived materials, etc. has been of interest for various technological reasons: recovery of precious and important metals (uranium and other radionuclides, gold, etc.), reclamation and remediation of contaminated sites, mining procedures, etc. This sorption process has variously been called bioconcentration, bioaccumulation, and biosorption, which is the most frequently used term. Metals can be sequestered by these substrates in living and dead organisms and metabolic activity is not necessarily required. An important mechanism for the accumulation of metal ions is their adsorption on the surface of the substrate through interactions with a wide variety of chemical functional groups on the cell wall such as amines, carboxyl groups, thiol groups and unmodified pectins, since the metals can bind to different kinds of sites. The mechanism of the complex phenomenon of biosorption depends on the type of substrate, on the element and even on its chemical form [1,2].

Yeast and fungi have been the most widely used biological substrates for metal accumulation. These cells take up metal ions by binding to the cell surface and intracellularly. The main structural component of fungal cell walls is either chitin or chitosan, which are polymers of N-acetylated or non-acetylated glucosamine, respectively. Other ligands include phosphate, carboxyl, amine acid and hydroxyl groups. A wide range of metals binds to fungal cell walls. Different cell forms of the same organism have different binding capacities [2].

Yeast are more widely used since they grow easily, are non-hazardous, have a high metal tolerance and a high cell-binding capacity. Unlike most microorganisms, yeasts tolerate a wide range of environmental conditions. Yeast grows in pHs ranging from below pH = 2 to above pH = 9, with an optimum usually between 4 and 7. The temperature range for growth of most yeasts is also broad (5-35°C), and some species grow above or below this range [3]. This is important since a yeast substrate allows work in a wide range of experimental condi-
tions with minimum risk of infection and contamination and it can be obtained cheaply as a by-product from many industrial sources. Ben Omar and co-workers [4] used *Saccharomyces cerevisiae* from the brewing industry for biosorption of La, Co, Mn, U, Pb, Ag, Zn, Cd, and Cr from aqueous solutions. The accumulated metals were further eluted using sodium carbonate as a desorbing agent.

Yeast can be used either free or immobilized on different supports and metal can be accumulated in both batch and continuous flow systems.

Maquiera [5] used *Saccharomyces cerevisiae* immobilized on CPG to preconcentrate Cu, Zn, Pb and Cd. Between pH 6.0 and 7.5, metal ions bound strongly to the cell surface and quantitatively desorbed when the pH was lowered to <2.0. The enhancement factors for Cd, Pb, Zn, Fe and Cu were 250, 125, 2000, 750 and 286, respectively, which were higher than those obtained using exchange chelating materials. The detection limits using an atomic absorption detector for Cd(II), Zn(II), Cu(II), Pb(II) and Fe(III) were 0.2, 0.1, 0.7, 0.8 and 0.6 ng mL⁻¹, respectively.

*S. cerevisiae* immobilized on sepiolite was used for on-line preconcentration of Cu, Zn and Cd followed by flame atomic absorption spectrometry [6]. The uptake efficiency was pH-dependent and the optimum values were 6 for Zn and 8 for Cu. The retained metal ions were eluted with 100 µL of 1 M HCl leading to preconcentration factors of 75 for Cd and Zn and 8 for Cu. This sepiolite was also cross-linked with formaldehyde for continuous removal of Cu, Zn and Cd from aqueous solutions at pHs 5.3, 6.0 and 6.7, respectively. The bound metals were completely recovered by washing the column biomass with 0.1 M HCl.

Baker's yeast has shown not only an ability to sequester heavy metals but also to differentiate species according to their toxicity or chemical oxidation states. It has been proposed as a substrate for differentiating Hg(II) and CH₃Hg⁺ [7]. The substrate has demonstrated a much higher affinity for organic mercury than for inorganic mercury and was successfully used to selectively and quantitatively separate methylmercury and Hg(II) under optimum conditions, i.e. 30 min incubation time at pH = 7 and 37°C. Methylmercury was immediately bound to the yeast over a wide pH and temperature range. The fraction of methylmercury bound was in all cases 100% and was unaffected by solution pH, temperature, incubation time, biomass and analyte amount and the presence of foreign ions. Later, the same authors developed a simple new on-line analytical method based on the use of a microcolumn filled with yeast immobilized on silica gel. In this case, both mercury species were retained in the pH range 2-12 (inorganic mercury on silica gel and organic mercury on yeast). Hg preconcentration and speciation was achieved by selective elution. The bound CH₃Hg⁺ and Hg²⁺ was removed with 0.02 M HCl and 0.2 M Na₂S₂O₃, respectively.

*S. cerevisiae* has also shown a higher potential for Se(IV) and Se(VI) than for Se(IV) and Se(V), respectively, in the pH 3-10 range [8]. In the presence of yeast, aqueous Se(VI) remained unaltered and in solution under all the experimental conditions tested, while Se(IV) was accumulated in the cell. The accumulation step may occur along with the Se(IV) transformation processes, probably reduction to Se(II) or Se⁰.

This paper describes the use of baker's yeast cells characterized as *S. cerevisiae* for the selective accumulation and preconcentration of Cr(III), in the presence of Cr(VI), in both batch and continuous flow systems. The substrate was employed either free or immobilized and was applied to preconcentrate chromium in natural waters and reme-

diate Cr from several industrial waste streams.

**Experimental**

**Instrumentation**

A Perkin-Elmer Model 1100B atomic absorption spectrometer equipped with an HGA-400 graphite furnace and a deuterium lamp background corrector was used. A Perkin-Elmer chromium hollow-cathode lamp was used as the light source for chromium determination. The monochromator spectral bandwidth required to isolate the 357.9 nm chromium line was 0.7 nm. An argon flow-rate of 300 mL min⁻¹ was maintained to purge air from the coated graphite tube, except during the atomization step, where stopped flow conditions were used.

A Crison Micro pH 2001 pH meter (Crisson Instruments, SA, Alella, Barcelona, Spain) was employed for measuring pH.

**Reagents**

All reagents were of analytical grade or higher purity and deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

A stock standard Cr(VI) solution (1000 mg L⁻¹) was prepared by dissolving 0.3735 g of potassium chromate (Merck, Darmstadt, Germany) in 100 mL of 0.03 mol L⁻¹ (Merck Suprapur). A stock standard Cr(III) solution (1000 mg L⁻¹) was provided by Merck. Working solutions were prepared daily. The Hepes buffer (2-(4-(2-hydroxyethyl)-1-piperazinyl)ethane-sulfonic acid) (Fluka) was prepared by dissolving the solid (5% w/v) in deionized water and adjusting to pH 13 with NaOH. A range of different pH values was prepared by adjusting with nitric acid or sodium hydroxide.

Baker's yeast supplied by a grocery shop was used for the selective accumulation of Cr(III) and Cr(VI). The yeast cells were microbiologically identified by the Department of Microbiology of the Complutense University as a pure culture of *Saccharomyces cerevisiae*.

**Immobilization of Saccharomyces cerevisiae**

Immobile yeast beads were prepared by external gelation as follows: ba-
ker's yeast (1 g) was hydrated with 3 mL of solution pH = 7.0. The resulting suspension was mixed into the alginate solution (7 mL of 2% w/v) until homogeneous. The alginate-yeast suspension was pumped through a syringe needle and dropped into a solution of 0.025 M CaCl₂·2H₂O where the alginate gelled as beads. The drop height from the capillary tip to the solution surface was adjusted to obtain spherical beads approximately 5 mm in diameter. After a 4 h hardening period, the beads were transferred to 0.005 M CaCl₂ solution and, when necessary, stored at 4°C prior to use. The biosorbent beads were then loaded into a home-made glass column which contained a total of 0.22 g of dried biomass.

**Cr(III) and Cr(VI) separation by the batch procedure.**

 Portions (200 mg) of the baker's yeast were weighed into individual centrifuge tubes. 250 ng of Cr(III) and 250 ng of Cr(VI) were then added separately in each tube, together with 1.0 mL of phosphate buffer (pH = 13). The resulting suspension was incubated at room temperature for 5 min and centrifuged at 4000 rpm for 2 min. The supernatant and suspension were adequately diluted and the amount of Cr(III) and Cr(VI) was directly determined by electrothermal atomic absorption spectrometry (ETAAS). To overcome matrix effects, the reported extraction efficiencies were derived by comparison with a reference which was prepared by the sample procedure but with the addition of analyte before the analytical measurement step.

**Cr(III) and Cr(VI) separation by the continuous procedure.**

 Standardized sample solutions of 25 mL each containing 250 ng of Cr(VI) and 250 ng of Cr(III) were prepared and the pH value was adjusted with HEPES solution to pH = 13. The solutions were separately run through the column by using a peristaltic pump adjusted to a flow-rate of 0.7 mL min⁻¹. Next, the retained Cr(III) was eluted from *S. cerevisiae* immobilized on alginate with 2.5 mL of 0.3 mol L⁻¹ HNO₃. The analytes in the eluates were determined by ETAAS. The recovery values were calculated from the ratio of the concentration found by ETAAS to that of the initial sample.

To check the effect of the experimental conditions, solutions containing Cr(III) and Cr(VI) were processed separately by both batch and continuous operation. Once the procedures were optimized, mixtures of Cr(III) and Cr(VI) were prepared at different ratios.

**Results and discussion.**

I. **Cr(III) and Cr(VI) uptake in the batch procedure.**

   1. a. Effect of experimental parameters on chromium uptake.

Using the experimental conditions described in the above section, the effects of time, temperature, pH, amount of substrate and analyte on the accumulation of Cr(III) and Cr(VI) by *S. cerevisiae* were investigated.

