CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

DEVELOPMENT OF SOLID PHASE EXTRACTION FLOW-BASED TOOLS FOR ENVIRONMENTAL MONITORING

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Chemistry

Tânia Cristina Ferreira Ribas Vaz Pedro

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Tânia Cristina Ferreira Ribas Vaz Pedro

Supervisor: António O. S. S. Rangel, Full Professor
Co-supervisor: Ildikó V. Tóth, Ph.D.

May 2020

To those who inspired it,

Ariana, Dinis, Frederico and Nuno

Abstract

Abstract

The development of new analytical tools can be considered a non-stop challenge due to the constant search for new improved features and also to the emerging environmental contaminants. Flow-based methodologies stand out in contributing for this analytical challenge, providing the automation and miniaturization of the analysis including sample pre-treatment.

This thesis was developed based on two major objectives, one of them was to develop new miniaturized and automated analytical tools based on flow analysis for environmental monitoring. When designing new methodologies, another essential objective was to simplify sample preparation by coupling these techniques, based on solid phase extraction (SPE), within the developed flow-based system. The developed methodologies were optimized based on the same principles: minimize the use of reagent, make greener choices of the reagents, minimize the effluent production, lower the limits of detection and quantification, simplify and minimize sample/reagent handling. The use of the in-line SPE strategy showed to bring advantageous features to the analytical method (lowering limits of detection and quantification). The in-line SPE was achieved by using commercial resins (NTA and Chelex 100) and also a lab-made polymer inclusion membrane (Chapter 3).

A biparametric sequential injection system for the determination of copper and zinc in water and soil leachates was developed (Chapter 3). The strategy was to use a non-specific coulour reagent (4-(2-Pyridylazo)resorcinol – PAR) and explore the use of two different sorbent materials to selectively separate the two different metal ions in the same manifold. A polymer inclusion membrane (PIM) and the commercial resin Chelex 100 were the chosen materials to selectively retain zinc and copper, respectively. It was the first time that a PIM was used with this purpose in a flow system.

A spectrophotometric method for iron quantification using a newly designed chromogenic chelator was developed (Chapter 4). This low toxicity iron chelator was a specially designed 3-hydroxy-4-pyrydinone functionalized with ethers. Furthermore, this reagent demonstrated to display high affinity and specificity for iron ions. With the main objective of quantifying iron in a variety of water samples (fresh and marine water) a strategy including SPE was added to the manifold. By using an in-line SPE process, resorting to a NTA resin column coupled to the flow system, sample matrix clean-up and also the enrichment of the analyte was achieved.

A method for the screening of biogenic amines in waters was developed (Chapter 5). The system was divided in two analytical parts. The first one was devoted to the pre-concentration of the analyte using a column packed with Chelex 100; the second was the derivatization of the biogenic amines using fluorescamine for the fluorescent detection of the analyte. This method intended to be a suitable and ease to operate system to obtain real-time information about biogenic amines content in water.

A flow injection system for the spectrophotometric determination of the total zinc content in plant digests was developed (Chapter 6). By using a NTA resin column, zinc pre-concentration and the removal of possible interferences was accomplished. A specially designed multi-reflection flow cell coupled with a light emitting diode was the chosen detection system for the spectrophotometric determination of zinc using Zincon as colour reagent. The physical configuration of the flow cell contributed to improve the limit of detection and minimize refractive index gradients produced by the mixture of the reagents.

Keywords: Flow analysis; solid phase extraction; green chemistry; water; plant.

Resumo

Resumo

O desenvolvimento de novas ferramentas analíticas pode ser considerado um desafio constante, devendo-se tal à busca incessante de características analíticas cada vez melhores e também ao surgimento de novos contaminantes ambientais. Os métodos em fluxo destacam-se ao contribuir para este desafio analítico, nomeadamente na automatização e miniaturização da análise, incluindo o tratamento da amostra.

A tese foi desenvolvida com base em dois objetivos principais, um dos quais se centrou no desenvolvimento de novos métodos analíticos em fluxo para a monitorização ambiental. No planeamento de novos métodos teve-se em consideração outro grande objetivo, a simplificação do tratamento da amostra, associando para tal técnicas de extração em fase sólida ao sistema de fluxo desenvolvido. A otimização dos sistemas analíticos teve por base os mesmos conceitos: minimizar o consumo de reagentes; fazer uma escolha mais ecológica relativamente aos reagentes; minimizar a produção de efluentes, melhorar limites de deteção e quantificação; simplificar e minimizar o manuseamento de amostras/reagentes. Ao recorrer a processos de extração em fase sólida em linha, conseguiu-se uma melhoria das características analíticas associadas ao método (baixando o limite de deteção e quantificação). De uma forma geral, a extração em fase sólida em linha foi realizada recorrendo à utilização de resinas comerciais (NTA e Chelex 100), mas também foi utilizada uma membrana produzida em laboratório (Capítulo 3; membrana de inclusão de polímeros – PIM).

Foi desenvolvido um sistema biparamétrico por injeção sequencial para a determinação de cobre e zinco em águas e lixiviados de solos (Capítulo 3). A estratégia usada para o desenvolvimento deste método envolveu o uso de um reagente de desenvolvimento de cor não específico - (4-(2-piridilazo)resorcinol – PAR) - e o explorar da utilização de diferentes materiais adsorventes para separar seletivamente os dois iões metálicos no mesmo sistema. Para tal recorreu-se a uma membrana de inclusão de polímeros (PIM) e a uma resina comercial (Chelex 100) com o intuito de reter e separar o zinco e o cobre, respetivamente. De salientar que foi a primeira vez que uma PIM foi utilizada com este objetivo num sistema de fluxo.

No Capítulo 4 foi desenvolvido um método espectrofotométrico para a determinação de ferro em águas naturais utilizando um quelante cromogéneo desenvolvido recentemente. O quelante de ferro de toxicidade baixa pertence ao grupo das 3-hidroxi-4-piridinonas funcionalizado com éteres. Este reagente demonstrou ainda ter uma elevada afinidade e especificidade para o ferro. Com o objetivo de aplicar o método à determinação de ferro em diferentes tipos de águas naturais (doces e salinas), foi incluído no sistema de fluxo um passo adicional de extração em fase sólida. Para tal, utilizou-se uma coluna empacotada com resina de NTA, a qual permitiu realizar a limpeza da matriz da amostra e também a possibilidade de se concentrar o analito de interesse.

Foi desenvolvido um método para o despiste de aminas biogénicas em águas (Capítulo 5). O sistema foi dividido em duas fases fundamentais. A primeira fase consistiu na pré-concentração do analito recorrendo a uma coluna empacotada com Chelex 100 acoplada ao sistema de fluxo; de seguida procedeu-se à derivatização das aminas com fluorescamina para a sua deteção fluorimétrica. O método desenvolvido tinha como principal objetivo ser de fácil execução, mas que desse uma resposta em tempo real sobre o conteúdo em aminas biogénicas em águas.

Foi desenvolvido um sistema por injeção em fluxo para a determinação de zinco total em plantas (Capítulo 6). Com a implementação de uma coluna de NTA no sistema de fluxo conseguiu-se a pré-concentração de zinco e também a remoção de possíveis interferentes presentes na amostra. Como sistema de deteção foi utilizada uma célula de fluxo multi-reflexão acoplada a um LED, visando a determinação espectrofotométrica do zinco utilizando Zincon como reagente de desenvolvimento de cor. Devido à configuração física da célula de fluxo, esta contribuiu para a minimização da influência da refração produzida pela mistura dos reagentes e para o melhoramento do limite de deteção do método.

Palavras chave: Análise em fluxo; extração em fase sólida; química verde; água; planta.

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To my person, my husband Nuno, for all the patience during all these years, for being always by my side.

To my little people, my children, who gave a new meaning and willingness to pursue my dreams.

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List of Abbreviations

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AAS – atomic absorption spectrometry

BAs – biogenic amines

Cad - cadaverine

CCD – charged coupled device

CE – capillary electrophoresis

D2EHPA – di-(2-ethylhexyl)phosphoric acid

Em Wl – emission wavelength

Ex Wl – excitation wavelength

ESI – electronic supplementary information

FIA – flow injection analysis

GC – gas chromatography

HC – holding coil

His - histamine

IC – injector commutator

ICP-OES - inductively coupled plasma – optical emission spectrometry

Int - fluorescence intensity

IV – injection valve

LC – liquid chromatography

LLE – liquid-liquid extraction

LOD – limit of detection

LOQ – limit of quantification

LOV – lab on valve

Me – metal ion

MRB13 - 1-(3’-methoxypropyl)-2-methyl-3-benzyloxy-4-(1H)pyridinone

MRC-LED – multi-reflection flow cell coupled with light emitting diode

MS – mass spectrometry

NTA - nitrilotriacetic acid

PAN – 1-(2-Pyridylazo)-2-naphthol

PAR - 4-(2-Pyridylazo)resorcinol

PIM – polymer inclusion membrane

PVC – poly(vinyl chloride)

RC – reaction coil

RSD – relative standard deviation

SD – standard deviation

SIA – sequential injection analysis

SPE – solid phase extraction

Spe - spermine

St – standard solution

SV – selection valve

S – sample

THF – tetrahydrofuran

TLC – thin layer chromatography

Try - tryptamine

Tyr – tyramine

UV – ultraviolet

2-PEA – 2-phenylethylamine

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CHAPTER 1

General Introduction

1.1. Environmental Analysis

1.1.1. The Environment

The concern about environment has been significantly increasing in the last decades. And so, the environment and its protection can be considered one of the biggest challenges nowadays. This challenge involves governmental agencies, academia and industry, which work together to accomplish all the guidelines of quality and minimize the impact of human activity into the surrounding environment. Society is then demanding higher quality for the environment (water, soil and air) to preserve the equilibrium of the ecosystems and consequently human health and well-being of living organisms (1,2).

In the past few years, a big effort has been made all around the world, involving several entities, creating groups focused on the environmental issues. Over the past 30 years, they have been trying to organize priorities and solve the existing problems to keep the normality of the environment and so reduce the impact of human activity.

The field of Green Chemistry was codified with 12 principles. These principles are a set of guidelines that aims to reduce the risks of the synthesis, processing and use of chemicals to humans and environment. In the last few years, there has been an effort for developing innovative processes in all the fields that involve chemistry. The ultimate goal is to accomplish a more effective, efficient and environmentally benign chemistry (3).

The twelve principles of Green Chemistry were firstly described by Anastas *et al* in 1998 (4) and they are focused on reducing risks and the impact of the chemistry (in all the fields that include chemistry and/or chemicals) to human life and the environment.

Those twelve principles are:

*"**1. Prevention.** It is better to prevent waste than to treat or clean up waste after is formed.*

***2. Atom Economy**. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.*

***3. Less Hazardous Chemical Synthesis**. Whenever practicable, synthetic methodologies should be designed to use generate substances that pose little or no toxicity to human health and the environment.*

***4. Designing Safer chemicals**. Chemical product should be designed to preserve efficacy of the function while reducing toxicity.*

***5. Safer solvents and Auxiliaries**. The use of auxiliary substances (e.g. solvents, separation agents, etc) should be made unnecessary whenever possible and, when used, innocuous.*

***6. Design for Energy Efficiency.** Energy requirements of chemical processes should be recognized for their environmental and economical impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.*

***7. Use of Renewable Feedstocks.** A raw material should be renewable rather than depleting whenever technically and economically practicable.*

***8. Reduce Derivatives.** Unnecessary derivatization (use blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.*

***9. Catalysis.** Catalytical reagents (as selective as possible) are superior to stoichiometric reagents.*

***10. Design for Degradation.** Chemical products should be designed so that at the end of this function they break down into innocuous degradation products and do not persist in the environment.*

***11. Real-time Analysis for Pollution Prevention.** Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.*

***12. Inherently Safer Chemistry for Accident Prevention.** Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions and fires."*

The awareness behind these guidelines leads to the improvement of the knowledge and development of new approaches in all the fields, including analytical chemistry. Under this context, there has been a continuous increase in the development of innovative methods, reactions conditions and analytical tools to accomplish the objective of a more environmentally-friendly analytical chemistry (3,5–9). Knowing that any substance is not completely safe, it is extremely important to obtain more information related to the chemical compounds, enabling the chemists the possibility of make informed and conscious choices. In the past few years, new methods and analytical tools have been developed to detect and quantify a variety of possible contaminants in the environment (water, soil and air). The new procedures also have the inherent objective of reducing the chemical impact of the overall analytical process.

A more recent publication, by Erythropel *et al* 2018, emphasises the use of these 12 principles of Green Chemistry in a new format, a tree format (Fig. 1.1 – adapted from the same publication). This tree is a result of the advances made along the years in the field of Green Chemistry. The ChemisTree diagram is a simple diagram that intends to simplify the interpretation of the principles and how these principles correlate with each other (branches and leaves).