The temperature and incubation time of *S. cerevisiae* had little influence on the Cr(III) and Cr(VI) uptake process in the ranges studied, 4-90 °C and 2-60 min, respectively. Therefore, for further studies, room temperature and a 5 min incubation time (the minimum time required to mix and centrifuge the suspension) were chosen. Figure 1 illustrates the effect of the amount of *S. cerevisiae* on the accumulation of the Cr(III) and Cr(VI). As can be seen, the retention of Cr(III) and Cr(VI) increased with increasing amount of yeast and reached a plateau at about 0.8 g of biomass. The optimum amount of biomass was 0.8 g, at which retention values of 70% and 37% for Cr(III) and Cr(VI), respectively, were achieved. Although this biomass enabled the maximum uptake of both chromium species, for the purposes of speciation 0.2 g of biomass is more suitable since a greater difference between Cr(III) and Cr(VI) accumulation is obtained. Since the main purpose of this work is the selective accumulation of Cr species, 0.2 g of yeast was selected for further studies.

One of the most important parameters affecting the accumulation process is the pH value. Accordingly, the influence of various pH values in the range of 1-13 was systematically studied. As shown in Figure 2, the retention versus pH relationship was different for Cr(III) and Cr(VI). There is barely any alteration in the retention of Cr(VI) as the pH varies, whereas Cr(III) seems to be highly pH-dependent.

![Figure 1. Effect of amount of *S. cerevisiae* on uptake of Cr(III) and Cr(VI). Temperature: room temperature; incubation time: 5 minutes; pH: 7; total amount of each analyte: 250 ng; sample volume: 1.0 mL.](image-url)
with maximum adsorption occurring at pH= 13. At pH 13 retention of 95% and 10% were achieved for Cr(III) and Cr(VI), respectively. The results shown in Figure 2 suggest that the mechanism is an ion-exchange process. The decrease in Cr(III) binding at lower pH values could be due to the competition of protons with the metal for negative active sites. However, at these pH values, the overall surface charge on the cells becomes positive and may inhibit the approach of the positively charged metal ions.

1.b. Isotherm adsorption studies.
Equilibrium batch adsorption isotherms were developed for optimum conditions and Cr adsorption data were fitted to both the Langmuir and the Freundlich models. In the Langmuir model, the simplest used for the adsorption of one component, the theoretical basis is that a postulated chemical or physical interaction (or both) takes place between solute and vacant sites on the adsorbent surface, and the heat of adsorption is independent of the fraction of the surface covered by the adsorbed solute.

\[ q_{eq} = \frac{a C_{eq}}{1 + b C_{eq}} \]  
\[ \text{Eq}(1) \]

where:
- \( a \) is the bulk liquid phase metal concentration (mg mL\(^{-1}\))
- \( b \) is the equilibrium constant
- \( q_{eq} \) is the bioadsorbant-phase (µg g\(^{-1}\))

The Freundlich model can be derived by assuming a logarithmic decrease in the heat of adsorption in the fraction of the surface covered by the adsorbed solute.

\[ q_{eq} = \frac{a C_{eq}^b}{1 + b C_{eq}} \]  
\[ \text{Eq}(2) \]

The adsorption isotherms of Cr(III) for yeast at the best retention pH are given in Figure 3, which shows the metal uptake equilibrium values predicted by the Langmuir and Freundlich model and the experimentally obtained values. Since a fixed cell biomass offers a finite number of surface binding sites, equilibrium uptake would be expected to, and in fact did, show saturation kinetics at high total metal ion concentration. The yeast had typical adsorption isotherms somewhat similar to those reported for other types of biomass.

When Eq(1) of the Langmuir expression was linearized, the correlation coefficient was determined to be 0.9985 and the adsorption constant obtained from the least-square fit was \( q = 1.26 \text{ mg g}^{-1} \) and \( b = 0.022 \text{ ppm}^{-1} \).

The Freundlich constants \( a \) and \( b \) were determined from the intercept
and slope of the linearized equilibrium equation 2. The correlation coefficient was 0.9071 and the Freundlich constants were 1.99 and 1.54 for a and b, respectively.

The behaviour of adsorption isotherms can be better approximated to the Langmuir type rather than the Freundlich type. The average percentage of errors (%E = \left(\frac{q_{\text{predicted}} - q_{\text{experimental}}}{q_{\text{experimental}}}\right) \times 100) between the experimental values predicted using the Langmuir model and Freundlich model for the entire data set were 8% and 20%, respectively. The correlation coefficients between experimental values and predicted values using the Langmuir and Freundlich models were 0.9984 and 0.8666, respectively.

1.c. Application to removing Cr(III) from a leather industrial wastewater.

The ability of S. cerevisiae to accumulate Cr was applied for removing Cr(III) from leather industrial wastewater. One of the most important applications of chromium is as a mordant dye in leather factories, although it is being gradually replaced by vegetable products. The removal of chromium ions from a leather industrial waste stream was examined by the batch method mentioned in the previous section. A 5.0 g amount of S. cerevisiae was added to 25 mL of wastewater at the optimum working pH. The suspension was centrifuged at 4000 rpm and the supernatant liquid was analyzed for chromium. The amount of Cr(III) bound by S. cerevisiae was calculated from the initial and final chromium concentrations in solution. The initial chromium concentration in the water samples was measured by Flame Atomic Absorption Spectrometry (FAAS) as 500 mg L⁻¹. After biomass treatment, the resulting concentration in the supernatant was 40 mg L⁻¹, which means that S. cerevisiae removed 92% of the Cr(III) from the leather industrial wastewater.

Although the batch procedure allows the bioextraction of high amounts of Cr, this process presents several disadvantages for remediation and analytical purposes: i.e., it is difficult, time consuming and requires multiple extractions in the case of metals that have low partition coefficients. With the aim of extending the applicability of baker’s yeast cells to both on-line speciation and preconcentration of chromium species, the substrate was immobilized in two different supports: silica gel (based on an adsorption process) and alginate (based on an entrapment process). The behaviour of the immobilized S. cerevisiae in Cr accumulation was studied and the experimental parameters affecting uptake efficiency, recovery of species and analytical applications were evaluated.

2. Cr(III) and Cr(VI) uptake for immobilized yeast in the continuous procedure.

2.a. Effect of experimental variables.

The first study carried out was the selection of the best support for immobilizing the substrate for its further application to the on-line accumulation of Cr(III) and Cr(VI). The two supports selected were silica gel and alginate and the immobilization procedures are described elsewhere and in the section below, respectively.

Unlike in the case of Hg(II), silica gel was unsuitable for Cr preconcentration since this analyte was accumulated in both silica gel and yeast to the same extent. To determine the contribution of the silica gel support to the uptake process, similar columns filled with only silica gel were prepared and solutions of Cr(III) and Cr(VI) were run under the same conditions with the immobilized yeast. Analysis of the eluate showed that Cr(VI) remained in solution in all cases (which agrees with the results observed in the batch procedure), whereas Cr(III) was retained on the silica gel or silica gel–yeast column, with a higher accumulation capacity in the latter.

The important contribution of the silica gel to Cr(III) accumulation made it necessary to use another support for immobilizing the yeast, and alginate was the one selected. The use of calcium alginate is one of the simplest methods and is used widely in the laboratory and pilot scale studies for bioremediation purposes. However, metal diffusion within gels is a major problem. A study was performed to establish the contribution of the support to Cr uptake. The results showed that no biosorption of Cr(III) occurred in beads composed entirely of alginate, demonstrating that the support by itself is not a metal sorbent. Therefore, this type of immobilization was selected for further studies of the parameters affecting Cr(III) accumulation, such as the alginate:yeast ratio, sample flow-rate, pH, sample volume and type of eluent.

The alginate:yeast ratio was studied by preparing several yeast beads containing a constant amount of alginate and increasing the amount of yeast from 0.2 to 2 g. Maximum uptake values were found for beads containing 1.0 g of yeast.

The influence of pH on the accumulation process was also evaluated in continuous mode. The pH effect was the same as that previously observed in the batch mode, with an optimal accumulation value of 90% for Cr(III) at pH = 13. With regard to Cr(VI), this analyte remained in solution over the whole pH range evaluated. Therefore, pH 13 was established as optimum for selective Cr(III) accumulation in the presence of Cr(VI).

The maximum amount of Cr(III) accumulated by the column was evaluated by running through the column a volume of 25 mL containing the analyte at increasing concentrations. As can be seen in Figure 4, the column is able to accumulate up to 11 μg of Cr/g alginate–yeast before saturation is reached. This value is notably lower than the
one provided by the batch mode. This decrease in loading capacity could be due to several reasons: a decrease in the effective amount of yeast present in the immobilized product, slow diffusion within the alginate gel and limited contact time that perhaps did not allow a true equilibrium loading.

For any proposed industrial or remediation application or analytical purposes (preconcentration of trace elements), uptake onto microbial biomass constitutes an initial step of the process but this must be followed by a recovery step. The simplest and cheapest method of recovering surface metals from microbial biomass is to wash or elute the metal from the surface by means of an appropriate desorbing agent.