Fig 1.1. The Green ChemisTREE highlighting the areas of inquiry and progress relevant to each of the 12 Principles of Green Chemistry, Abbreviations: crit. – critical; eff. – efficiency; haz. mat. – hazardous materials; metr. – metrics; prod. – production; solv. – solvent; ADME-absorption, distribution, metabolism, excretion; HTS-high throughput screening; (Q)SAR-(quantitative) structure–activity relationship. Adapted from a Journal of the Royal Society of Chemistry, 2018 (Green Chemistry, 20, 2018, 1929 – 1961).

It is obvious that analytical chemistry has a huge impact in the environment, leading to new goals for chemists when developing new analytical methods and strategies. The real-time analysis plays an important role in environmental contamination prevention. With this strategy, it is possible to assess in real-time any change in some parameter in the environment (water, soil or air), allowing a fast answer to a probable or detected problem.

The development of new analytical tools and processes also plays an important role in the Green Chemistry point of view. The main goal when developing new methods are: ideally, not use reagents; however, when needed, reduce quantities and make conscious options of the reagents used; minimize the volumes of sample needed per analysis; reduce the production of effluents. New goals are now involved in the design of new analytical methodologies that do not only involve sensitivity, precision and throughput.

The awareness about the environment began in the 90s, not only with the Green Chemistry concept and guidelines, but also with the Sustainable Development Goals that were firstly discussed at the Earth Summit in Rio de Janeiro. Here, a partnership composed for more than 178 countries developed a comprehensive plan focused on the environment. This plan was built looking to the urgency of improving human lives quality and protect the environment. Sustainable Development Goals were organized in a total of seventeen guidelines (Fig. 1.2) that were proposed in the United Nations Sustainable Development Summit in September 2015. These goals can also be applied to the everyday laboratory practice in analytical chemistry.

Fig. 1.2. Sustainable Development Goals adapted from (10).

Therefore, the Green Chemistry overall concepts and the Sustainable Development Goals are guidelines that look for the sustainability of the planet and the minimization of the impact of the human activity.

In analytical chemistry, it would be perfect, in a Green Chemistry point of view, if analysis could be performed directly upon an untreated sample. It would be also desirable to obtain as much as possible information about the sample without the need of a treatment before measurement. However, in most of the analytical methods, this is not possible due to the complexity of sample matrix or even the concentration and/or availability of the analyte (11,12). The development of new methods is an important field, in which a lot of work remains to be done to reduce the risks and the impact of human activity, helping to preserve the natural equilibrium of the environment.

1.1.2. Environmental Analysis – Water and Plant Analysis

The determination of chemical species in the different environmental matrices presents different challenges depending on the nature of the sample (water, soil and plants). The main challenges are the ultra-low analyte concentration and the complexity of the sample. These sample characteristics can affect quality assurance of the data with regard to accuracy, thus compromising the analysis (13–15).

Water is vital for life in all aspects, not only for consume but also for recreational purposes. The vital importance of water makes it indispensable for monitoring. The important chemical processes that alter water composition vary on the water source (groundwater, river, lake and seawater), consequently varying the expected elements present. Human activity can also interfere with water quality. So, analytical chemists must be aware of the possible existing problems and analytical tools should ideally be developed to assess any substances (changed element content and/or element contamination) that may interfere with water quality and consequently with life (14,16,17).

When developing analytical methods, it is necessary to consider the water source where different challenges may be encountered. As an example, when analysing an estuarine water, as it is a dynamic system, a high variability, spatial or temporal, in matrix composition can be found. Salinity can differ depending on the sampling site (proximity of the sea) and also depend on the weather conditions (rainy or dry weather), thus affecting directly sample composition. Therefore, the water source and referred conditions may also affect the analyte concentration, and so, the analytical method should be directed for the sample and its specificities (13,15).

Generally, to perform water analysis, two different approaches can be used: (i) in situ analysis; (ii) water sampling followed by sample storage, handling and analysis at the laboratory. The i*n-*

situ analysis offers some advantages over the other approach as it simplify the overall analysis process. This occurs because, when a laboratory analysis is performed, there is the need to guarantee that the sample integrity is maintained since the sample is collected until is analysed. Furthermore, an appropriate protocol should be adopted to minimize any possible change in the sample composition that may affect the analysis. Additionally, an in-situ analysis can give the possibility of act earlier to revert a possible problem.

Plant analysis is used to identify the constituents of the plants, playing a major role in detecting mineral nutrition problems. Plant analysis is also used to identify and monitor any potential toxic species that can affect plant growth or can enter into the food chain for humans or animals (18).

Plants are solid samples and so other difficulties are associated to plant analysis. For elemental analysis, plant sample preparation involves decomposition/destruction of organic matter through a digestion or extraction (19). And so, plant analysis is usually carried out on plants after some preparation process.

1.2. Sample Analysis – Flow-based Methods

The need to consider the development of automatic methods of analysis arose in the 1950s, when clinical tests started being increasingly used for diagnostic purposes in medicine. A contribution to the solution for this problem was provided by segmented flow analysis (SFA), which provided elevated throughput and substantial saving in samples and reagents (20–22).

This technique, SFA (Fig. 1.3), was proposed by Skeggs in 1957 (23). The equipment, used in SFA, comprises: peristaltic pumps for continuous aspiration of the sample and reagents; plastic tubes to carry liquid streams and the detector. After aspiration of the samples, air bubbles are introduced into the liquid stream, thus dividing it into separated compartments. These air bubbles serve various purposes: avoid mixture between samples; prevent dispersion of the sample plug; and facilitate the formation of a turbulent flow to homogenize the mixture sample/reagent in the plug between two bubbles. In SFA, the mixture of the sample and reagents needs to reach physical homogenization and chemical equilibrium before passing through the detector, where the signal is continuously monitored and recorded (20,23). In this technique, the liquid streams flow under a turbulent flow regime and the detection should be performed once the reaction reaches the steady state.

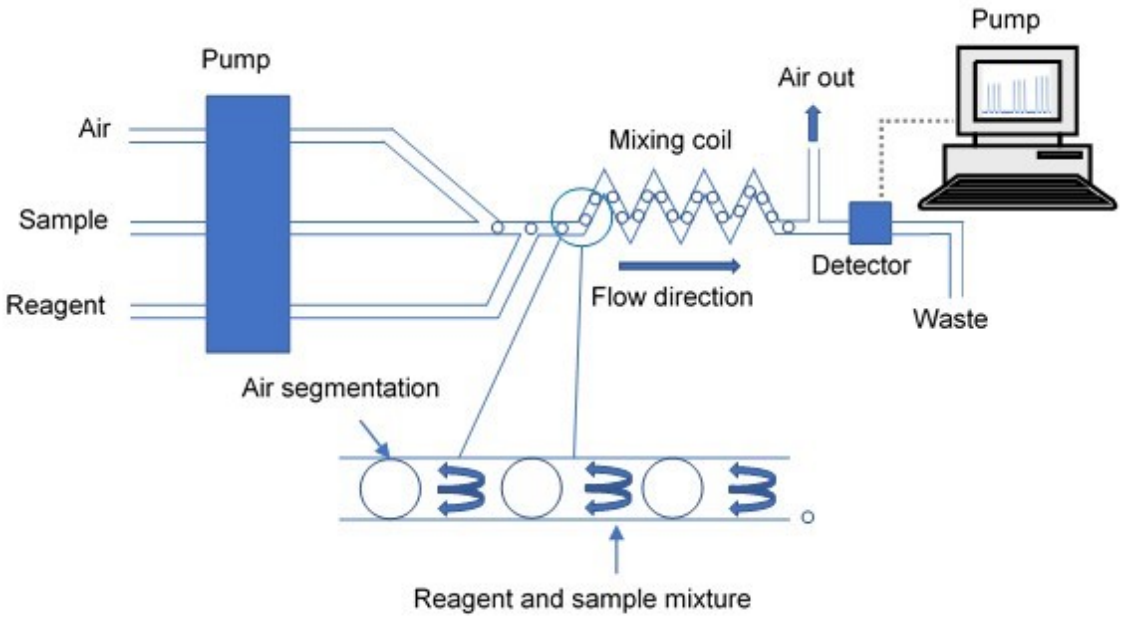

Fig. 1.3. Schematic representation of a generic manifold of a segmented flow analysis system.

SFA was widely used in the laboratory practice, medical, environmental and more. However, new flow-based methodologies have been emerging along the years; in the 70´s, a new concept emerged, flow injection analysis (FIA). This method is a flow-based method that brought a lot of novelties to flow methods and also to the analytical chemistry field.

1.2.1. Flow Injection Analysis

In 1975, J. Ruzicka and E. H. Hansen proposed a technique that initially resembled SFA, named flow injection analysis (24). The basic components of a FIA system (Fig. 1.4) are the same as in SFA, including also peristaltic pumps, a series of plastic tubes and the detector. However, unlike SFA, this methodology is based on the injection of a constant volume of sample into a non-segmented and continuous carrier stream via an injection valve. The injected sample forms a zone, which is then transported to the detector that continuous records the physical parameter (absorbance, potential, or other). The signal changes as the sample passes through the flow cell. In a FIA system, a transient signal is obtained, which is caused by the concentration gradient formed by the dispersion of the solutions through the tubing (20). Unlike SFA, in a FIA method a laminar flow is observed inside the tubbing, which reduces likelihood of carry-over between successive samples.

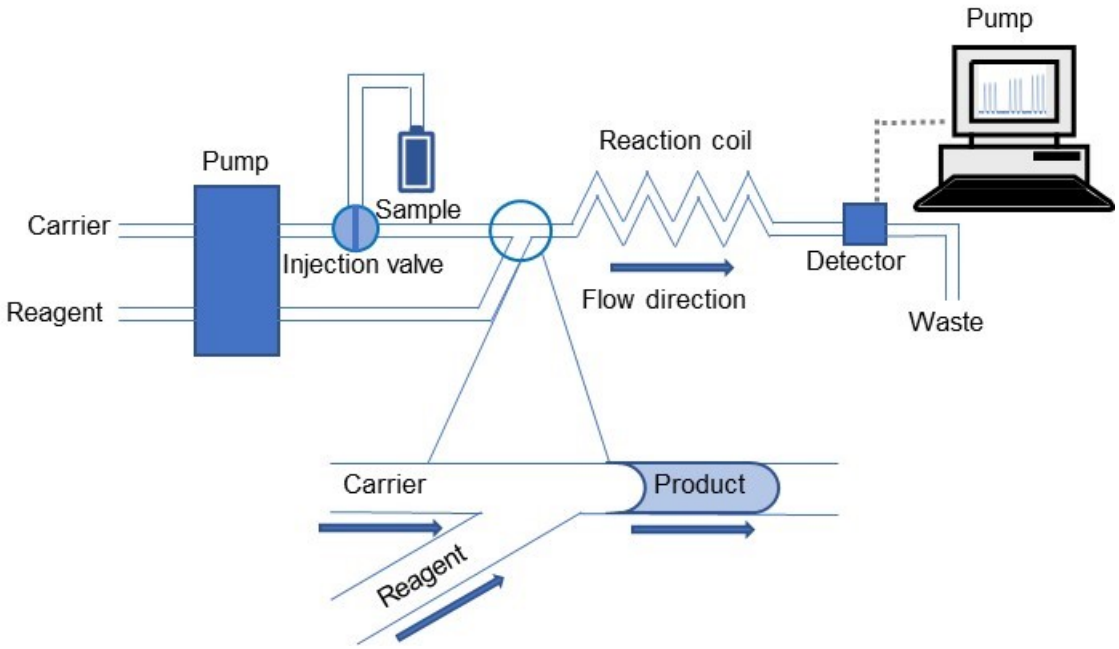

Fig. 1.4. Schematic representation of a generic manifold of a flow injection analysis system and a description of a merging zone at the confluence point.

FIA and related techniques rely on the combination of three basic principles: (i) reproducible sample injection, (ii) controlled dispersion of the sample zone and (iii) reproducible timing between the injection and the detection. The biggest difference between this new generation of flow methods in comparison with other analytical methods, is that the chemical reaction is taking place while the sample is dispersing within the reagent along the reaction coil until reaching the detector, with no need to reach chemical and/or physical equilibrium. If the three principles are guaranteed, the conditions are maintained for every single injection, and consequently the equilibrium phase of the mixture (sample and reagent) that reaches the detector is maintained.

The main goals behind the flow-based methods development are: the use of low sample volumes, minimize reagent consumption, reduce effluent production, increase the degree of automation, simplify and avoid sample handling, reduce contamination risks and increase sample throughput (25).

1.2.2. Sequential Injection Analysis

Although successful as a laboratory technique and a versatile tool for the enhancement of instrumental analysis, the application of FIA has been hampered by the use of increasingly complex manifolds and by the limited applicability (25,26). Sequential injection analysis (SIA) was developed in 1990, by Ruzicka and Marshall (26) as an alternative to FIA, intending to answer to some associated FIA difficulties, such as the continuous consumption of carrier and reagents caused by the continuous flow. SIA (Fig. 1.5) is based on the same three principles as FIA: sample injection, controlled dispersion and reproducible timing. However, in a SIA system, a single pump allows precise flow control in both directions within a single flow channel, incorporating a selection valve (instead of injection valve). By means of this valve, precisely measured volumes of sample and reagents are aspirated into the holding coil. After this, the valve position is switched to direct the flow through the detector. The mixture of the consecutive aspirated solutions is accomplished by means of flow reversal when the flow direction is switched. The complexity of a multi-channel FIA system is reduced using a selection valve. In a SIA system is also possible to reduce the volumes of sample/reagent and consequently the production of effluent is reduced (13).

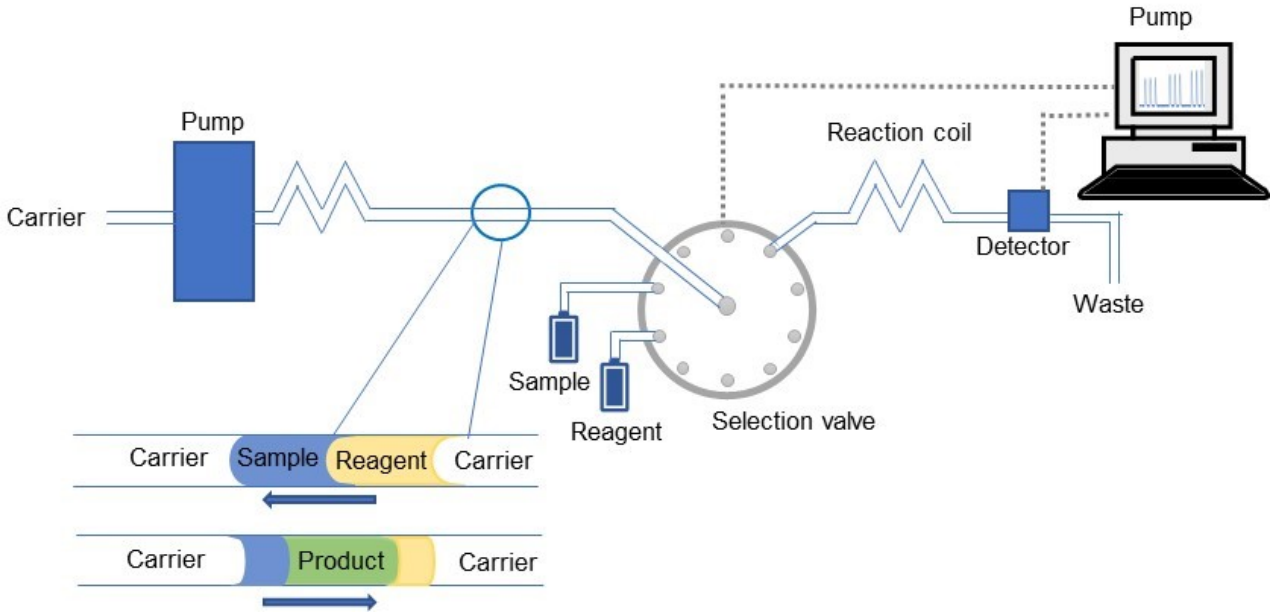

Fig. 1.5. Schematic representation of a generic manifold of a sequential injection analysis system. Description of a merging zone of the two consecutive aspirated plugs of sample and reagent followed by inversion of the flow thorough to the detector.

1.3. Separation and Pre-concentration

In analytical chemistry, one of the biggest challenges, which has a direct impact in the determination, is the sample preparation. The challenges associated with the analysis can be due to the sample matrix complexity that can interfere with the final concentration and/or to the concentration of the analyte itself, that can be at a trace level. These challenges can affect the detection of the target analyte and consequently the analysis. Trying to overcome the difficulties during the quantification process, sample preparation is often necessary. Several preparation techniques are available to make a sample suitable for analysis, being the extraction techniques the most common. The basic concept is the use of some kind of affinity to separate one or more species from a complex matrix and/or provide the enrichment of the target analyte/s. The most used extraction techniques are liquid-liquid extraction (LLE) and solid phase extraction (SPE). More recently, membrane-based extraction has gained a large interest in this area of extraction. During this thesis the topics that will be mainly discussed are about solid phase and membrane-based extraction techniques.

1.3.1. Solid Phase Extraction

Solid phase extraction is a sample pre-treatment extensively used in analytical chemistry. It is a versatile technique in sample preparation for sample matrix removal and/or analyte enrichment. The principle of SPE is similar to the one of LLE, in which a partitioning of solutes between two different phases is observed. However, unlike LLE, where this partitioning is observed between two immiscible liquids, in SPE this process occurs between a liquid phase (usually the sample matrix) and a solid phase (the sorbent material). This way, it enables the enrichment of the target analyte and/or clean-up of the sample matrix (27–30).

The first SPE experiments date back to 1950s (27,31,32); however, due to its advantages over LLE, the development of SPE and related techniques have been expansively growing during the past few years.

A general SPE method consist in, at least, four basic steps (Fig. 1.6): (i) conditioning of the sorbent material – crucial step that enables the wetting and the solvation of the functional groups of the sorbent material. The chosen solution to perform the conditioning of the sorbent depends on the sorbent and/or the target analyte; (ii) loading of the sample – this step consists on the percolation of the sample through the sorbent material, where the concentration of the analyte or the elimination of possible interferences is achieved, it depends on the final objective of the extraction; (iii) washing the sorbent – this step intends to eliminate any sample component, not desirable, that may be retained by the sorbent; (iv) elution of the target analyte/s from the sorbent – at this stage a elution solution percolate the sorbent material to elute the target analyte, this fraction is collected for further analysis. At stage (ii) the analyte should have stronger affinity to the sorbent material then to the sample matrix. However, at stage (iv), the opposite should occur and so the target analyte should have a stronger affinity to the elution solution than to the sorbent material. The mechanism of retention and elution of the target species depends on the nature of the sorbent material and this could be simply an adsorption mechanism, chelation or ion-exchange (27). If the major goal of the SPE method is the enrichment of the analyte, the loading sample volume should be the higher possible and the collected fraction should be the minimum possible volume, favouring the increment of the concentration of a trace element.

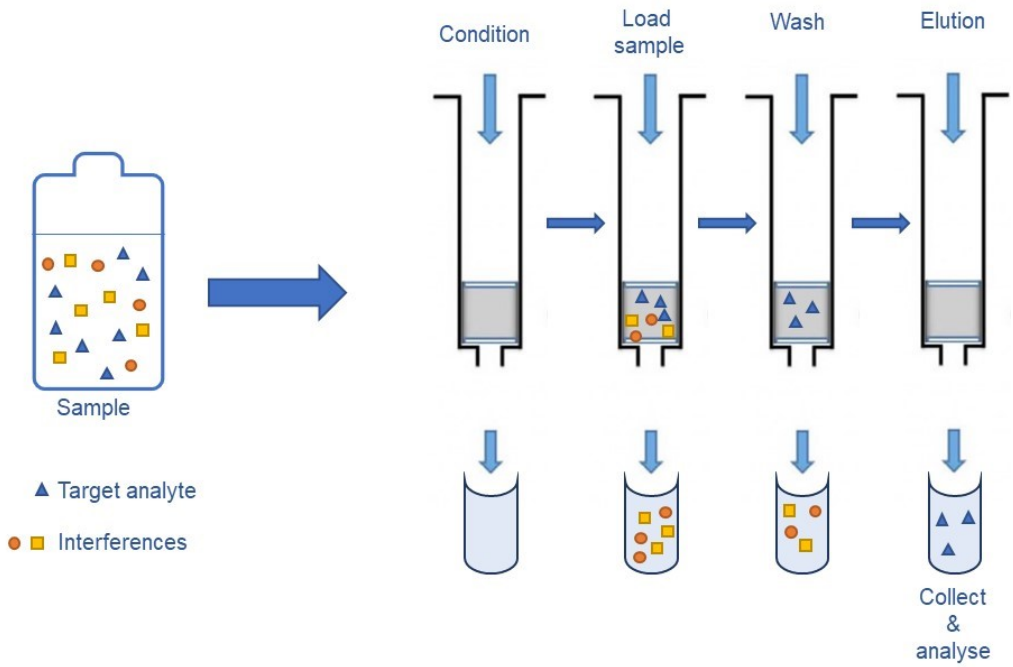

Fig. 1.6. General steps involved in a solid phase extraction process.

SPE offers advantages over LLE, as in the latter the procedures usually involve large volumes of organic solvents, methodologies in general are time-consuming and also require strict control of the procedure conditions (pH, temperature and ionic strength). On the contrary, in a SPE method, the use of organic solvents is reduced or eliminated. Usually, SPE methods are faster, have higher analyte recoveries, are relatively low-cost and the sorbents have the possibility of being reused. The ease of automation and coupling to other techniques, such as chromatographic methods (HPLC and GC), atomic absorption spectrometry, flow-based analytical methods among others, is also an advantage associated with SPE. In addition, the high number of available sorbent materials (with intrinsic different affinities) for selective analyte removal, makes this technique an attractive choice in sample pre-treatment.

1.3.2. Emerging Sorbent Material – Liquid Membranes

A membrane is defined, in a simple way, as a barrier between two phases and, when some or more elements of a mixture move through the membrane, a separation is accomplished (33–35).

Membranes have been used for a long time in analytical chemistry as sensing elements in analytical sensors (such as in potentiometry) and for separation processes. In the last few years, there has been a big effort involving new applications in membranes science, including extraction for sample preparation. The main goal of this research is to take advantage of the already known associated features of the membranes, such as the specificity, stability and sustainability, and use them in another field, the sample preparation. A lot of advantages that will be discussed further on are associated with the use of membranes.

A liquid membrane can be described as a thin organic layer. It is an immiscible liquid between two different solutions - donor and acceptor - acting as a barrier between these solutions (33).

Different approaches have been proposed for liquid membranes. And so, liquid membranes can be divided into two major groups: non-supported liquid membranes, if they are composed only by liquid phases, or supported liquid membranes, if they additionally include a solid support in their composition. Non-supported liquid membranes embrace bulk liquid membranes (BLMs) or emulsion liquid membranes (ELMs). When a solid support is in the composition of the membrane, we have the supported liquid membrane (SLMs) and more recently polymer inclusion membranes (PIMs) (33).

The overall apparatus for BLMs is simple; the membrane is a thick layer of immiscible liquid that separates the two phases (donor and acceptor). The thickness of the membranes influences the amount of the target element that is transported across the membrane. At the end, the efficiency of this technique is very low. ELMs are based on an emulsion, composed by a liquid membrane with an inner receiving phase, being the emulsion stability the major problem.

In SLMs, the carrier or extractant (selectivity agent) dissolved in an organic solvent is impregnated on porous film (polymeric porous support) by capillary action. These liquid membranes operate also with two aqueous phases (donor and acceptor), and the target species move through it. The organic phase is immiscible with the aqueous phases and sometimes, these membranes, also contain a modifier to favour the extraction process. The stability of the membrane is the major drawback associated with SLMs. Poor mechanical stability of the film or low chemical stability of the carrier is often observed, resulting in the loss of the carrier (33,34,36).

Nevertheless these weaknesses associated with the use of liquid membranes for extraction purposes, many advances in membrane science have been done to understand and improve membrane stability. This is due to the rising awareness of searching more environmentally friendly processes. Consequently, the growing concern about the environment leads to the need to develop new separation processes and minimize the use of conventional separation processes, thus minimizing the use of toxic organic solvents usually used.

This continuous investigation resulted in the development of PIMs, a type of supported liquid membranes formed by a liquid phase and a base polymer. PIMs have been used to provide the liquid separation of metal ions and small organic molecules from a solution. These membranes can serve many purposes in analytical chemistry, such as a sensing component of an ion-selective electrode, as optodes and, more recently, for membrane-based extraction in sample preparation. PIMs can combine high selectivity and ease of operation as SLMs; however, with PIMs, the stability and the durability of the membrane was improved (34,37). The higher lifetime can be explained by the fact that the extractant is entrapped in the base polymer, decreasing this way possible extractant loss, thus increasing the membrane stability and robustness.

The interest about the use of PIMs and their wide range of different applications has been rising in the last few years. In Fig 1.7, the number of published papers about polymer inclusion membranes is shown, the first one dates back in 1996 (search made on ISI Web of Knowledge – Web of Science on January 10th 2020. However, due to its advantageous properties, a lot of research remains to be done to increase the applicability of PIMs in the field of sample treatment.