In order to determine the appropriate eluent type for the elution of Cr(III) retained on the yeast surface, different elution solutions were examined. For this purpose, several concentrations of HNO₃ solutions (0.001, 0.1, 0.3 and 0.5 mol L⁻¹) were tested. As shown in Figure 5, 0.3 mol L⁻¹ HNO₃ allows the elution of chromium (III) with a desorption efficiency of 98 ± 2%. The next step was to determine the minimum volume of eluents needed to release Cr(III) quantitatively from the columns. Different volumes of 0.3 mol L⁻¹ HNO₃ for Cr(III) elution were evaluated and, as shown in Figure 6, 95 ± 2% of the retained Cr(III) was eluted with 2.5 mL of 0.3 mol L⁻¹ acid. Although HNO₃ allows quantitative stripping of the Cr retained in the column, the prolonged use of this mineral acid destroyed the integrity of the yeast beads, leading to loss of biomass. The alginate beads were apparently unaffected by the mineral acids used over times up to 3 h. With the aim of increasing the uses of the columns, HEPES solution was tested as a potential stripping agent. HEPES was selected since this compound is involved in the immobilization process and is unlikely to alter the integrity of the beads. For this study 2.5 mL and 5 mL of HEPES solution were run through the column. In all cases the recovery obtained was about 60% of the retained Cr(III). The use of HEPES as an eluent increased the lifetime of the column but reduced the efficiency of Cr removal.

To determine whether the method described can be used as a concentration stage for highly diluted solutions, different water volumes (25, 50, 100, 150 and 200 mL) containing the same total amount of Cr(III) were run through the column and the eluate was measured. Figure 7 shows that a constant recovery of 98 ± 2% was achieved up to 25 mL.
Figure 6. Effect of HNO$_3$ volume on the elution of Cr(III) retained on yeast-alginate column. Sample volume: 25 mL, sample pH: 13, total amount of Cr(III): 250 ng.

Figure 7. Influence of sample volume on Cr(III) retention by yeast-alginate column as a function of nature of the sample. Total amount of Cr(III): 250 ng.

of sample, while the efficiency of detection fell drastically for higher sample volumes. According to these results, Cr(III) can be preconcentrated by a factor of 10. Nevertheless, when more complex matrices (tap, river and sea and wastewater) were analysed, the volume that could be run without a decrease in Cr(III) retention was much lower than in deionized water. Figure 7 shows the retention efficiency as a function of the sample nature and volume. As can be seen, the recovery obtained depends on the type and complexity of the matrix. In sea, tap and river water samples, the uptake decreases after running 25 mL, leading to retention values of 14%, 65% and 57% for sea, tap and river water, respectively. In contrast, the decrease in the retention of Cr(III) with increasing sample volume was less pronounced for wastewater samples.
(from industrial sources) in which a constant recovery of 91 ± 3% was achieved up to 25 mL of sample (similar behavior to that of deionized water). All these factors together suggest that the retention of Cr(III) is affected by the complexity of the matrix. This could be explained by competition for retention sites on the substrate by other concomitant elements in the sample and/or could simply be attributed to a washing effect of the substrate.

2.b. Application to Cr decontamination and on-line preconcentration.

The system developed was applied for a dual purpose: remediation of contaminated sites and online preconcentration for Cr determination. For the remediation of contaminated systems two wastewater samples, urban and industrial, were selected for decontamination. The urban wastewater sample was provided by a municipal treatment plant and the industrial wastewater was obtained from a paint factory. The initial chromium concentration in the urban and industrial water samples was measured by ETAAS as 30 μg L⁻¹ and 14 μg L⁻¹, respectively. The study was carried out by running 25 mL of water samples through the column under the previously determined optimum conditions. After biomass treatment, the resulting concentration in the eluate was not detected by ETAAS. This implies that the S. cerevisiae immobilized in alginate can remove the Cr in both samples and can be regarded as an alternative for on-line reclamation and remediation of Cr contaminated waters and applied to clean contaminated effluents or receiving waters, providing a rapid, cheap and semiquantitative check monitoring tool for use in routine industrial settings.

The method was also applied as a preconcentration stage prior to trace Cr determination in a natural river water reference material (SLRS-3, National Research Council of Canada) with a Cr reference value of 0.30 ± 0.04 μg L⁻¹. The study was carried out by running 25 mL of water sample and eluting with 3 mL of 0.3 mol L⁻¹ HNO₃. The eluate was analysed by ETAAS. The concentration obtained, after applying the preconcentration factor (25/3), was 0.28 ± 0.09 μg L⁻¹ (n=4). These results showed no significant differences between the value found and the certified value at 95% confidence level. These results also support the suitability of S. cerevisiae for Cr on-line preconcentration.

Conclusions

Inexpensive baker’s yeast cells characterized as Saccharomyces cerevisiae, either free or immobilized on alginate, have been shown to selectively accumulate Cr(III) from water samples. The percentage of the Cr(III) fraction bound depends only on the pH, with maximum accumulation occurring at pH 13. In contrast, aqueous Cr(VI) remained unaltered and in solution under all the experimental conditions tested. The use of immobilized yeast on alginate offers several advantages compared to its use in the batch procedure: it is less time-consuming and has better reproducibility, and the whole procedure is easily automated.

References

Simultaneous determination of nutrients (ammonium and phosphate) in marine aquaculture effluents by flow analysis

Abstract

In this work, a flow analysis method with spectrophotometric detection has been developed for the simultaneous determination of phosphate (P-PO$_4^{3-}$) and ammonium (N-NH$_4^+$) in marine aquaculture effluents. The method is based on the dual combination of a reversed flow injection system and a continuous flow system without injection. The performance of the system was optimised by using both an univariate optimisation and a modified simplex method. The method meets the requirements of marine fish farms for monitoring the quality of input and output water streams, particularly in terms of simplicity and the high sampling rate allowable. Besides, it has a limit of detection of 3.6 µg L$^{-1}$ P-PO$_4^{3-}$ and 19.8 µg L$^{-1}$ N-NH$_4^+$ and relative standard deviations (RSD) of 6.9% and 3.6%, respectively.

Keywords: Aquaculture, nutrients, phosphate, ammonium, simultaneous determination, continuous determination, flow injection, seawater.

A. Tovar, C. Moreno*, M. P. Manuel-Vez and M. Garcia-Vargas

Department of Analytical Chemistry, Faculty of Sea Sciences, University of Cádiz.
11510 Puerto Real, Cádiz (Spain)

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Introduction

In recent decades, aquaculture has been used as an alternative fish production. When possible, these activities are carried out in an intensive regime, obtaining very high yields and commercial profit. As a consequence, fish farms effluents contain very high amount of pollutants as both solid and dissolved particles. One of the most important impacts of aquaculture is the high concentration of nutrients which are introduced into the environment [1, 2]. These nutrients have their origin in fish excretions and uneaten fraction of the food, and mainly consist of nitrogen and phosphorous compounds, ammonia and phosphate being two of the nutrients originated in higher concentration.

Actually, attention is paid in minimising the environmental impacts of aquaculture. Thus, the use of low-pollutant foods is extended and, in some cases a cleaning step is done to the output streams prior to be poured [3, 4]. However, it is generally accepted that higher efforts are still needed in this field, and several countries are becoming aware of the problem. For example, the Spanish Government has published recently a White Book on Aquaculture, where it is noted that deeper studies on environmental impacts of aquaculture are needed [5]. In many cases, these studies are not carried out because of the complexity of analytical methods, which frequently have to be applied by non-expertise workers. Thus, simple analytical tools allowing in-situ measurements of nutrients (especially ammonium and phosphate) in marine aquaculture effluents are desirable.

Although the continuous determination of nutrients in seawater has been studied by many authors [6, 7], the works dealing with the simultaneous determination of more than one nutrient are very scarce, nitrite and nitrate being the couple of nutrients mainly studied [6].

Several flow injection methods have been used for the individual determination of ammonium and phosphate both in fresh and seawater [8-10], but no method has been found describing the simultaneous determination of these two nutrients. In the present work the spec-
trophotometric methods based on the formation of indophenol blue [11–12] and phosphomolybdate blue with salinity compensation [10], have been adapted and applied to the continuous simultaneous determination of ammonium and phosphate, respectively, in seawater. The proposed method must be low cost and easily applicable to allow its implementation in the fish farms. Optimisation of the variables controlling the performance of the method have been carried out by using both the univariate and modified simplex methods. The optimised method was used to analyse the ammonium and phosphate concentrations in the effluents of a fish farm devoted to the intensive culture of gilthead seabream.

**Experimental**

1. **Apparatus**

The flow injection manifold used in this work is shown schematically in Figure 1. It consisted on a model Perimex 12 eight-path peristaltic pump (Spetec, Germany) equipped with Tygon tubing, which was used to manipulate the flows of reagent and sample solutions, a Model 1106 injection valve (Omnifit, UK), a Model 5014 switching valve (Rheodyne, USA), a thermostated bath WB2001 (Heidelberg, Germany) and a Lambda 11 UV/VIS spectrophotometer (Perkin-Elmer, Germany) equipped with a quartz flow cell with a 10 mm pathlength (Hellma, Germany). Transport lines and reaction coils were made using 0.8-mm i.d. PTFE tubing (Omnifit, UK). Connections were made of polypropylene (Omnifit, UK).