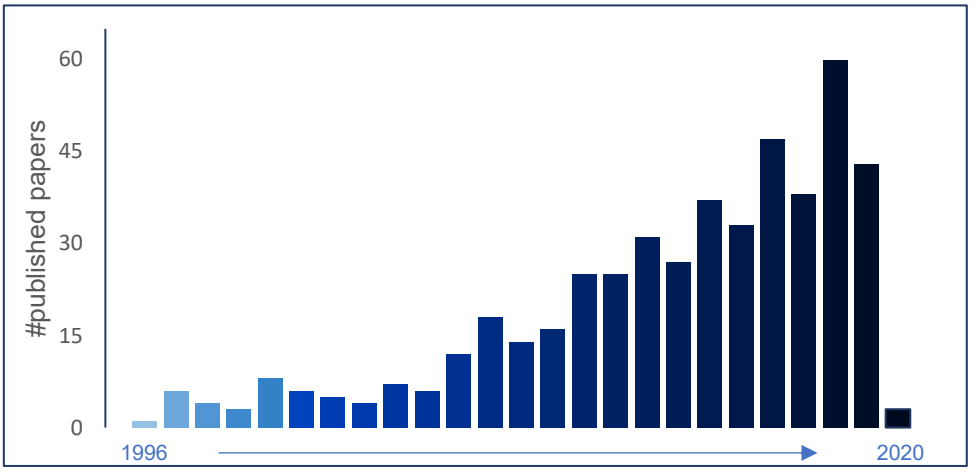

Fig. 1.7. Evolution of published papers about polymer inclusion membranes since 1996. Data collected on Web of Knowledge site (search made on January 10th 2020).

A search for the published papers was made on ISI Web of Knowledge – Web of Science (search made on January 10th 2020), describing PIMs and its use. The distribution of the scientific literature is depicted in Fig. 1.8. A large number of the published information about PIMs is about its use in the extraction processes due to the intrinsic characteristics of these membranes. When the search combined the keywords PIMs and extraction, a large number of the cited papers were devoted for metal extraction. From these, only a few studies (6%) are focused on sample pre-treatment in analytical chemistry.

As mentioned before, sample pre-treatment is a challenging step of major importance in analytical chemistry. The continuous demand for lower limits of detection, the increasing concern of conventional analytical chemistry techniques and the impact of the chemistry in the environment (lower the use or no use of toxic reagents), makes sample pre-treatment an important issue in analytical chemistry. PIMs have shown good capabilities to assist in this non-stop challenge sample pre-treatment for analytical chemists (Table 1.1).

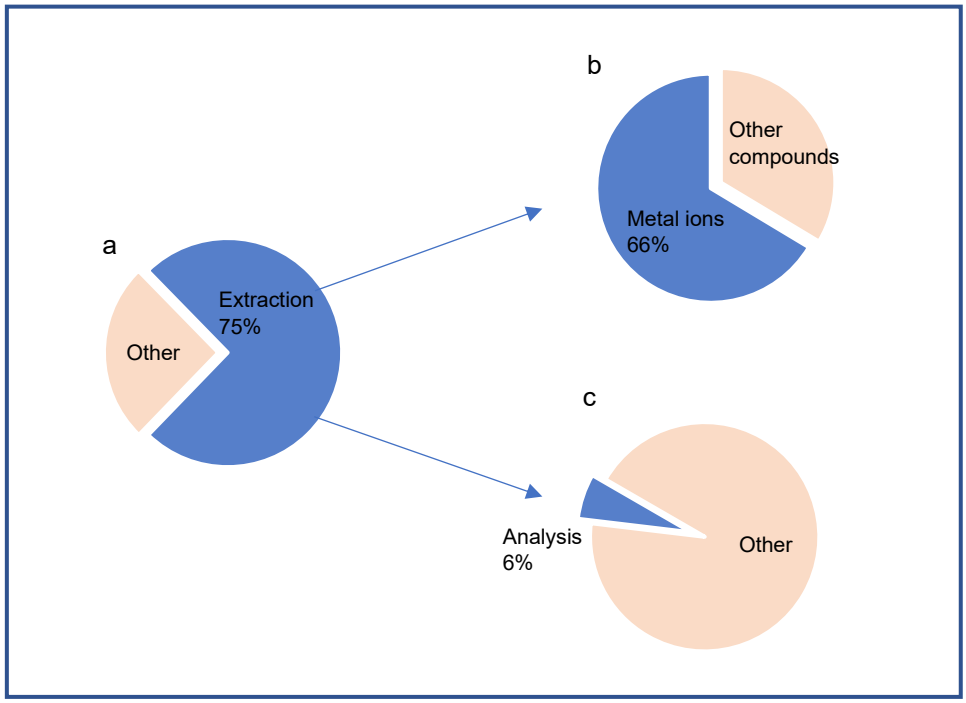

Fig. 1.8. Distribution of the published papers devoted for the development of PIMs and its use. a – Polymer Inclusion Membrane or Polymer Inclusion Membranes search (479 papers), in blue the percentage of works devoted for the use of PIMs for extraction purposes are presented (357 papers); b and c – number of papers for the combined search of the keywords PIM and extraction (357 papers); the blue part corresponds in b to those devoted to metal ions (237 papers); the blue part corresponds in c to those devoted to chemical analysis (23 papers). Data collected on ISI Web of Knowledge – Web of Science (search made on January 10th 2020).

Table 1.1. Analytical characteristics of developed analytical methodologies involving polymer inclusion membranes for the extraction process (presented in descending chronological order).

Target species	PIM composition		Detection	Application	Features	Reference
	Extractant	Base Polymer/Plasticizer				
Hg	PAR, thiourea, CCS and dithizone	PVC	X-ray fluorescence	Water		(38)
CN^-	Aliquat 336	PVC/Mg-Al-CO3 LDH	UV spectrometry	Water	LOD = 1.4 μg L^{-1} Dynamic range – 5 – 500 μg L^{-1}	(39)
As (V)	Aliquat 336	Poly(vinylidene fluoride-co-hexafluoropropylene)		Drinking water	LOD = 3.0 μg L^{-1}	(40)
Cd	THTDPCl	CTA/NPOE		Seawater	LOD = 10.0 μg L^{-1}	(41)
As (V)	Aliquat 336	CTA and PVC	colorimetry	Groundwater		(42)
Pesticides		CTA/NPOE	GC-MS	Water	Determination range – 50-1000 ng L^{-1}	(43)
Naproxen	Aliquat 336	CTA/oNPOE	UV spectrometry	Urine	Dynamic range – 5 – 200 μmol L^{-1}	(44)
Cu	D2EHPA	PVC/DOP	Spectrophotometry	River water	LOD = 0.10 mg L^{-1}	(45)
V(V)	THTDPCl	Poly(vinylidene fluoride-co-hexafluoropropylene)/2NPOE	Spectrophotometry	Water and dietary supplements	LOD = 0.08 mg L^{-1}	(46)
Hg	TOMATS	-	X-ray fluorescence	River, sea, ground and tap water	LOD = 0.2 μg L^{-1}	(47)

Al		PVC/Triton X-100	Spectrophotometry	Aqueous solutions	LOD = 1.04 x 10^{-6} mol L^{-1}	(48)
Thiocyanate	Aliquat 336	PVC	Spectrophotometry	Fertilizer	0.014 mg L^{-1}	(49)
Al	Aliquat 336	PVC/2-NPOE	Spectrophotometry	Aqueous samples	0.2 – 50 mg L^{-1}	(50)
As	Aliquat 336	PVC	Spectrophotometry	Groundwater	4.5 µg L^{-1}	(51)
Orthophosphate	Aliquat 336			Natural waters	LOD = 0.5 µg L^{-1}	(52)
Zn	D2EHPA	PVC/DOP		Pharmaceuticals and galvanizing industrial samples	LOD = 0.04 µg L^{-1}	(53)
Molecular iodine	PVP	CTA	Spectrophotometry	Aqueous samples	LOD = 0.3 µg L^{-1}	(54)
Alpha emitting actinides	D2EHPA	CTA		Tap water and sewater		(55)
Cr(VI)	Aliquat 336	CTA or PVC/2-NPOE	X-ray fluorescence	Electroplating water	LOD = 0.3 mg L^{-1}	(56)

PAR – 4-(2-Pyridilazo) resorcinol; CCS – calconcarboxylic acid; Aliquat 336 – tricaprylmethylammoiium chloride; Mg-Al-CO3 LDH - Mg-Al-CO3 – layered double hydroxide; CTA - cellulose triacetate; THTDPCl – trihexyl(tetradecyl)phosphonium chloride: NPOE – nitrophenyl octyl ether; PBAT – poly(butylene adipate-co-terephtalate); DNNSA – dinonylnaphthalene sulfonic acid; DOA – dioctyl adipate; DBP – dibuthylphtalate; NTA - nitrilotriacetic acid;; PVDF – polyvinylidene fluoride; PVP – polyvinyl pyrrolidone;; D2EHPA – di(2-ethylhexyl)phosphoric acid; DOP – dioctylphtalate; PVP – poly(vinylpyrrolidone)
*different LODs correspond to different flow analysis strategies

Generally, a PIM (Fig 1.9) is composed by a base polymer, an extractant and some membranes also contain a plasticizer in their composition. A PIM is a thin, flexible and stable polymeric film that has the property of selectively separate solute/s of interest depending on the composing extractant.

The base polymer is the solid support that entraps the liquid and provides the mechanical strength to the membranes. Although the existence of a variety of polymers that can be used with this purpose, poly(vinyl)chloride (PVC) or cellulose triacetate (CTA) are still the most usually used polymers. With both polymers, a relatively simple procedure based on its dissolution in an organic solvent is necessary to produce a thin membrane (37,57), thus explaining the extensively use of these two polymers. Furthermore, more investigation needs to be done, to try to get more information about the intrinsic characteristics of the base polymer and how a proper choice of a polymer can improve the stability of the membrane.

Fig. 1.9. PIM composed by PVC (base polymer) and D2EHPA (extractant).

Some PIMs also have a plasticizer or modifier in their composition, to increase the flexibility of the membrane. A plasticizer can also help in the extraction process, making the species more soluble within the extractant phase (57).

As referred before, the component that confers selectivity to the PIM is the extractant or carrier. Many studies have been done during the last few years, focused on the use of new extractants to increase selectivity of the PIMs. However, the most documented extractants are Aliquat 336 and di-(2-ethylhexyl)phosphoric acid (D2EHPA).

Aliquat 336 (tricaprylmethylammoiium chloride) is a basic carrier and is one of the most referred carriers used in a PIM development for quantification purposes (33,40,42,44,49–52). Aliquat 336

is a commercial mixture of quaternary ammonium chlorides. When using Aliquat 336, no use of plasticizer is required, because of its plasticizing properties (58). Heidarbeigi *et al* 2019 developed a PIM flexible and efficient for cyanide extraction. The PIM was composed by Aliquat 336 as extractant and reinforced with Mg-Al-CO3 layered double hydroxide (LDH) to promote the extraction efficiency of the membrane (39). With the purpose of applying a PIM for the determination of As(V), Vera *et al* 2019 developed PIM composed of poly(vinylidenefluoride-cohexafluoropropylene), Aliquat 336 and a microporous polytetrafluoroethylene (PTFE) gas-permeable membrane. This PIM was used in a flow-based mode for the automatic extraction and quantification of As(V) in drinking water (40). In another study, Vera *et al* 2014 proposed a PIM with the same extractant for the extraction and preconcentration of As(V). This PIM was successfully applied for the determination of the target analyte in groundwater (51). A PIM with the same extractant was developed by See *et al* 2018 for the extraction and quantification of naproxen in urine samples (44).

Another extractant that has been widely used is D2EHPA. This extractant has been used for the extraction of metal ions (45,53,55). D2EHPA is classified as an acidic carrier and can also act as a bidentate chelating agent (37). Using this carrier, the extraction process occurs by the exchange of the metal ions for the protons present in the carrier.

Generally, a PIM is prepared by dissolving the correspondent quantity of all components with a minimal volume of a volatile solvent and finally let the solvent evaporate onto a surface (depending on the application). The overall apparatus for a PIM preparation makes these membranes physically and chemically versatile sorbent options. The possibility of choosing and adapt the PIM constituents (extractant, base polymer and plasticizer) to the target species, make PIMs an attractive subject for analytical chemist to develop new strategies to improve sensitivity and selectivity in an extraction procedure (59).

1.4. In-Line Solid Phase Extraction

As mentioned, SPE is a technique that can easily be coupled with a variety of other techniques used in analytical chemistry. Flow-based methods are appealing to implement this type of strategies for the in-line sample preparation due to the associated apparatus versatility. Additionally, the high versatility of a SPE strategy, makes it to be fairly easily implemented into a flow-based manifold.

When the two techniques are coupled, flow-based methods with in-line sample pre-treatment, the system gains the inherent advantages of the two techniques. The automation and miniaturization of the sample extraction, in a flow-based mode procedure, decrease the time needed per analysis, thus improving sample throughput, minimize the operator sample handling, improve method precision and minimize the overall reagents/sample volumes consumption (15,36).