2. **Reagents and solutions**

Stock solutions of 1000 mg L⁻¹ N-NH₄⁺ and 200 mg L⁻¹ P-P₂O₅ were prepared by dissolving the necessary amount of ammonium chloride, NH₄Cl, and potassium dihydrogen phosphate, KH₂PO₄, of analytical grade (Merck, Germany), respectively. Further dilutions were made daily as required, keeping the salinity of the solutions at 35 g L⁻¹ with sodium chloride, NaCl, of analytical grade (Merck, Germany). All the optimisation studies were carried out with 400 μg L⁻¹ N-NH₄⁺ and 40 μg L⁻¹ P-P₂O₅ solutions. For the preparation of reagents, three different solutions were prepared for each analyte. For the determination of phosphate, reagents were prepared as follows:

Reagent R1: 20 g of ammonium molybdate, (NH₄)₂MoO₄, 4H₂O (Merck, Germany) were dissolved in 700 mL of bidistilled water, 40 mL of sulphuric acid, H₂SO₄, (Scharlau, Spain) were added and then, the solution made up to 1000 mL. Reagent R2: 56 mL of H₂SO₄ were added to 700 mL of bidistilled water and made up to 1000 mL. Reagent R3: 20 g of L(+)-ascorbic acid, C₆H₇O₆ (Merck, Germany) and 1 g of antimony potassium oxide (++)-tartrate 0.5 hydrate, K₂SbO₆C₆H₄O₆·1/2H₂O, (Merck, Germany) were dissolved in 700 mL of bidistilled water, then, 50 mL of H₂SO₄ were added and the solution made up to 1000 mL. All these reagents were prepared daily. The reagents used for the determination of ammonium were prepared as follows: Reagent R4: a 2.5% (W/V) solution of sodium citrate, C₆H₅Na₃O₆·2H₂O, (Scharlau, Spain). Reagent R5: 2 g of sodium hydroxide, NaOH, (Merck, Germany), 2 g of sodium tetraborate decahydrate, Na₂B₄O₇·10H₂O, (Merck, Germany) and 4.5 mL of sodium hypochlorite, NaClO, 5% of active chlorine, pure (Panreac, Spain) were dissolved in bidistilled water and made up to 100 mL. Reagent R6: 15 g of phenol, C₆H₅OH, (Scharlau, Spain), 5 g of NaOH, and 9·10⁻⁷ g of sodium nitroprusside, Na₃Fe(CN)₆NO

![Figure 1. Schematic representation of flow analysis manifold. R1-R6: Reagent streams; S1-S2: Sample streams; PP: Peristaltic pump; IV: Injection valve; SV: Switching valve; RC1-RC3: Reaction coils; TB: Thermostatted bath; W: Waste. Optimum operational conditions as indicated.](image-url)
H₂O, (Panreac, Spain), were dissolved in bidistilled water and the solution made up to 100 mL. All chemicals, except sodium hypochlorite, were of analytical grade.

3. Procedure

For the optimisation of the flow system depicted in Figure 1, the absorbances of the phospomolybdic and indophenol complexes obtained in the reactions with phosphate and ammonium, respectively, were chosen as the responses to be maximised. A two-position switching valve was used to select the analyte to be measured. Thus, when the valve was in position I, phosphate was determined, while ammonium was quantified with the switching valve in position II. For the determination of phosphate, a certain volume of ammonium molybdate solution (R1) was injected into a sulphuric acid carrier solution (R2) and merged with the sample stream (S1). Once the formation of phosphomolybdic acid has taken place, it is reduced to phosphomolybdenum blue by ascorbic acid with amonia as a catalyst (R3), and then, its absorbance at 640 nm is measured. For the determination of ammonium, a sample stream (S2) was mixed with a nitric acid solution (R4). Then it joins with the hypochlorite solution (R5) and, finally, the resultant stream is mixed with the solution containing phenol (R6) and, with the switching valve in position II, goes through the detector and the absorbance at 640 nm is measured.

When the determination of one analyte is being performed, the other one was derived to waste by using the switching valve. The temperature of the thermostated bath was always kept at 60°C.

The signal recorded when analysing phosphate and ammonium concentrations in a sample is shown in Figure 2. With the switching valve in position I, the ammonium molybdate solution was injected and then, a peak was recorded, its height being proportional to the concentration of phosphate in the sample. Once the baseline was re-established, the valve was switched to position II and then, the signal experienced a vertical displacement, proportional to the concentration of ammonium in the sample.

To search the best operational conditions of the manifold, two optimisation methods were used successively. On the one hand, we used the classical univariate method, where one variable is varied by maintaining constant the rest. Thus, the analytical signals obtained for both ammonium and phosphate complexes were studied as a function of: length of the three reaction coils (0–4.75 m), injection volume (126–626 μL), pumping rate (3.5–15.5 rpm) and volume of sample, studied as the ratio between sample and reagent flows. This univariate procedure is frequently used to optimise flow systems, but it does not take into account the interactions between the studied variables. These interactions are especially important in flow systems due to the interdependence of the variables and they can produce wrong optimum experimental conditions. In our case, and once the univariate optimisation was performed, a second multiparametric optimisation process, based on the use of a modified simplex with two response variables (absorbance of phosphate and ammonium complexes) was carried out to eliminate the interaction effects. In our case the simplex represented a hexahedron, since five variables were optimised simultaneously: pumping rate, injection volume and the lengths of the three reaction coils used in the system. For simplex optimisation, calculations were done by using the software MultiSimplex.98 (MultiSimplex AB, Sweden).

Finally, and after reaching the optimum experimental conditions, the determination system was applied to the quantification of phosphate and ammonium concentrations in several real samples of seawater from the effluents of a marine fish farm devoted to the intensive culture of seabream.

Results and discussion

Maximum absorbances of coloured complexes of phosphate and ammonium used in this work are typically measured at 690 nm and 640 nm, respectively. With the aim of using a single detector (to simplify the method) a single wavelength was selected to quantify both nutrients simultaneously. Thus, the absorbance spectra were developed and plotted as shown in Figure 3. As can be observed, the band of absorption of phosphate complex is wider than the spectrum of ammonium complex and then, the lowest loss of sensitivity will be achieved by measuring the absorbance of both analytes at 640 nm (maximum absorption
of the ammonium derivative). For this reason, 640 nm was chosen as the single analytical wavelength.

The chemical conditions used in this method were obtained from the bibliography. Thus, the reagents needed for the determination of ammonium and their concentrations were described by Cerdá et al. [13]. Nowadays, when tested, an important instability was observed when analysing a sample containing ammonium. We attributed this fact to the very high concentration of phenol in the reagent solution (24 g L\(^{-1}\)). Thus, the effect of the concentration of phenol (within the range of 0.24-24 g L\(^{-1}\)) in the absorbance of the complex was examined. This absorbance increased linearly when increasing phenol concentration up to 15 g L\(^{-1}\) and then the response became unstable, due to the formation of precipitate in the liquid stream. Thus, a concentration of 15 g L\(^{-1}\) was chosen as optimum value, and used henceforth.

**Optimisation of the manifold**

The optimisation of the system consisted of two steps. On the one hand, an univariate pre-optimisation process was carried out. This methodology does not give the optimum conditions for the performance of a flow manifold, due to the interaction effects caused by the interdependence existing between the variables optimised. Thus, it is necessary to perform a second optimisation process, based on the use of a multivariate method, to reach the optimum conditions.

1. **Univariate method**

In order to obtain the optimum signals all the factors controlling the spectrophotometric response were firstly studied by using the univariate method. We started studying the variables affecting to the determination of only one nutrient and later, we optimised the variables controlling the determination of both ammonium and phosphate. Thus, firstly, the effect of the length of the reaction coils RC1 and RC2 (see Figure 1) in the determination of phosphate and ammonium, respectively, were studied by varying them within the ranges 0.08-4 and 0.25-4 m, respectively. The results obtained are shown in Figure 4. As can be observed, the formation of phosphate complex is very rapid, and then, the absorbance decreased when the length of the re-
action coil RC1 increased due to the dispersion of the sample. As optimum length, 0.25 m was selected, instead of 0.08 m, due to the instability of the signal obtained with this reaction coil. The formation of ammonium complex is slower, a minimum length of the reaction coil RC2 of 1.8 m is needed, then an increment in the length presented lower influence in the absorbance of the complex, a plateau being reached between 1.8-4 m, where a coil of 2 m was used as optimum.

Immediately after, the optimum volume of sample to be used was investigated. This study was carried out by varying the volume ratio sample/reagent, by changing the inner diameter of the tubing used for pumping the sample. Thus, the different absorbances of phosphate and ammonium complexes were obtained within the ranges 0.45-14.5 (S1/R2) and 0.125-2.1 (S2/R4) respectively. The results are plotted in Figure 5, where we can see that the absorbance of phosphate increased gradually with volume ratio. Due to the instability of the signal observed at ratios higher than 8, the optimum value was selected at 7.6. In the same figure we represent the variation of the absorbance of ammonium complex, which became maximum and almost independent of volume ration between 1.0 and 1.3, where an optimum ratio of 1.2 was chosen.

Finally, the effect of injecting different volumes of reagent R1 (126-626 μL) on the absorbance of phosphate complex was examined. The results represented in Figure 6 show that after an initial increase, the absorbance reaches a maximum between 176-206 mL. Higher injection volume originated, in these conditions, poorer mixing and, as a consequence, lower signal. Optimum injection volume was 206 μL.

Once the variables controlling the determination of only one nutrient were optimised, the study of the influence of the variables affecting the determination of both phosphate and ammonium was undertaken. In this case, without forgetting the signal obtained for ammonium complex, for the selection of optimum values, priority was given to the increment of the signal of phosphate, due to its lower sensitivity.

The length of the reaction coil rinsed into the thermostated bath (RC3) was varied within the range 0-4.75 m. As can be seen in Figure 7, the absorbance of both phosphate and ammonium increased with re-
From 3.5 to 15.5 rpm. Once more, the optimum rate was chosen when the absorbance obtained for the phosphate complex was maximum at a pumping rate of 6.8 rpm.

Optimum operational conditions obtained from the univariate optimisation process were: length of reaction coil RC1: 0.25 m, length of reaction coil RC2: 2 m, injection volume: 206 μL, length of reaction coil RC3: 2.75 m, pumping rate: 6.8 rpm and sample volume ratios: S1/R2 = 7.6 and S2/R4 = 1.2.

2. Modified Simplex method

Once the system was optimised by using the univariate method, a second procedure based on simplex optimisation was carried out. In this second procedure, only five variables were studied (sample volumes were kept constant). Besides, the conditions of the initial simplex (shown in Table 1) were selected by using the knowledge obtained from the univariate optimisation. Because of the difference between the sensitivities of phosphate and ammonium determination methods, a weighted average function combining the individual responses, was used as the experimental function to be maximised [14]. The influence values assigned to the individual response variables (1 and 0.67 respectively) were used as weights.

The increment of absorbance (sample minus blank) was calculated as an average of, at least, three replicates. As indicated in Table 2, a total of 32 different experiments were required to decide the optimum conditions and then, the system did not need further experimentation to improve the response. The criterion chosen for stopping the search was based on the measurement of response variation due to the simplex. This variation was estimated by means of sample variance of all the responses of each simplex. A small response variation means different response values at the vertices are due only to random error. This estimation was carried out by

Figure 7. Dependence of absorbances of ammonium and phosphate on thermostated reaction coil length.

Figure 8. Dependence of absorbances of ammonium and phosphate on pumping rate.

action coil length. In this case, the optimum value was chosen at 2.75 m, where maximum signal for phosphate, together with a good signal for ammonium, was obtained.

The last variable optimised was the pumping rate. Figure 8 shows the variation of absorbance for the phosphate and ammonium complexes, with the rotational speed of the peristaltic pump, which was varied...
Table 1. Variables and corresponding values of initial simplex. RC1-RC3: reaction coils lengths; IV: injection volume; PR: pumping rate

<table>
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<tr>
<th>Vertex</th>
<th>RC1, m</th>
<th>RC2, m</th>
<th>I.V., μL</th>
<th>RC3, m</th>
<th>P.R., rpm</th>
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Table 2. Simplex optimisation of variables controlling the determination of ammonium and phosphate. Legends as in a table 1

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<td>0.02</td>
<td>0.184</td>
<td>0.387</td>
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</table>

(1): First reevaluation; (2): second reevaluation
F: first simplex; R: reflection; E: expansion; C-: negative contraction; C+: positive contraction
calculating the F-values for the ratio of simplex variance and a variance of the method, which was experimentally calculated [15]. Trial 29 gave the highest value of the response function, the optimum conditions being: length of reaction coil RC1: 0.08 m, length of reaction coil RC2: 2.12 m, injection volume 263 μl, length of reaction coil RC3: 2.46 m and pumping rate 3.2 rpm.

As expected, by using simplex optimisation a better selection of experimental conditions can be done and then, an improvement in the spectrophotometric signals was obtained. The calibration lines for ammonium (0-1500 μg L⁻¹) and phosphate (0-120 μg L⁻¹) were measured by using the experimental conditions described as optimum in both optimisation procedures (univariate and simplex). The results showed that, in the case of ammonium, the conditions obtained by means of univariate optimisation were almost optimum, and then, calibration plots are very similar: y = 0.04 + 4.3E⁻⁴ x; (R²=0.999) and y = 0.03 + 4.4E⁻⁴ x; (R²=0.999) for univariate and simplex, respectively. Due to the lower signals given by phosphate complexes, small variations in experimental conditions can make sensitivity to increase notably. Thus, the slope of the straight line obtained by simplex optimisation was more than twice higher than the slope obtained with the optimum conditions of univariate process: y = -4.9E⁻⁴ + 3.2E⁻⁴ x; (R²=0.993) and y = 6.2E⁻³ + 7.1 E⁻⁴ x; (R²=0.999) for univariate and simplex, respectively.

Limits of detection and precision of the method were estimated within the range studied. For phosphate and ammonium, the limits of detection (calculated as 3S₀/m, where S₀ is the standard deviation of the intercept and m is the slope of the calibration curve) were 3.6 μg L⁻¹ P-P₅O₅²⁻ and 19.8 μg L⁻¹ N-NH₄⁺, the relative standard deviations being 6.9% (for a real sample containing 46.1 μg L⁻¹ P-P₅O₅²⁻) and 3.6% (for a real sample containing 226.5 μg L⁻¹ N-NH₄⁺).

Application

Once optimised, the method was applied to the continuous determination of ammonium and phosphate in several real samples of seawater taken from the outputs of a marine fish farm devoted to the intensive culture of seabream. The results obtained were compared with those obtained by applying the standard batch methodology mostly used for the determination of ammonium and phosphate in seawater [16, 17]. The determinations (both continuous and batch) were repeated three times.

As observed in Table 3, the results obtained by the proposed method are in good agreement with those used as reference. The precision obtained with the proposed method was of the same order, or even lower, than the precision of the standard method. The accuracy of the results was also estimated, the average relative errors being 2.8% and 7.0% for the determination of ammonium and phosphate, respectively. Besides, the absence of systematic errors was confirmed by applying the t-test with a probability of p=0.05. As can be observed in the results shown in Table 3, all the results obtained with the new method were accepted. Therefore, an accurate and rapid determination of ammonium and phosphate in seawater can be carried out with the proposed flow method, by using a simple and low-cost methodology, easily applicable to the on-line monitoring of fish farm effluents.

Acknowledgements

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References


Table 3. Results of the analysis of ammonium and phosphate in real seawater samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>NH₄⁺ (μg L⁻¹)</th>
<th>PO₄³⁻ (μg L⁻¹)</th>
<th>Eᵣ (2)</th>
<th>t-test (b)</th>
<th>Found (a)</th>
<th>Known (a)</th>
<th>Eᵣ (2)</th>
<th>t-test (b)</th>
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<tr>
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<td>PO₄³⁻</td>
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<tr>
<td>1</td>
<td>149.4±6.0</td>
<td>158.2±6.3</td>
<td>-5.6</td>
<td>t=2.54</td>
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<td>t=1.59</td>
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<td>230.5±8.3</td>
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<td>43.6±1.8</td>
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<td>t=1.55</td>
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<td>3</td>
<td>164.3±14.4</td>
<td>163.4±0.4</td>
<td>0.6</td>
<td>t=0.11</td>
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<tr>
<td>4</td>
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<td>251.0±13.7</td>
<td>8.7</td>
<td>t=4.26</td>
<td>47.8±9.6</td>
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<tr>
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<td>4.1</td>
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</table>

Average Eᵣ: 2.8

(a): Mean (n=3) ± standard deviation (b): relative error (c): experimental t values (critical value: t=4.30)
Purification of cadmium wastewater: characterization and electrochemical behaviour of cadmium-bearing ferrites

Abstract

This investigation was designed to verify the efficiency of the "ferrite process" for the purification of wastewater heavily contaminated with cadmium, and to characterize the cadmium-bearing ferrites produced using five starting \( \text{Fe}^{3+}/\text{Cd}^{2+} \) molar ratios (20/1, 15/1, 7/1, 5/1 and 3/1). The five ferrite products were subjected to XRF, chemical analysis (ICP-AES and potentiometric titration), XRD and DSC, indicating \( \text{Cd}_x\text{Fe}^{III}_{1-x}\text{O}_2 \) (\( x = 0.04, 0.06, 0.15, 0.25 \) and 0.33 respectively) as the most probable structure. Accordingly, the general formation reaction proposed for these compounds is: \( (2 + (1-x))\text{Fe}^{2+} + x\text{Cd}^{2+} + 4\text{OH}^- + \text{O}_2 \rightarrow \text{Cd}_x\text{Fe}^{II}_{1-x}\text{O}_2 + 2\text{H}_2\text{O}. \)

Electrochemical analysis of the solid cadmium ferrites was performed using a carbon paste electrode in HClO\(_4\) and HCl medium. In each case, the first cyclic voltammogram showed the participation of solid species in the electrochemical transformation process, since the shape of the spectra could be related to the structure and stoichiometry of the ferrites. In second and successive scans, the voltammograms indicated the redox couples \( \text{Fe}^{3+} + 1\text{e}^- \rightarrow \text{Fe}^{2+} \) (E = 0.525 V vs. AgCl, Ag) and \( \text{Cd}^{2+} + 2\text{e}^- \rightarrow \text{Cd} \) (E = -0.700 V) in HClO\(_4\) and \( \text{FeCl}_2^{\text{aq}} + 1\text{e}^- \rightarrow \text{FeCl}_3^{\text{aq}} + \text{Cl}^- \) (E = 0.475 V) and \( \text{CdCl}_2^{\text{aq}} + 2\text{e}^- \rightarrow \text{Cd} + 2\text{Cl}^- \) (E = -0.750 V) in HCl. Chronopotentiometric data were consistent with these mechanisms.

Keywords: Cadmium, decontamination, cadmium-ferrites, voltammetry, carbon paste electrode.

E. Barrado\(^1\), F. Prieto\(^2\), J. Medina\(^3\) and R. Pardo\(^1\)

\(^1\) Departamento de Química Analítica 3.- Departamento de Física de la Materia Condensada, Cristalografía y Mineralogía, Facultad de Ciencias. 47005 Valladolid (Spain).
\(^2\) Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Hidalgo, Crta. Pachuca-Tulancingo, Km 4.5, 42076 Pachuca, Hidalgo (México).