The features of a final analytical system with in-line sample extraction are more advantageous in comparison to a batch mode sample pre-treatment. In addition, when a SPE strategy is incorporated in a flow-based technique, usually offers the advantage of easily reuse the sorbent material and also lower the quantity of the sorbent material needed for the sample preparation.

A high number of strategies to implement SPE in a flow-based method have been developed during the last few years as a response to the inherent advantages (Fig. 1.10). A review was published in 2018 by Rocha *et al* describing the applications of solid phase extraction in flow analysis, and the synergic development made in this area (60). Another review was published by Calderilla *et al* 2018, in which recent advances in the automation of solid phase extraction are described (30). A search for the published papers (since these two last reviews in 2018) was made on ISI Web of Knowledge – Web of Science describing the hyphenation of solid phase extraction and flow-base methods. The features of the developed methodologies are summarized in Table 1.2.

As referred, a PIM is a supported liquid membrane and so, the partitioning of the solutes occurs between two liquids. However, based on the solid support, an option was made, and this type of membrane was considered as a SPE strategy.

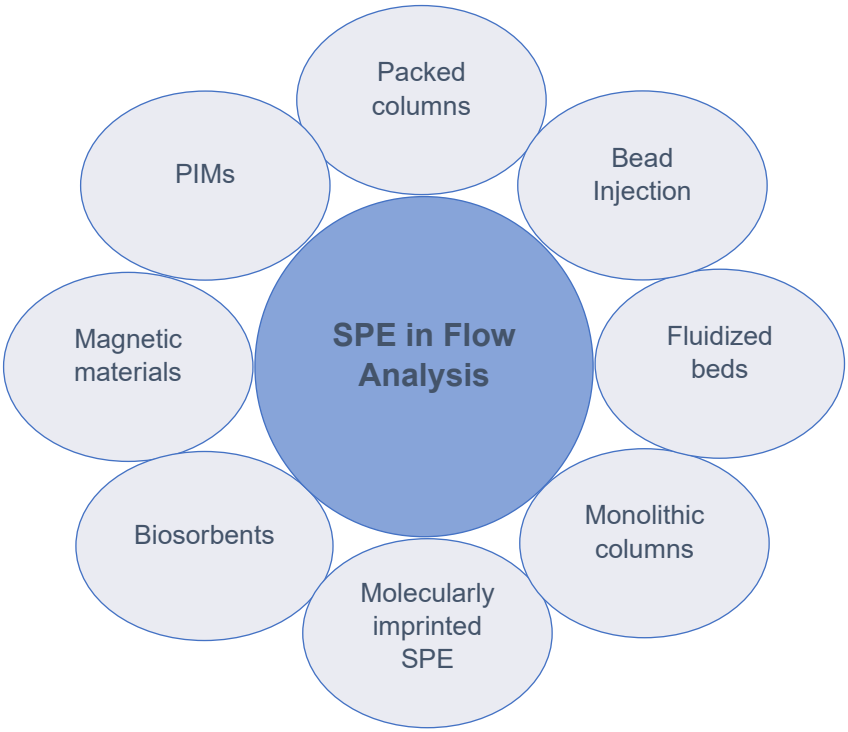

Fig. 1.10. Developed sorbent and/or strategies to implement solid phase extraction in flow-based methodologies.

Most of the documented applications of SPE in a flow-based system involve the use of packed columns coupled with the manifold, FIA or SIA systems. This strategy of using packed columns for the in-line SPE involve trapping the sorbent material into a commercial mini-column, cartridges, or another type of bed reactor (61,62). Solid phase extraction resorting to commercial resins has been widely used and the most used sorbents are based on silica or organic polymers functionalized with different ligands. The selection of the sorbent depends on the target analyte and its reaction with the sorbent material (30,60).

The incorporation of the packed column within the system can be made in different modalities, depending on the system and also the purpose of the extraction. Miranda *et al* 2016 developed a sequential injection system for iron determination in natural waters. In this system, a packed column of NTA resin was incorporated in a side port of the selection valve with the purpose of matrix clean-up (63). Paluch *et al* 2018 developed a bi-parametric sequential injection system with in-line SPE. A packed column with Chelex 100 resin was the chosen strategy to retain copper to perform zinc determination (64). The column was placed in a side port of the selection valve and the sample was aspirated directly through this port. Another system, developed by Ribas *et al* 2019 (described in this thesis), used a packed column with the purpose of pre-concentrate the

analyte. A column packed with the same commercial resin, Chelex 100, had the objective of analyte enrichment to lower limits of detection (65).

The main challenges of using packed columns can be the excessive backpressure formed with the packed material, low contact area, preferential pathways, the clogging of the flow and also the leakage of the fluids (61). As the advantages of using SPE in flow analysis are greater than the associated drawbacks, efforts have been made to overcome the related limitations. Some alternatives to packed columns have been designed with the main goal of applying sample treatment to flow-based systems (Fig 1.10).

One of those alternatives is bead injection. The procedure of a bead injection analysis involves the introduction of a defined quantity of the sorbent (as suspension) into the flow system and trapping it in the flow-cell (66,67). Most of the documented flow systems with bead injection were implemented in a SIA system. The selection valve in a SIA system can provide versatility and the possibility of flow reversal, thus simplifying the bead suspension handling (60).

A lab-on-valve (LOV) system developed by Vidigal *et al* 2011 used the bead injection mode for the quantification of total iron content in wines. A suspension of NTA resin was used as sorbent material for iron complexation. A defined quantity of the resin was aspirated and packed in the flow cell. After this, the sample was aspirated and passes through the resin at the flow cell where the iron is retained. The final step was the aspiration of the reagent that passes through the flow cell and reacts with the retained iron, at this point the absorbance was measured (67). Another approach was developed by Yu *et al* 2012, where the beads of Sephadex QAE-A25 previously immobilized with Zincon (colour reagent) were used for determination of copper in waters. This system was also developed in a LOV format (68).

Fluidized beds were developed as a SPE strategy, aiming to improve the solid-liquid interfacial interaction, thus increasing the efficiency of analyte extraction. In fluidization, the sorbent material is not static, but on the contrary it should be movable. The fluidization of the particles is attained by a pulsed stream that passes through the chamber where the sorbent material is in. The fluidization is accomplished by mechanical stirring, air inlet, sonication or magnetic beads (60,69).

A recent review paper focused on the applications of the fluidized beds was published by Dias *et al* 2018. In here, the potentialities and the limitations of the technique (61) were also discussed. Fontes *et al* 2008 developed a multi-pumping flow system for the determination of sulphate and chloride in natural water. The strategy was to resort to an in-line column with Bio-Rex 70 cation exchanger mini-column with fluidized beds for the sample matrix clean-up (70).

In general, flow-based analytical systems do not allow the separation of the analytes for multi-component analysis (71). However, to overcome this weakness, an attempt was made in this direction, coupling low pressure chromatographic columns (monolithic columns) to flow-based systems. A monolithic column is a column composed by macropores and mesopores. The objectives for having different, but well-defined pore-size, are: mesopores act as retention phase

and the macropores make possible the through flow, providing good separation power and high chemical stability with low flow pressure (71–74). Coupling the use of monolithic columns in a flow-based system, a faster, selective, low-cost separation and determination in a multi-component analysis is accomplished. (73–75).

Santos *et al* 2016, developed a simple and high throughput low pressure chromatography method for monitoring the biodegradation of fluoroquinolones (ofloxacin and ciprofloxacin). A monolithic column was coupled efficiently to a flow system for the simultaneous determination of the two different analytes. The developed method required less expensive instrumentation, short time of analysis and low consumption of solvents and production of effluent, when compared with conventional HPLC method (74).

Another format for SPE in flow analysis, is the molecularly imprinted SPE. This technique is based on the use of molecularly imprinted polymers, what results in a more selective sorbent material. This selectivity occurs by the impression of the target analyte (template) in the imprinted polymer, forming recognition sites after analyte removal (60,76). Serrano *et al* 2017 developed a flow injection SPE system for the determination 1-hydroxypyren in human urine. SPE using a molecular imprinted polymer was the authors chosen strategy to perform matrix clean-up and pre-concentration of the target analyte (77).

Biosorbents work as an alternative for the extensively used synthetic sorbents and can be obtained from plants and microorganisms. The use of biosorbents are mainly explored in the analysis of metal ions. As an example for the use of this particularly sorbent material, filamentous fungi loaded on TiO_2 nanoparticles was successfully used for the separation and pre-concentration of lead in tap and seawater (60,78).

Magnetic materials have also been used as sorbent material in SPE. The use of this magnetic sorbent is based on its dispersion on the sample and separation of the sorbent material by means of an external magnetic field after extraction process. The following steps of the SPE procedure (washing and elution) are based on the same concepts, using the magnetic characteristics of this sorbent (30,60,79). Frizzarin *et al* 2016 proposed a flow-based methodology using magnetic porous carbon for the determination of anionic surfactants in waters (80). González *et al* 2017 used the same magnetic sorbent in a flow-based mode for the determination of estrogens in wastewater (81).

Table 1.2. Analytical features of flow-based analytical methodologies with in-line sample extraction. Data presented in descending chronological order until the review presented by Rocha *et al* 2018 (60) (search made on ISI Web of Knowledge – Web of Science January 24th 2020).

Flow System	Analyte	Sorbent material	Sample	Sample Volume	Sample throughput (h^{-1})	LOD	Reference
SIA	Hydrazine	Oasis HLB	Pharmaceuticals	200 µL	12	0.9 µg L^{-1}	(82)
LOV	Flavonoids	C18 resin	Citrus juices	8 mL	-	0.1 µg mL^{-1}	(83)
SIC	Phenolic acids	DSC - SAX	-	200 µL	-	0.0075 – 0.03* mg L^{-1}	(84)
SIA	Uranium (VI)	3D printed device coated with TEVA resin	Water	0.1 – 9 mL	-	0.5 µg L^{-1}	(85)
FIA	Cu (II)	IIP-HEMA-BSA	Milk	20.0 mL	20	1.1 µg L^{-1}	(86)
FIA	Tl	PTFE	Water/ urine	12.4 mL	40	1.93 µg L^{-1}	(87)
FI	Pb	MNPs coated with ionic liquid	Drinking water	14 mL	-	4 µg L^{-1}	(88)
FIA	Ti	NTA	Seawater	25 mL	8	0.10 nmol L^{-1}	(89)
SIA	Biogenic amines	Chelex 100	Water	1.0 mL	10	1.4 µmol L^{-1}	(65)
SIA	lovastatin	MIP	Dietary supplements	0.25 mL	8	0.150 µg mL^{-1}	(90)

FI	Cr(III)/Cr(VI)	PTFE	Water	5 mL	30	0.26/0.30 μg L^{-1}	(91)
SIA	Zn/Cu	Chelex 100	Soil leachates and water	413 μL	3	1.4/3.0 μg L^{-1}	(64)
FIA	Sr	MSPE**	Water	1 mL	13	0.59 μg L^{-1}	(92)
FIA	As/Sb/Hg	DTPH-MNPs	Water	10 mL	16	0.25/0.003/0.22 μg L^{-1}	(93)
FIA	naproxen	PIM (Aliquat 336)	Urine	10 μL	-	2 μmol L^{-1}	(44)
SIA	Sr/Ni	Sr resin extractant Ni resin extractant	Water	3 – 10 mL	5 - 9	0.25/3.56 μg L^{-1}	(94)
Flow-based	F	LDH	Water	42 mL	-	15 μg L^{-1}	(95)

SIA – sequential injection analysis; Oasis HLB – Hydrophilic-Lipophilic based material, universal polymeric reversed phase sorbent (Waters); SIC – sequential injection chromatography; DSC – SAX. strong anion-exchanger resin (Discovery®); FIA – flow injection analysis; IIP-HEMA-BSA – copper-imprinted poly(allylthiorrea) modified with 2-hydroxyethyl methacrylate and bovine serum; PTFE – poly-tetrafluoroethylene; MNPs – magnetic nanoparticles; NTA – nitrilotriacetic acid resin; MIP – molecularly imprinted polymer: FI – flow injection; MSPE – magnetic solid phase extraction; DTPH-MNPs – magnetic nanoparticles functionalized with 1,5-bis(di-2-pyridil) methylene thiocarbohydrazide; Sr resin extractant – composed of 4,4'(5')-di-t-butylcyclohexane 18-crown-6 (crown ether chromatographic resin); Ni resin extractant – composed of dimethylglyoxime polymethacrylate (DMG); LDH – layered double hydroxide.
*different LODs correspond to different phenolic acids
**Micro-magnetic silica-based particles chemically immobilizing diethyl sulfone functional groups

Mrecently, PIMs have also been successfully used as sorbent material in an automatic in-line sample extraction procedure. Vera *et al* 2019 described for the first time the use of a PIM for the extraction and separation of trace levels of arsenate in drinking water, in a flow system (43). Yaftian *et al* 2018 developed also a flow injection analysis system using a PIM for the extraction and determination of vanadium. The system was efficiently applied to water and dietary supplements (46).