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Introduction

Metal ions and particularly those of heavy metals are not potentially dangerous when they occur in the environment at trace or ultra-trace levels. However, uncontrolled industrial, agricultural and other practices have led to the accumulation of these contaminants. The main sources of air and water contamination are steel production, nonferrous metal production, refining, and cement, pigment and battery manufacturing [1], although over recent years the use of metal ions in batteries, anticorrosive coatings, or as stabilizers in PVC and alloys is on the increase, whereas their use in electroplating and pigments is diminishing [2]. Other anthropogenic activities resulting in metal ion emission to the atmosphere, water and land are: ashes from combustion, oil combustion, waste incineration, coal combustion and the use of phosphate fertilizers [3]. The growing concern of increasing environmental levels has prompted rigorous restriction measures including the establishment of limit indices for each metal. In the case of Cadmium, 0.1 mg L\(^{-1}\) is the limit for residual waters and 5 mg L\(^{-1}\) is the maximum permissible level established for drinking water. These limits reflect the highly toxic nature of Cd since it accumulates in the liver, kidney and pancreas [4]. Cadmium is carcinogenic and non biodegradable and can provoke nausea and vomiting. In adults not
frequently exposed to this metal, the body burden can be 9–40 mg/Kg, with 10 to 50 mg/Kg in the kidney cortex, while blood and urine levels may reach 5 mg/l and 3 to mg/l respectively.

It has been, nevertheless, established that the toxicity and the particular behaviour of trace metals are determined by their specific forms rather than their total concentration [5]. Indeed, the absorption of cadmium is dependent on particle size and type of compound [2]. Cadmium shows high affinity for the functional groups of the active centre of many enzymes (amino, imino, sulphydril etc.) [6]. In soft tissues it is trapped primarily by bonding to sulphur rich proteins such as metallothionein and to the HS group of other proteins [3].

Prompted by the need to ensure that permissible levels of heavy metals are not surpassed, in previous works [7] we optimised a procedure for the removal of heavy metals from wastewater involving their precipitation from alkaline solutions containing Fe (II) [8]. The ultimate aim was to achieve maximum purification and an end-product, or "ferrite sludge", with magnetic properties [9]. Using a 5 L reactor, the conditions giving rise to a high purification efficiency and a ferrite sludge of maximum magnetic permeability were: pH 10, temperature 60 °C, airflow 30 L/min, stirrer rate 560 rpm and treatment time 1 h. The total amount of Fe (II) added to the solution to be treated had a notable effect on the degree of purification and also on the stoichiometry and properties of the ferrite [7].

The present investigation was designed to confirm the efficiency of the ferrite process for the purification cadmium-bearing wastewater, and to characterize the resultant cadmium ferrites produced using five starting Fe2+/Cd2+ molar ratios (from 20/1 to 3/1), as previously described for Pb and Cu [10,11]. In order to acquire knowledge on the stability and possible reuse of these residues, the composition and stoichiometry of the ferrites were determined by chemical analysis, XRF, XRD and DSC. The electrochemical behaviour of the solid species was also analysed using a carbon paste electrode (CPE) and an electrolyte binder [12]. Voltammograms of the cadmium ferrites were obtained in both HClO4 and HCl medium, and the oxidation and reduction peaks were interpreted by comparison with spectra corresponding to a pure iron ferrite sludge [13], iron oxides [14], magnetite [15] and a cadmium oxide. Chronopotentiograms were obtained in the same media.

Experimental

1. Equipment and Reagents

The equipment used in the ferrite-forming treatment process and the instruments for XRD, XRF and DSC have been described elsewhere [7,10,11].

Electrochemical characterization of the samples was performed using an EG&G 273-A Princeton Applied Research potentiosstat/galvanostat, equipped with model 270/250 Research Electrochemistry software (v. 4.23). A carbon paste electrode (prepared as indicated in the Procedures section), a Pt rod and an AgCl/Ag/KCl (Ag = 0.222 V vs. SHE, [KCl] = 3 M) electrode were used as the working, auxiliary and reference electrodes respectively. All potential values are expressed with respect to this reference electrode.

2. Procedures

The cadmium ferrite samples were obtained by placing 5.489 g of Cd(NO3)2-4H2O in a 5 L reactor and diluting to 2 L with water (1000 ppm of Cd2+). Subsequently, 98.940, 74.205, 34.629, 24.735 and 14.840 g of FeSO4.7H2O were added to the Cd solution to yield Fe/Cd molar ratios of 20/1, 15/1, 7/1, 5/1 and 3/1 respectively. The pH was adjusted to 10 by the addition of NaOH solution. The reactor was then closed and the electric stirrer, temperature probe, pH electrode and air-supply tube connected. After 1 h, the liquid was separated from the solid ferrite by decanting and pressure filtration. The resulting solids were desiccated at 100 °C for 2 h and powdered in an agate mortar. These solid products are hereafter referred to as FexCd.

The Cd (II) (λ = 214.438) and total Fe (λ = 247.926) levels remaining in the treated solutions and those of dissolved solid ferrite samples were determined by ICP-AES. The Cd (II) and total iron contents of the solid ferrites were also estimated by XRF. For the determination of Fe (II), a 0.200 g sample of cadmium ferrite was dissolved in 10 mL of 25% (v/v) HCl in a nitrogen atmosphere to avoid oxidation to Fe(III), and diluted with 20 mL of deionized water. The solution was then treated with 5.00 mL of 10-3 M Ce(SO4)2 (standardised with sodium oxalate), and the excess Ce (IV) determined by potentiometric titration using a standard solution of Fe (II) prepared from ferrous ammonium sulphate (NH4)2Fe(SO4)2·6H2O.

The J-shaped carbon paste electrode was prepared using ultra pure graphite powder (particle size 200 mesh) supplied by Carbone Lorraine. Graphite powder (ca. 100 mg) and a small amount of ferrite (1-2 mg) were mixed with 1-2 drops (5-100 mL) of electrolyte binder. The mixture was then thoroughly homogenised and placed in the electrode reservoir.

Voltammograms were obtained at a scan rate of 0.5 mV/s; potential slits were set by the supporting electrolyte. A linear scan was performed from the open-circuit potential in the positive direction (LSV), followed by successive cyclic scans towards negative potentials and back (CV1, CV2 etc.). Chronopotentiograms were obtained by preparing a similar electrode and the application of constant intensities in the range -0.1 to -6 mA or 0.1 to 6 mA.

Results and discussion

The residual Cd (II) concentration of the processed wastewater solutions determined by ICP ranged from 0.098 to 0.404 mg L-1 and corresponded to a Cd removal efficiency of 99.99 to 99.95%. These values are of the same order as those obtained for other heavy metals removed from wastewater samples
from educational laboratories [7], and demonstrate the suitability of the ferrite precipitation method for the purification of wastewater highly polluted with cadmium.

1. Characterization of the cadmium ferrites

To determine the Cd(II) concentration and total Fe in the solid ferrites, 0.100 g of the solid product was dissolved in 20.00 mL of 25% HCl and diluted to 100.00 mL in deionized water. Cd(II) and total Fe (Fe(III)+Fe(II)) contents were determined by ICP-AES, and Fe(II) levels were estimated by potentiometric titration as described in the experimental section. Table 1 shows the values obtained and also provides the percentage total Fe(II) plus III and Cd(II) levels of the solid samples directly determined by XRF. Based on these values, stoichiometries and formula weights were proposed for the cadmium-bearing ferrites and are shown in Table 2. The general reaction proposed for the formation of these compounds during the wastewater purification process is accordingly:

\[(2 + (1-x)) \text{Fe}^{2+} + x \text{Cd}^{2+} + 4 \text{OH}^- + \text{O}_2 \rightarrow \text{Cd}_x\text{Fe}^{\text{III}}_{1-x}\text{Fe}^{\text{II}}_3\text{O}_4 + 2\text{H}_2\text{O}\]

To confirm the stoichiometry of these ferrites, solid samples were subjected to XRD and DSC. Figure 1 shows the XRD patterns corresponding to three compounds Fe15Cd, Fe7Cd and Fe3Cd respectively. As for the others, these examples show the presence of a major magnetite (Mgn) phase, and when washing is not exhaustive, tenardite may appear in small proportion. Figure 1 also shows the displacement of the peaks corresponding to this phase reflecting a slight increment in interplanar distances as the Fe(II)/Cd(II) relationship diminishes. This occurs since Cd(II) ions progressively substitute the Fe(II) ions occupying the octahedral positions of the magnetite structure, tending towards the stoichiometry of the cadmium ferrite CdFeO4. However, it is impossible to obtain this ferrite stoichiometry via this pathway.

The DSC curve of the cadmium bearing-ferrite shows an endothermic and an exothermic peak. The endothermic peak recorded from room temperature to 160 °C is associated with a loss in humidity (the presence of absorbed water suggests the hygroscopic nature of the ferrite, since the solid was dried at 100 °C). TGA demonstrated that the mass transition from room temperature to 150 °C accounts for a weight loss of 1.0%.

The exothermic peak occurs from 160 to 280 °C, with a maximum at 230 °C, which is most likely due to the oxidation of the magneti-
te phase (Fe₂O₃) to form maghemite (γ-Fe₂O₃) by atmospheric oxygen:

\[ \text{[Cd₄Fe₃xO₄y]₋₃[2Fe₂O₃] + 1/2O₂ → [Cd₄Fe₃xO₄y]₋₃[3g-Fe₂O₃]} \]

This exopeak shows a shoulder associated with the magnetite fraction (Fe₂O₃) appearing in the cadmium ferrite (Cd₄Fe₃xO₄y), which is oxidised according to:

\[ 2n[Cd₄Fe₃xO₄y] + n/2O₂ \rightarrow 2nx[CdFe₂O₄] + 3n(1-x)\gamma\text{-Fe}_2\text{O}_3 \]

The total integrated area of this 160 to 280 °C-peak, corresponds to the total transformation of magnetite to maghemite according to:

\[ 2\gamma\text{-Fe}_2\text{O}_3 + 1/2O₂ \rightarrow 3\gamma\text{-Fe}_2\text{O}_3 + 85 \text{ J g}^{-1} \]

2. Electrochemical analysis

Electrochemical analysis of the cadmium ferrites was performed using a J-shaped carbon paste electrode [12]. The voltammetry peaks were compared with those corresponding to an iron ferrite [13], iron oxides [14], magnetite [15], and also with scans corresponding to cadmium oxide. The voltammetry peaks shown in successive scans were labelled Rₓ and Oₓ, where R and O refer to reduction and oxidation, and the subscripts x and y indicate the figure and peak number respectively.