The use of PIMs coupled to flow systems is a very recent application of liquid membranes, for the in-line sample extraction; however, due its features and promising high analyte application more research remains to be done.

As previously referred, the determination of chemical species in environmental samples are addressed with a variety of challenges, and the most frequent are the complexity of the matrix and the trace content of the analyte. To overcome these challenges, SPE strategies showed to offer many attractive features. These features can be even more advantageous when SPE process is coupled to a flow-based system.

1.5. Objectives

The main objective of this thesis was to develop miniaturized and automatic analytical tools for environmental monitoring based on flow analysis methods.

The idea was to design methods with no need for off-line treatments. Therefore, when required, in-line sample extraction would be included aiming also the automation and miniaturization of the sample treatment. Extraction process resorting to different sorbent material could serve various proposes, such as the elimination of sample matrix and/or enrichment of the target analyte.

Furthermore, the development of the flow-based techniques resorting to low toxicity reagents was a priority. When the use of low toxicity reagents was not accomplished, the idea was to minimize the quantity of reagents needed per analysis. The overall process for the development of new analytical methods was carried aiming to accomplish some of the Green Chemistry principles, minimizing the impact of the chemistry into the environment.

1.6. Structure of the thesis

This thesis was organized in seven chapters.

In chapter 1, a general introduction about environmental analysis and related challenges, is presented. The main concepts of flow-based methodologies, in-line sample treatment and its contribution to environmental analysis, is also discussed.

In chapter 2, a brief presentation of the general material and methods used throughout the experimental work, is presented. However, material and methods specificities of the developed analytical methodologies are referred in the respective chapter.

In the subsequent four chapters (from chapter 3 to chapter 6), a detailed description of the developed flow-based systems for environmental analysis is presented. In each chapter, the results, their discussion and respective conclusions of the developed method, is also described. Chapters 3, 4 and 5 are devoted to the development of analytical tools for water analysis and, in chapter 6, to plant analysis.

The developed work of this thesis that is published in international scientific periodicals with referees is identified in each chapter. The information presented in this thesis corresponds to the published information. However, an option was made to present it in the format of a thesis.

In chapter 3, a description of the work entitled “Use of a polymer inclusion membrane and a chelating resin as sorbents for the flow-based determination of copper and zinc in water and soil leachates” is presented. This work is now in preparation for publication.

In chapter 4, a description of the work entitled “Greener and wide applicability range flow-based spectrophotometric method for iron determination in fresh and marine water” is presented. This work is in the submission process for publication.

In chapter 5, a work already published is presented: A sequential injection fluorimetric methodology with in-line solid phase extraction for biogenic amines screening in water. This work was published at the International Journal of Environmental Analytical Chemistry, volume 99, 2019 (270-281).

In chapter 6, a published work is presented: A solid phase extraction flow injection spectrophotometric method for the zinc determination in plants. This work is published at the Microchemical Journal, volume 130, 2017 (366-370).

The final chapter of this thesis (chapter 7) is devoted for the general conclusions of the developed work during this thesis. Additionally, in this chapter, some possible future work is proposed.

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CHAPTER 2

General Materials and Methods

2.1. Introduction

The general considerations related with reagents and sample preparation are described along this chapter.

In this chapter, it is also described the general characteristics and components of the developed flow-based manifolds.

Additionally, several aspects of optimization procedures and statistical treatment are also described in this chapter.

2.2. Reagents and Solutions

All solutions were prepared with analytical grade chemicals and MilliQ water (resistivity > 18MΩ cm, Millipore, USA).

In chapters 3, 4 and 6, the stock solution for each metal was prepared by dilution of the respective 1000 mg L^{-1} atomic absorption standard solution (Spectrosol, England). Working standards were prepared by dilution of the respective stock solution in 0.01 mol L^{-1} nitric acid solution.

In chapter 5, stock solution of BAs were prepared by dissolution of the correspondent quantity of the solid with water. Working standards were prepared by dilution of the stock solution.

A 0.01 mol L^{-1} nitric acid solution was prepared by dilution of the commercial solution (d = 1.39, 65%, Merck, Germany).

A buffer solution of 0.50 mol L^{-1} boric acid (Aldrich, Germany) was prepared by dissolution of the correspondent quantity of the solid in a solution of 0.2 mol L^{-1} sodium hydroxide (Panreac, USA). The final pH of the buffer solution (depending on the reaction) was adjusted with sodium hydroxide.

When required, a combined glass pH electrode (Crison potentiometer, model 2002, Spain) was used to measure the pH of the solutions.

2.3. Sample Collection and Preparation

Water samples (chapter 3, 4 and 5) were collected and filtered with Acrodisc 25 mm syringe filters 0.45 µm (Pall, USA) and acidified to pH 2 with nitric acid. Samples were kept refrigerated until analysis.

In chapter 3, soil leachate samples were produced in the laboratory. For that purpose, soil samples were collected using an acrylic cylinder. To produce the samples, rain simulations were performed by passing previously collected rain through the soil columns. Both collected rainwater and soil leachates were filtered with Acrodisc 25 mm syringe filters 0.45 µm (Pall, USA) and the soil leachates acidified to pH 2 with nitric acid. Characteristics of these soil leachates samples are detailed in chapter 3.

Plant samples, in chapter 6, were sampled and cleaned with flowing tap water, deionized water and finally oven dried until constant weight. Microwave assisted digestion was performed in the dried plan samples as follows: two hundred milligrams were mixed with 5 mL of 65% nitric acid in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. The resulting clear solutions were transferred to 25.00 mL volumetric flasks and the volume made up with ultrapure water.

All the sample solutions were diluted in a multistep approach in order to fit their composition to the range of the established calibration plot.

2.4. Flow-Based System Components

2.4.1. In-Line Extraction Columns

Laboratory made packed columns (Fig. 2.1) were prepared for the in-line solid phase extraction described in chapters 3, 4, 5 and 6.

The column was made of Tygon tube (Gilson, France), and the respective resin packed inside the tubing. To prevent the packed column to get out from the tubing, two pieces of dishwashing foam were placed in each extremity of the column. The column was subsequently coupled to the flow-based system. The resins were chosen according to the correspondent targeted extraction and their characteristics are detailed in each chapter.

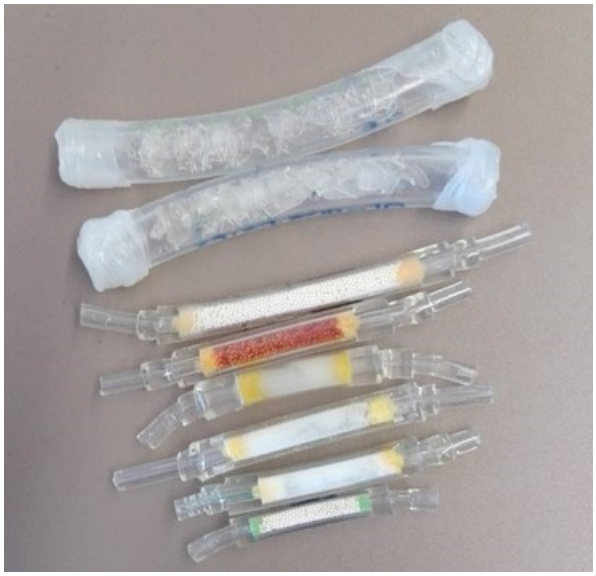

Fig. 2.1. Laboratory-made in-line extraction columns

2.4.2. Propulsion Devices

The propulsion devices used during this work are depicted on Fig. 2.2. These devices provided the movement, aspiration and propelling of the solutions inside the system tubing.

A multisyringe pump (Crison, Spain) was the device used in chapters 3, 4 and 5. In chapter 6, a Minipuls 3 peristaltic pump (Gilson, France) was used as propulsion device.

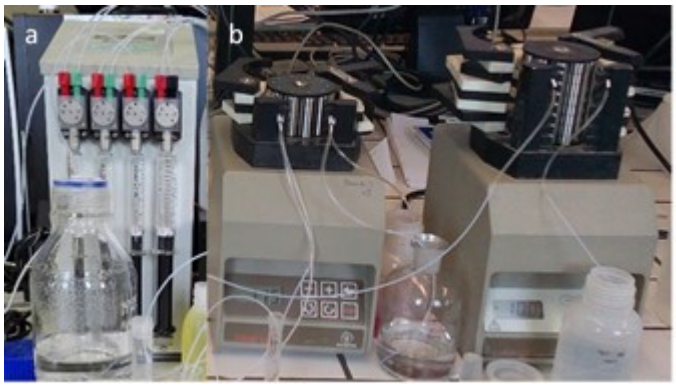

Fig 2.2. Photographs of the propulsion devices: a – Crison multisyringe pump; b – Minipuls 3 peristaltic pump.

2.4.3. Valves

The development of the different methodologies was conducted resorting to different flow-based strategies. To accomplish the individual proposed analytical strategy, different valves were used (Fig. 2.3).

In chapters 3 and 5, a ten-port electrically actuated selection valve was used (Valco VICI cheminert C25-3180D 06B – 0699C, USA). In chapter 4, the selection valve was connected to an injection valve (Valco VICI Cheminert 60736-E45 230, USA). Both the selection and the injection valve were controlled by an AutoAnalysis Station 5.0 computer software (Sciware, Spain).

In chapter 6, a laboratory-made injector commutator was used so that it could comprise an injection volume (loop) and an extra loop to perform the SPE step of the method.

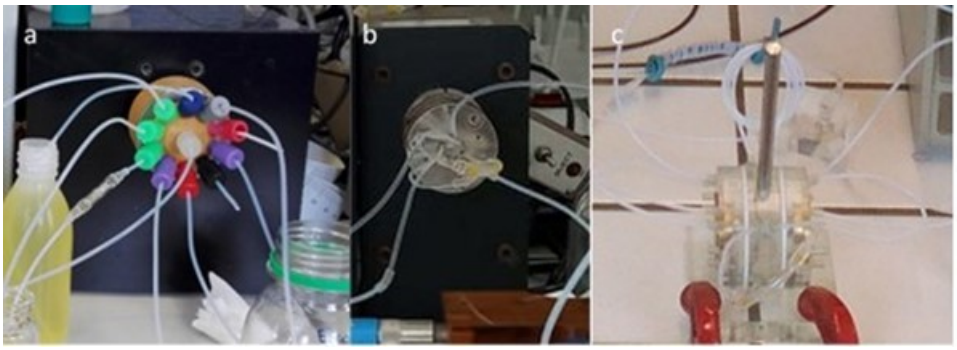

Fig 2.3. Valves: a – ten-port selection valve; b – eight-port injection valve; c – injector commutator.

2.4.4. Detection Systems

The detection system used in chapter 3 and 4 comprised an Ocean Optics (USA) USB 4000 charged coupled device (CCD) detector equipped with a pair of 600 mm optic cable and a Mikropack DH-2000_BAL deuterium halogen light source and an Ultem® flow cell (SMA-Z-50) with 50 mm optical path. The analytical signal was recorded by an OceanView software.

As detection system, in chapter 5, a fluorescence spectrophotometer (LS 55, Perkin Elmer, USA), equipped with a flow cell made of quartz for fluorescence measurement was used. The analytical signal was recorded by BioLight Studio Software version 1.03.01.

In chapter 6, the detection system consisted in an especially designed multi-reflection flow cell, equipped with a red-light emitting diode as a light source connected to a 12 V power supply regulated to a 5 V using a multimeter. The analytical signal was recorded by a Kipp and Zonen (Delft, Holand) BD chart recorder.

2.4.5. Tubes, Connectors and Other Devices

All the tubing that connected the components of the flow-based systems were made of 0.8 mm i.d. PTFE from Ominifit (UK).

In chapter 6, the Minipuls 3 peristaltic pump was equipped with Tygon pumping tubes (Gilson, France). The remaining tubes that connected all the components were of 0.8 mm i.d. PTFE from Ominifit (UK).

2.5. Study and Characterisation of the Method

All the parameters were optimized in order to reach the targeted dynamic working range, accomplish the highest possible sensitivity and lowest interception point on the calibration curve. Additionally, the optimization process was carried out using a univariate process, in order to minimize the reagent consumption and maximize the determination rate.

Calibration curves were established by injecting working standard solutions into de developed system and the corresponding signal registered: absorbance (chapter 3 and 4); fluorescence intensity (chapter 5); and peak height (chapter 6). The relationship between signal and concentration was linear in all the developed methodologies.

Afterwards, the developed flow-based systems were characterized as follows: limits of detection and quantification, application range, determination rate, in-line extraction, reagents consumption and applicability of the method to environmental samples.

The limits of detection and quantification were calculated according to IUPAC recommendations, corresponding to the concentration of the sum of three and ten times, respectively, the standard deviation to the mean value of ten consecutive blank signals.

The determination rate was calculated for one cycle and comprised a three-replica analysis of a sample.

The reagents consumption was calculated per cycle, composed by three replicas of a sample.

The repeatability of the developed methods was evaluated in terms of relative standard deviation (RSD), expressed in percentage. This value was calculated from consecutive determinations of the same standard solution. The repeatability was also evaluated by performing calibration curves in the same day and different days (inter and intraday repeatability of the method).

2.6. Accuracy Assessment

For accuracy assessment, in chapters 3 and 4, the results were compared with those obtained by ICP-OES (Perkin Elmer Optima 7000 dv, USA).

For comparison purposes, in chapter 6, the determination of zinc was carried out using the atomic absorption method (equipment: Solaar 969 AA Spectrometer, Unicam, UK) as reference procedure.

The results obtained with the developed methods were compared with those obtained with reference procedure by establishing a linear relationship between the two set of results with a 95% confidence interval.

Additionally, to evaluate also the accuracy of the developed methodologies, certified reference materials were analysed (chapters 3, 4 and 6). The analysis of the certified reference material was performed with the developed flow-based system and the results were compared with the certified values.

CHAPTER 3

Use of a Polymer Inclusion Membrane and a Chelating Resin for the Flow-Based Sequential Determination of Copper(II) and Zinc(II) in Natural Waters and Soil Leachates

Use of a Polymer Inclusion Membrane and a Chelating Resin for the Flow-Based Sequential Determination of Copper(II) and Zinc(II) in Natural Waters and Soil Leachates

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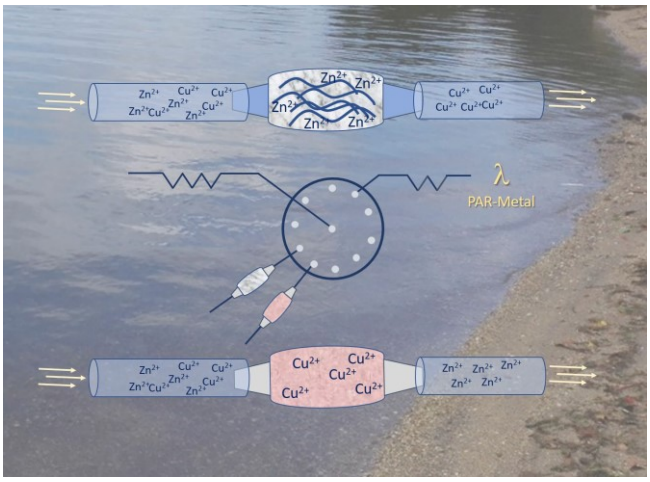
In submission process

Graphical Abstract



Use of a Polymer Inclusion Membrane and a Chelating Resin for the Flow-Based Sequential Determination of Copper(II) and Zinc(II) in Natural Waters and Soil Leachates

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Abstract

A biparametric sequential injection method for the determination of copper(II) and zinc(II) when present together in aqueous samples was developed. This was achieved by using a non-specific colorimetric reagent (4-(2-pyridylazo)resorcinol – PAR) together with two ion-exchange polymeric materials to discriminate between the two metal ions. A polymer inclusion membrane (PIM) and a chelating resin (Chelex 100) were the chosen materials to retain zinc(II) and copper(II), respectively. The influence of the flow system parameters, such as composition of the reagent solutions, flow rates and standard/sample volume, on the method sensitivity were studied. The interference of several common metal ions was assessed, and no significant interferences were observed (< 10% signal deviation). The limits of detection were 3.1 and 5.6 µg L-1, for copper(II) and zinc(II), respectively; the dynamic working range was from 10 to 40 µg L-1 for both analytes. The newly developed SIA system was applied to natural waters and soil leachates, and the results were in agreement with those obtained with the reference procedure.

Keywords: bi-parametric method, sequential injection analysis, micronutrients, polymer inclusion membrane, Chelex 100.

3.1. Introduction

Sample preparation is considered an essential part of the analytical process. Some of the most commonly used sample pre-treatment methods are extraction techniques, namely solid-phase extraction (SPE), liquid-liquid extraction (LLE), and more recently, membrane-based extraction techniques, among others (1).

The basic principles of both SPE and LLE can involve adsorption, partition or ion-exchange of solutes between the two different phases. In SPE this occurs between a liquid phase (i.e., aqueous sample) and a solid phase (e.g., sorbent material) while in LLE this occurs between two immiscible liquid phases (2–5). In recent years, these techniques have been evolving towards their miniaturization (i.e., solid-phase/liquid-phase microextraction), and different types of membranes have also been used as a support for the microextraction (1).

Separation techniques using solid resins or liquid membrane-based materials offer: good selectivity; ease of operation; low use (or no use at all) of organic solvents; low cost; fast rates of analyte extraction; and the possibility of being reused (2,3,6). These factors can be even more advantageous when these techniques are conducted in an on-line fashion as part of flow analysis methods. Therefore, these on-line separation techniques have gained high interest in the last decade (3,7).

In the present study, the strategy was to explore different polymeric materials to selectively separate and determine both copper(II) and zinc(II). A sequential injection system for the direct determination of these metal ions using the same colour reagent was proposed. The chromogenic reagent selected was 4-(2-pyridylazo)resorcinol (PAR). PAR is a very commonly used chromogenic chelator for the spectrophotometric determination of various metal ions (8). This reagent was selected because it is water soluble and does not need the use of organic solvents in the preparation of its solutions, unlike some chromogenic chelating agents used for metals quantification (9).

According to a previous work (9), Chelex 100 efficiently retains copper at pH of 2. Thus, Chelex 100 was the chosen resin to avoid copper(II) interference in the zinc(II) determination. Chelex 100 resin is a styrene divinylbenzene copolymer, weakly acidic due to its carboxylic acid groups thus allowing cation exchange. This sorbent material acts as chelating resin to bind metal ions and its selectivity is closely related to the pH of the chelating process. Advantage was taken from this property to retain the target analyte.

For the selective detection of zinc(II), a polymer inclusion membrane (PIM) was used (6,10). PIMs are considered as a type of liquid membranes which have attracted considerable attention in recent years (11). These membranes are usually fabricated by casting a solution containing an extractant, a base polymer, a plasticizer (if necessary), and a volatile solvent which dissolves all

PIM components. After casting and evaporation of the volatile solvent a thin, flexible and stable polymeric film is formed. PIMs have the ability to selectively separate a species of interest depending on the extractant used (6). Kolev et al. reported a PVC-based PIM with di-(2-ethylhexyl)phosphoric acid (D2EHPA) as the extractant, capable of extracting Zn(II) selectively (12). This PIM composition was thus chosen for the zinc(II) extraction from the sample matrix, thus enabling the copper(II) determination.

The target analytes, copper(II) and zinc(II), are important micronutrients essential for the proper functioning of living organisms, however, in high concentrations both become toxic. The presence of these metal ions in ground and surface water are a direct result of using soil fertilizers or of other anthropogenic activities influencing water quality. In this scenario, it is important to monitor these metal ions in natural waters, as they act as pollution indicators. Some research has already been done for the determination of these two metal ions in water samples based on flow systems (9,13–18). However, to accomplish both determinations with a single sequential injection analysis (SIA) system, a mathematical discrimination treatment of the experimental results had to be used (9,14–18), except for the system developed by Santos et al (13). A different approach is here proposed which involves the use of two on-line columns containing different ion-exchange polymeric materials for the biparametric determination of copper(II) and zinc(II) in natural waters and soil leachates.

3.2. Experimental

3.2.1. Reagents and Solutions

All solutions were prepared with analytical grade chemicals and MilliQ water (resistivity > 18 MΩ cm, Millipore, USA).

A stock solution of 50.0 mg L^{-1} of copper(II) and zinc(II) were prepared by dilution of the respective 1000 mg L^{-1} atomic absorption standard solutions (Spectrosol, England). An intermediate solution of 500 µg L^{-1} of each metal solution was prepared by dilution of a 50.0 mg L^{-1} stock solution. Working standards, in the range 10-40 µg L^{-1} in 0.01 mol L^{-1} of nitric acid were prepared weekly by dilution of a 500 µg L^{-1} intermediate solution with a 0.01 mol L^{-1} nitric acid solution.

A 0.01 mol L^{-1} nitric acid solution was prepared by dilution of the commercial concentrated nitric acid solution (d = 1.39; 65%, Merck; Germany).

A buffer solution of 0.50 mol L-1 boric acid was prepared by dissolution of the solid (H3BO3, Aldrich, Germany) in a solution of 0.2 mol L^{-1} NaOH (Panreac, USA), with the final pH adjusted to 11.0 with a sodium hydroxide solution.

A 2 mmol L^{-1} stock solution of PAR ($C_{11}H_8N_3NaO_2 \cdot H_2O$, Sigma-Aldrich, Germany) was prepared by dissolving the corresponding quantity of the monosodium salt hydrate in water. A PAR reagent solution of 25 µmol L^{-1} was prepared weekly by dilution of the stock solution with MilliQ water.

3.2.2. Preparation of the PIM Column

PIMs were produced by dissolving a mixture of 8.25 g of PVC and 6.75 g of D2EHPA in 165 mL of tetrahydrofuran (THF). Approximately 2.75 mL of this solution was cast into a glass ring with a 76 mm diameter position of a flat glass plate. All rings were covered with filter paper, a glass plate and a foil tray to control the evaporation of THF which was completed within a period of 48-72 h. The resulting PIM composition was 45 wt % D2EHPA and 55 wt % PVC.

PIMs were subsequently cut into strips of approximately 2 mm in width. A laboratory made column (5.5 cm length of Versilon 2001 tubing with 4.8 mm i.d.) was packed with the PIM stripes (approximately 100 mg) between two female luer Tefzel connectors (P-624; Thermo Scientific, USA). The column was connected to one of the ports of the selection valve of the SIA utilised in this study and subsequently used for zinc(II) retention.

3.2.3. Preparation of the Chelex 100 Column

A laboratory made column with 25 mm in length of Tygon tubing (Gilson, France) with 1.85 mm i.d. and 67 µL inner volume was used to pack the chelating resin. Approximately 75 mg of Chelex 100 (mesh 200-400, Bio-Rad, USA), previously suspended in water, was introduced into the column between two pieces of dishwashing sponge. The column was connected to one of the ports of the SIA selection valve and used for copper retention.

3.2.4. Apparatus

Solutions were propelled in the experimental SIA system (Fig. 3.1) by a syringe pump with a 5 mL barrel (Crison, Spain). The pump was connected to the central channel of a ten-port electrically actuated selection valve (Valco VICI Cheminert C25-3180D 06B – 0699C, USA) with a polytetrafluoroethylene (PTFE) tubing. PTFE tubing (0.8 mm i.d., Omnifit, UK) connected all the components of the SIA system. The syringe pump and the selection valve were controlled by AutoAnalysis Station 5.0 computer software (Sciware, Spain). As detection system consisted of an Ocean Optics (USA) USB 4000 charged coupled device detector (CCD) equipped with a pair of 600 mm optic cables, a Mikropack DH-2000-BAL deuterium halogen light source and an Ultem® flow cell (SMA-Z-50 cell) with 50 mm optical path (130 µL inner volume).

3.2.5. Flow Manifold and Procedure

The sequence of steps for the determination of copper(II) and zinc(II) is shown in Table 3.1. It was divided in two parts, one corresponding to the Zn(II) determination using solid-phase extraction for the removal of Cu(II), and the other to the Cu(II) determination using a PIM to retain Zn(II). For the Zn(II) determination (steps A - D), reagent (Port 1, Fig. 3.1), buffer (Port 2) and sample/standard (Port 5) were sequentially aspirated into the holding coil (HC). The sample/standard was aspirated through the Chelex 100 column (C2) via Port 5. Then the staked zones were propelled to the flow cell (FC) where the absorbance was continuously monitored at 490 nm corresponding to the absorption maximum of the Zn(II)-PAR coloured complex. For the Cu(II) determination (steps E – M, Table 3.1), sample/standard was aspirated to the holding coil via Port 3, and then propelled through the PIM column (C1) to eliminate possible Zn(II) interference (steps E and F). After passing through the PIM column, the flow was reversed twice to promote Zn(II) retention. After this procedure, reagent (Port 1), buffer (Port 2) and the Zn(II) free sample/standard (Port 1) were aspirated into the holding coil (HC). Then these three zones were propelled to the flow cell (FC) for absorbance measurement of the Cu(II)-PAR coloured complex at 490 nm.

At the end of the cycle, the PIM column was washed and reconditioned with 0.5 mol L-1 nitric acid (Port R4) and ultrapure water (carrier solution), sequentially.

Each absorbance value was calculated as the difference between the absorbance at 490 nm (wavelength of maximum absorption) and that at 800 nm; this subtraction aimed at minimizing the schlieren effect (19).