2.1. HClO₄ medium

Linear scans from the open circuit potential to +1.0 V, and several cyclic scans from +1.0 V to negative potential (-1.0 V) and back to +1.0 V were recorded using CPEs. These were prepared by loading the reservoir with a mixture of approximately 0.020 g solid cadmium ferrite, 0.100 g graphite powder and 2 drops of electrolyte binder (HClO₄), and immersing the electrode into a solution of the same electrolyte.

Figure 2a shows the voltammetry scans obtained using electrodes containing Fe₅Cd and 1 M HClO₄. One oxidation peak, Oₓ, was observed in the LSV, while three reduction peaks Rₓ, Rₓ₋₋ and Rₓ₋ were observed in the first cyclic voltammogram (CV1). Successive cyclic scans (CV2 etc.) yielded the peaks Rₓ₋₋, Rₓ₋₋, and Oₓ₋₋ only. Previous authors [13-15] have established that the peaks Oₓ₋₋, Rₓ₋₋, Rₓ₋, and Oₓ₋₋ correspond to the Fe(III)/Fe(II) couple:

\[ \text{Oₓ₋₋ peak} \]
\[ \text{Fe}^{n+} \rightarrow \text{Fe}^{n+} + \text{e}^- \]

\[ \text{Rₓ₋₋ and Oₓ₋₋ peaks} \]
\[ \text{Fe}^{n+} \rightarrow \text{Fe}^{n+} + \text{e}^- \]

The potential of the system Fe^{n+}/Fe^{n+} was calculated from the half-peak potentials according to the expression:

\[ E = \frac{E_{\text{ox}} - E_{\text{red}}}{2} \]

giving a value of 0.525 V. This value is comparable to the thermodynamic value obtained for this system (+0.770 ± 0.002 (vs. SHE)) by Whittemore and Langmuir [16].

The Rₓ₋₋ peak corresponds to the electrochemical solubilization of the ferrite [17]:

\[ n\text{Cd}^{2+} + m\text{Fe}^{n+} + n\text{H}_2\text{O} \rightarrow \text{qCd}^{2+} + m\text{Fe}^{n+} + n\text{H}_2\text{O} \]

The Rₓ₋₋ and Oₓ₋₋ peaks are specific to cadmium ferrite. Their potential -0.700 V corresponds to the reduct oxidation couple Cd²⁺/Cd⁺ of potential -0.403 V vs. SHE according to Okinawa [18]. Thus, the most probable electrochemical reaction, which may be assigned to these peaks, is:

\[ \text{Cd}^{2+} + 2\text{e}^- \rightarrow \text{Cd}^{2+} \]

Figure 2b shows the voltammetry scans obtained using electrodes containing Fe₃Cd and 6 M HClO₄ as electrolyte and binder. The only difference with the result obtained in 1 M HClO₄ is that, this time, the Rₓ₋₋ peak does not appear. This is due to the higher solubility of the compound in 6 M HClO₄. Nevertheless, it is possible to observe an increase in the size of the Cd peaks at the expense of that of the Fe peaks. Indeed, the intensity and area of the Oₓ₋₋ and Rₓ₋₋ peaks increase with the amount of cadmium in the ferrite. Thus, peak areas of 17, 21, 84, 135 and 180 mC respectively were recorded using the same quantities of
Fe20Cd-Fe3Cd to prepare the electrodes. These areas are consistent with the stoichiometry proposed for these solids.

In order to confirm this mechanism, a voltammogram of a cadmium oxide solution in 1 M perchloric medium was obtained. This scan only showed the $O_{33}/R_{33}$ peaks at -0.720 V, i.e., the system assigned to the Cd$^{2+}$/Cd$^{0}$ couple. From the i-t curve, the peak areas were shown to be practically the same; 580 mC for an electrode containing 0.0011 g of Cd$^{0}$ and 0.1064 g of graphite.

2.2. HCl medium

The voltammograms shown in Figure 3 were obtained using an electrode prepared as described for the perchloric medium, but this time 1 M HCl was used as the electrolyte and binder. Voltammograms corresponding to Fe15Cd, Fe7Cd and Fe3Cd are shown in Figure 3a, b and c respectively.

All the peaks produced were similar to those recorded for the iron ferrite (magnetite) in the same medium [13-15], with the exception of $O_{33}$ and $R_{33}$, corresponding to the cadmium, as seen in the perchloric medium. The potential of the $O_{33}/R_{33}$ system corresponding to Fe$^{8+}$/Fe$^{2+}$ is 0.475 V, showing a shift with respect to the value of -55 mV obtained in the perchloric medium. This may be explained by the participation of chloride ions in the electrochemical reactions. The different morphology of the $R_{33}$ peak may be attributed to the greater solubility of the cadmium ferrites in HCl compared to 1 M HClO$_4$, the participation of the chloride ion in the electrochemical process [13], and to the difference in the crystalline structure of the compounds [13-14]. These results are in accordance with those obtained for similar compounds [10,11], and suggest the following reactions related to the peaks:

$\text{FeCl}^{+}\text{ad} + \text{Cl}^{-} \rightarrow \text{FeCl}^{+}\text{ad} + 1\text{e}^{-}$

$\text{FeCl}^{+}\text{ad} + 1\text{e}^{-} \rightarrow \text{FeCl}^{+}\text{ad} + \text{Cl}^{-}$

$n\text{Cd} + n\text{Fe}^{2+} + m\text{O}_{4}^{-} + p\text{H}^{+} + q\text{e}^{-} \rightarrow g\text{Cd}^{2+}\text{ad} + h\text{FeCl}^{+}\text{ad} + j\text{H}_{2}\text{O}$

and in the second and subsequent scans:

$\text{FeCl}^{+}\text{ad} + 1\text{e}^{-} \leftrightarrow \text{FeCl}^{+}\text{ad} + \text{Cl}^{-}$

The $O_{33}/R_{33}$ peaks reflect the presence of cadmium in the ferrite. Their potential in 1 M HCl was -0.750 V, showing a 50 mV shift towards more negative values with respect to the value obtained using the perchloric medium. Moreover, it was confirmed that the shift was dependent on the concentration of chloride ions present in the solution. This is clearly shown in Figure 4a obtained using 2 M HCl (E = -0.760 V). In Figure 4b, which corresponds to the use of 6 M HCl, the reduction of the protons masks the cadmium peaks. The participation of chloride ions in the electrochemical process may consequently be expressed as:

$\text{CdCl}_{x}^{(x-2)} + 2\text{e}^{-} \leftrightarrow \text{Cd}^{0}\text{(s)} + x\text{Cl}^{-}$

with the following possible mechanisms: CdCl$_{2}$ + 2 e $\leftrightarrow$ Cd(s) + Cl$^{-}$; CdCl$_{2}$ (s) + 2 e $\leftrightarrow$ Cd(s) + 2 Cl$^{-}$; CdCl$_{2}$ + 2 e $\leftrightarrow$ Cd(s) + 3 Cl$^{-}$; CdCl$_{3}$ + 2 e $\leftrightarrow$ Cd(s) + 4 Cl$^{-}$.

although the values of the complex constants [19] would seem to indicate that the former is the most likely mechanism.

2.3. Chronopotentiometry

As for voltammetric determinations, when potentiometric scans
are performed, the results obtained using a freshly prepared CPE differ to those recorded by a reused electrode. Further, the processes involved do not obey the laws of linear diffusion and thus preclude the application of Sand’s equation. To obtain reproducible results, it is best to alternate positive and negative current scans since this avoids the need for equilibration periods.

Using a freshly prepared electrode containing 0.021 g Fe3Cd, 0.1042 g graphite, and HClO₄ as the electrolyte and binder, several chronopotentiograms were obtained. When the electrode was first prepared, the scan towards negative potentials gave rise to a signal reflecting the electrochemical rupture of the solid, while successive scans only yielded peaks corresponding to the Fe³⁺/Fe²⁺ and Cd²⁺/Cd⁰ couples. Figure 5a shows the chronopotentiogram obtained with the compound Fe3Cd in 6 M HClO₄ at -2 mA, -3 mA and -5 mA. In each case, two transition times were observed: t₁ corresponding to the Fe(III)/Fe(II) couple, and t₂ to the Cd(II)/Cd(0) couple at 0.530 V and -0.720 V respectively. These potentials are consistent with the voltammetric data. The amounts of current involved were: 428 mC, 378 mC and 315 mC for the iron system, and 170 mC, 120 mC and 75 mC for the Cd system (at -2, -3 and -5 mA respectively for each system). A reduction of approximately 50 mC may be seen in each case and can be explained by the fact that during the experiment, diffusion of dissolved species takes place from the electrode toward the bulk of the solution. This was confirmed by performing qualitative assays for iron and cadmium in both positive cases.

The chronopotentiograms in Figure 5b correspond to the ferrite Fe3Cd in 6M HCl. Here it may also be observed that the transition time decreases as the intensity is increased, but the main difference is that, in this medium only one jump corresponding to the Fe system may
be seen. This result confirms the previous suggestion based on the voltammetric findings that the Cd system disappears due to overlapping by the protons barrier. The participation of chloride ions in the electrochemical reaction was also confirmed but it is not possible to determine which reaction is predominant in the case of Cd. The chronopotentiograms performed at positive and negative intensities (±1 and ±2 mA) depicted in Figure 5b show that the cross-over points corresponding to the potential of the iron system obtained in the voltammetry experiments.

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References

Increasing titration speed by using an end point anticipator device

Abstract

A simple device is described for use in many titrations with the objective of rapidly locating the vicinity of the end point of a titration. The device stores inside about 10% of a 10 ml titrant solution. The titration itself proceeds with rapid addition of titrant until the end point is passed. The anticipator device now starts to rotate, using a dc motor, which is turned on by a microcomputer. The solution stored in the device is mixed with the already titrated solution and the property being monitored returns to a reading before the end point. The titration is now continued, knowing that the volume (or mass) necessary to reach the true end point is about 10% greater than the value found in the pre-titration. The computer estimates this volume and controls the last part of the titration, calculating the volume/mass increments. This considerably speeds up the titration process and obtains the necessary number of points for a precise and accurate localization of the end point using the titration curve. The device has been evaluated in potentiometric titration of HCl and of acetic acid with NaOH as titrant, and using a gravimetric burette. The results demonstrate that a titration can be performed in about 1.5 minutes by using the anticipator device, with no significant difference when compared with the conventional, stepwise and slower titrant addition procedure.

Keywords: Titration speed, end point detection, titration equipment.

J. J. R. Rohwedder, E. V. Aquino, S. H. F. Scafi, and C. Pasquini*

Instituto de Química, Universidade Estadual de Campinas, UNICAMP. C.P.: 6154 – CEP: 13093-970 – Campinas – SP (Brazil).

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Introduction

Titration is still a widely employed analytical procedure in many laboratories. The advantages of titration over other direct methods are in the use of a fundamental chemical property, stoichiometry, as well as the high accuracy that arise from statistical treatment of the titration curve in order to locate the end point. However, direct methods are usually faster than titration, even when both are automated by using modern resources based on the use of microcomputers as key devices.

The addition of titrant can be performed by measuring its volume, mainly using automated burettes (the most common case) or by measurement of mass. This last method has been recently automated [1,2] with a gravimetric burette which keeps all the advantages of gravimetric titration [3,4] with the possibility of full automation and scale reduction of sample and reagent consumption [1]. On the other hand, both the volumetric and gravimetric approach to titrant addition are a limiting factor with respect to the speed with which a titration can be performed. This is consequence of the fact that the end point can not be passed without the penalty of losing the titration and needing to re-start the determination with a new aliquot.

Therefore, many potential procedures can be found in the literature with have as their goal anticipating the end point of a titration. Anticipating the volume or mass of the titrant necessary to reach the end point of a titration can be used to accelerate titrant addition up to the vicinity of that point. Then its delivery rate is slowed down in order to avoid kinetic problems and to obtain a reasonable number of titration points that permit accurately locating the end point. Most of the anticipation methods described in the literature are based on mathematical algorithms that uses titration results from a first trial to fit into equations that predict the end point. Such procedures include the Gran’s plotting method [5] while others employ more complex equations [5,6]. These methods have in common the disadvantage of low versatility in relation to the type of titration and/or in relation to the concentration range of the samples to which the procedure can be applied.
Another way to control a titration in the vicinity of the end point is based on the analysis of the behaviour of the titration curve in the vicinity of the end point [6-8]. These procedures also depend on the type of titration and on the characteristics of the titration curve and do not improve the titration speed very much, as a reasonable number of points needs to be obtained before the end point, in order to predict the tendency of the titration curve and to infer its proximity.

On the other hand, only a few efforts can be found in the literature reporting on mechanical (hardware) devices employed to anticipate the titration end point. Carlson [9] has described such a device, based on a titration cell which can isolate a fixed volume of the titrand and automatically deliver it, by a pump/valve controlled system, near the vicinity of the end point. The disadvantage of this device is the relatively complex titration cell necessary to implement the methodology.

This work presents a simple device employed to locate the proximity of the titration end point which is intended to be of universal application while allowing a considerably reduction in the time necessary to perform a titration. The proposal has been evaluated using the previously described gravimetric burette [1,2] because this kind of titrant delivery is more prone to slow titration procedures. The overall goal of such approach to titration can be ascribed to the more general trend presently found in Analytical Sciences by which the analytical methods are developed aiming accessory analytical properties, mainly their expeditiousness [10].

**Experimental**

The end point anticipator device. The device constructed for anticipation of the end point of a titration is described in Figure 1. It was made of PTFE and contains two parts. A rod that connects a reservoir (about 1.0 mL inside volume) to a dc motor that can be turned on/off by the microcomputer that also controls the gravimetric burette. The reservoir is attached to the rod through a thread and it looks like a small cup. In its bottom and upper side, four orifices of 1 mm diameter were drilled. The inner wall of the cup contains a helicoidal thread which ensures that, under clockwise rotation imposed by the dc motor (at 60 rpm), the liquid will circulate through the cup promoting its mixing with the external solution present in the titration flask.

Gravimetric titration with the anticipator device. A gravimetric titrant addition arrangement was used to perform titration with the aid of the anticipator device. Details on the gravimetric burette and on the microcomputer interface used to effect the titration, to control the burette and the dc motor have been described elsewhere [1,2].

The titration procedure and the principle of operation of the device are very simple. 10 mL of the sample to be titrated is placed in a beaker. The cup of the anticipation device is placed alongside the glass combination and a magnetic bar. About 1.0 mL of the sample is retained inside the cup of the anticipation device and it is then isolated from the external solution during the pre-titration. The magnetic stirrer is turned on. The movement of the magnetic bar is not capable of promoting mixing with the solution that is inside the cup of the device because this can only be done by diffusion through the bottom holes. The microcomputer starts the titration, delivering the titrant to the flask as rapidly as possible, following the change in the monitored property, pH in this case. When an abrupt change is observed, indicating the pre-titration end point was roughly attained, titrant delivery is stopped and the computer accesses the mass delivered. This value is used to estimate the mass still necessary to achieve the real end point. The user can select how many points he wants to use to construct the titration curve in the vicinity of the stoichiometric equivalence point. The motor is turned on and the stored volume of titrand mixes with the previously titrated solution causing the pH to return to a value prior to the end point. The motor is kept on and the microcomputer proceeds with the titration, adding a number of equally spaced mass quantities of titrant. Any algorithm to locate the end point from the titration points, such as first or second derivative, can be used. In the present case the second derivative was always employed.

**Results and Discussion**

Figure 2 shows a typical result obtained for a titration curve of a strong acid, 0.0100 mol L⁻¹ HCl, titrated with NaOH of the same concentration. It is possible to observe
the effect of the use of the anticipator device and the instant the motor is turned on, returning the pH to a value prior to the real end point.

The gravimetric burette allows an additional advantage in the titration because it can manage up to four solutions with the same balance [1]. In the present example, the precision of the automatic titration made with the help of the anticipator can be enhanced by using two concentrations of titrant. The more concentrated one (0.0100 mol L⁻¹) is employed in the fast titration step to achieve the vicinity of the end point. From that point, the titration is completed using a titrant solution that is ten fold more dilute. Relative differences between the automatic titration and the manual standard procedure were, on average, reduced two time by employing the two solution approach to the gravimetric titration.

The gravimetric titration of acetic acid (0.01 mol L⁻¹) was also performed, in order to access the accuracy of the titration procedure using the anticipator device with a weak acid. Table 1 summarises the results obtained and presents good accuracy for the proposed methodology.

Although was not the objective of this work, the use of the anticipator device for titration of mixtures of acid could be investigated. It is possible to expect that the device could be used for those mixtures containing acids presenting significant differences in their pKas. This enable to include a decision based on two pH values where the first and second end point would be anticipated. The device should be stopped

| Table 1: Comparative results for titrations of HCl and acetic acid with NaOH. |
|---------------------------------|--------|------------------------|
| Titrated type                   | HCl (mol L⁻¹) (±rsd)¹ | Acetic Acid (mol L⁻¹) (±rsd)¹ |
| Manual                          | 9.83 x 10⁻¹(± 0.3%)   | 9.91 x 10⁻¹(± 0.3%)         |
| Gravimetric with anticipator/    | 9.83 x 10⁻¹(± 0.4%)   | 9.89 x 10⁻¹(± 0.3%)         |
| two titrant solution             |                    |                        |
| Relative difference (%)²        | 0.0                | -0.2                    |

¹ Relative standard deviation of 6 titrations.
again to anticipate the second end point.

The most significant gain found by using the end point anticipator device is in the reduction of the time necessary to perform a potentiometric titration without any significant loss in accuracy. The titration could be performed in about 1.5 min. Such speed can jeopardise direct methods, even automated ones using, for example, the flow analysis principle.

**Conclusions**

The proposed end point anticipator device demonstrates its usefulness to accelerate the titration procedure mainly when employed with gravimetric burettes. The gravimetric titration could be performed at a very high speed keeping all the advantages of that kind of titrant delivery.

Although only potentiometric titrations have been tested in the present work it is possible to anticipate the use of the proposed device in any kind of titration monitored by any kind of intrinsic solution property, with the same benefits achieved in the present study. In this way, the device present a more robust and versatile way to accelerate the titration procedure which can, in principle, be applied to any kind of titration employing titration curves or even employing end point location through a change in indicator property.

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**References**

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Units in use with the International System

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