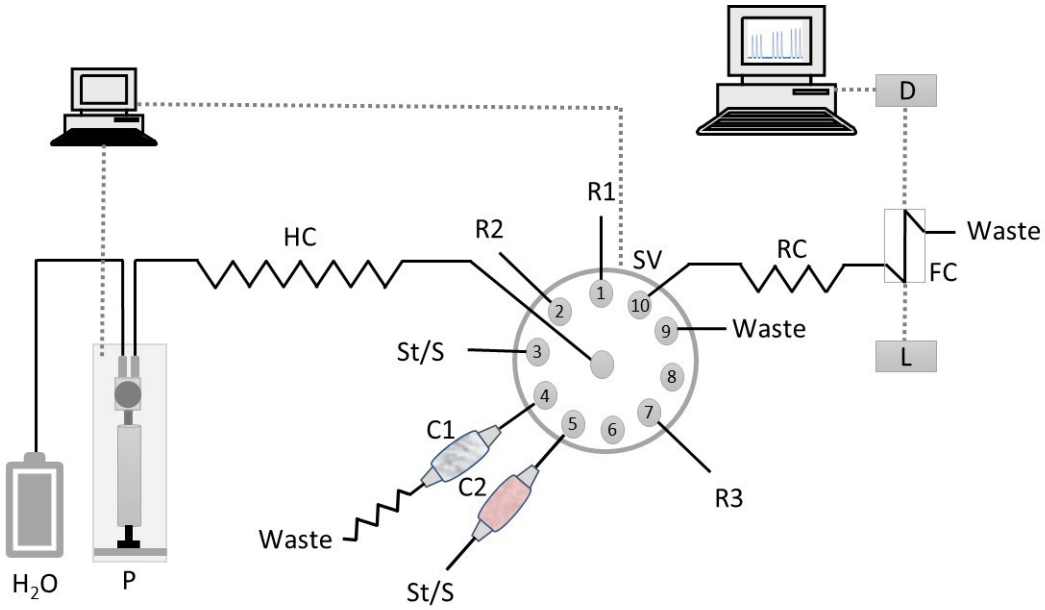

Fig 3.1. Flow manifold for Cu(II) and Zn(II) determination in waters and soil leachates. St/S – standard solution or sample; R1 – PAR reagent (25 μmol L^{-1}); R2 – boric acid buffer solution (pH 11); R3 – nitric acid solution (0.5 mol L^{-1}); C1 – PIM column; C2 – Chelex 100 resin column; P – syringe pump; SV – selection valve; HC – holding coil (300 cm); RC – reaction coil (10 cm); D – CCD detector; L – light source; FC – Z flow cell (50 mm path length); W – waste.

Table 3.1. Experimental protocol sequence for the copper(II) and zinc(II) determination.

Step	Selection valve position	Volume (mL)	Flow-rate (mL min^{-1})	Description
Preliminary steps before starting consecutive cycles		5.000	-	Syringe reset position – syringe fill with carrier
		1.000	5.000	Propel carrier (water) to waste
A	1	0.250	3.529	Aspirate PAR solution
B	2	0.020	3.529	Aspirate boric acid buffer solution
C	5	0.550	2.000	Aspirate standard/sample trough the Chelex 100 column to eliminate copper(II) interference
D	10	2.100	3.529	Propel through the spectrometer for Zn(II) quantification
				Fill the syringe with carrier
E	3	0.550	2.000	Aspirate standard/sample
F	4	0.600	2.000	Propel through the PIM column to eliminate Zn(II) interference by retaining Zn(II)
G	4	0.250	2.000	Aspirate standard/sample trough the column to promote retention of Zn(II)
H	4	0.250	2.000	Propel standard/sample trough the PIM column to promote retention of Zn(II)
I	9	0.250	3.529	Dispense to waste the left residues in the holding coil
J	1	0.250	3.529	Aspirate PAR solution
K	2	0.020	3.529	Aspirate boric acid buffer solution
L	4	0.550	2.000	Aspirate Zn(II) free standard sample solution
M	10	2.100	3.529	Propel through the spectrometer flow cell for Cu(II) quantification
				Fill the syringe with carrier
R	7	0.500	5.000	Aspirate HNO_3 solution
S	4	1.500	5.000	Propel through the PIM column – cleaning step

3.2.6. Sample Collection and Preparation

3.2.6.1. Water Samples

River water samples (S1 - S9) from various locations in the Porto area were collected, filtered (Acrodisc 25 mm syringe filters 0.45 μm, Pall, USA) and acidified to pH 2 with nitric acid, according to the reference procedure (20). Samples were kept refrigerated at 4 °C until analysis.

When copper(II) concentration was above 40 μg L^{-1}, the water samples were diluted in order to fit the respective linear working range. In some samples the zinc(II) concentration was below the detection limit and in those cases the samples were spiked with zinc(II) (samples S1 - S3).

3.2.6.2. Soil Leachates Samples

The soil samples were collected in the northwest of Portugal using an acrylic cylinder that was pushed into the ground to collect a superficial soil core (about 20 cm depth). Tow soil cores were collected.

To produce soil leachates, rain simulations were performed by passing 50 mL of previously collected rain-water (pH ≈ 6.6; conductivity ≈ 8.4 μS cm^{-1}) through each soil core. Both the collected rain-water and the obtained leachates were filtered (Acrodisc 25 mm syringe filters 0.45 μm, Pall, USA) and acidified to pH 2 with nitric acid.

Several simulations of rain were performed (for 5 consecutive days) and the soil leachates were collected. Samples were kept refrigerated until analysis. The soil leachates samples (S9 – S14) were diluted when zinc(II) concentrations were above 40 μg L^{-1}, in order to fit in the linear range of the zinc(II) calibration.

3.2.7. Reference Procedure

For validation purposes, the determinations of Cu(II) and Zn(II) in soil leachates and natural waters were carried out by inductively coupled plasma – optical emission spectrometry, ICP-OES (Perkin Elmer Optima 7000 dv, USA), and the results were compared with those obtained with the newly developed SIA method.

Additionally, the SIA system developed for the quantification of Cu(II) and Zn(II) was applied to the analysis of a certified water sample, ERM-CA011 (hard drinking water, LGC, UK). The certified water sample was diluted in a multi-step fashion so that its concentration would fit within the linear range of the corresponding calibration curves.

3.3. Results and Discussion

3.3.1. Preliminary Studies

Two different colorimetric reagents for metal ions, namely PAR and 1-(2-pyridylazo)-2-naphthol (PAN) were initially studied via wet/bench chemistry, in order to determine which one could be more advantageous for the spectrophotometric quantification of both Cu(II) and Zn(II). Using the same conditions for both reagents (1 mL of 0.1 mmol L-1 of reagent solution, 1 mL of 0.5 μg L-1 of metal solution and 1 mL of 0.6 mmol L-1 of carbonate buffer solution pH 10), a spectrum for each metal-reagent complex was obtained (ESI Fig. 1). By assessing the wavelength of maximum absorption of both metal complexes, it was observed that the signal was higher for the metal-PAR complexes, therefore PAR was chosen as the metal indicator to develop the SIA method. Additionally, PAR is water-soluble which makes it easier to use.

3.3.2. Development of the SIA System

The development of the SIA system involved a number of optimization studies to assess the influence of some chemical and physical variables on the system's analytical performance. As both complexes of PAR, with copper(II) or zinc(II), showed similar sensitivity under the same conditions (concentration of PAR, metal and buffer), copper(II) was chosen as the model analyte to conduct the optimization of the colorimetric reaction in the SIA system. The parameters assessed were: the volumes of the PAR, sample and buffer solutions; the pH of the buffer solution; the reaction coil length; and the concentration of the PAR solution. These parameters were optimized in order to attain the highest sensitivity (calibration curve slope), the lowest reagent consumption and most effective sampling rate.

3.3.2.1. Study of the Reaction Conditions

The first study to be carried out was the PAR, sample and buffer solution volumes. Different volumes of PAR (250-350 µL), buffer (10-30 µL) and sample/standard (300-650 µL) solutions were studied to evaluate their impact on the calibration curves parameters (Fig. 3.2). The increase in the reagent volume resulted in an increase of the intercept (≈ 15%) but no increase of sensitivity (calibration curve slope); so, the lowest volume was selected to ensure minimal reagent consumption. The sensitivity increased with increasing the buffer volume up to 20 µL, with almost no variation in the intercept (< 5% variation). A similar behavior was observed for the sample volume, as the sensitivity increased with the increase in the sample volume up to 550 µL, but in this case the intercept decreased. For higher sample volumes, the slope decreased slightly (≈ 10% variation) and the intercept increased slightly (≈ 10% variation).

The chosen volumes were 250 µL for PAR, 20 µL for the buffer, and 550 µL for the sample/standard solutions, as this combination displayed the highest slope and lowest intercept values for the calibration curve, indicating better reaction sensitivity with potentially lower detection limits for both determinations.

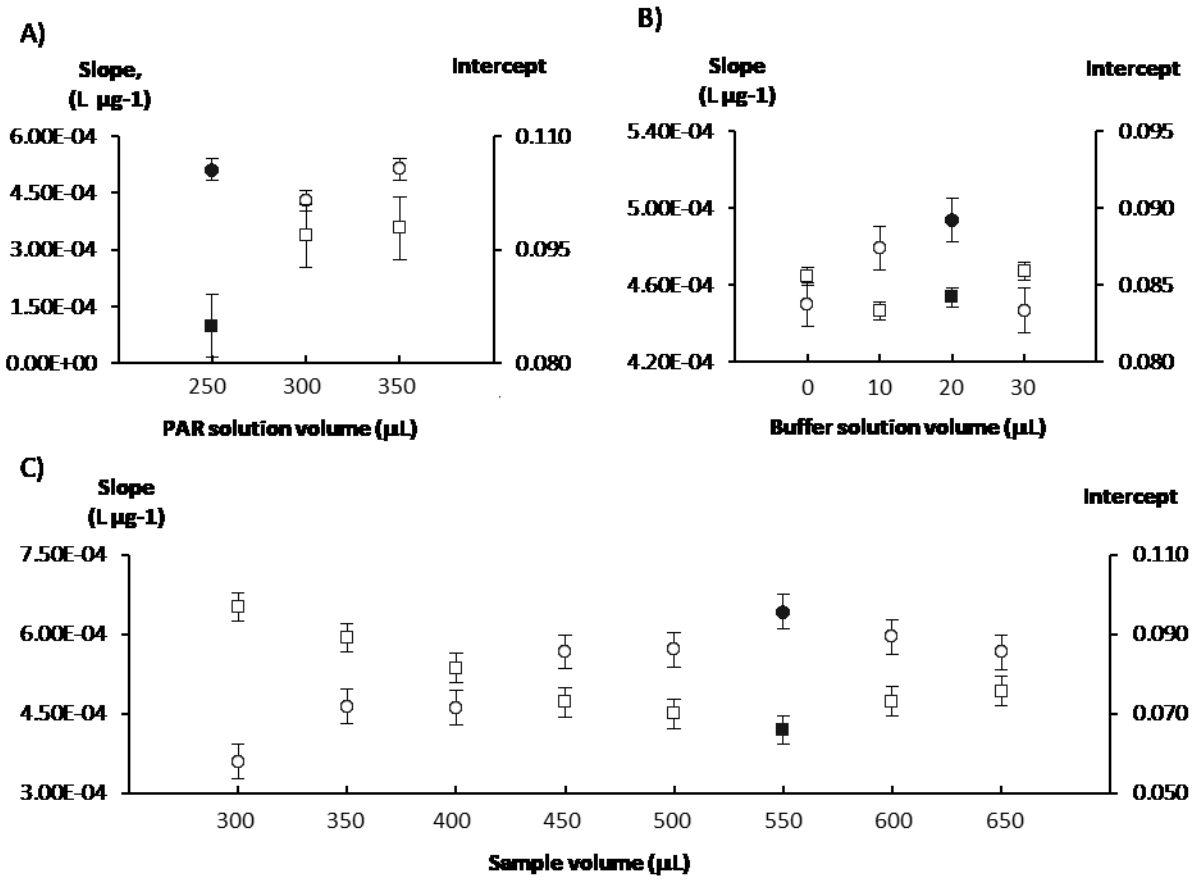

Fig. 3.2. Study of the influence of the reagents (A and B) and sample (C) volumes on sensitivity expressed as the calibration curve slope (circles) and on the calibration curve intercept (squares); the chosen values are represented in black; the error bars represent the standard error.

The reaction PTFE coil length was initially kept to a minimum (10 cm) allowing to physically connect the central port of the selection valve to the flow cell. As there was no significant difference (less than 10%) in the absorbance signal when the coil length was increased to 20 cm, the 10 cm length was used in the remaining experiments.

The influence of the buffer solution composition and pH was studied by comparing the sensitivity obtained when using a boric acid buffer (0.5 mol L^{-1}) or a carbonate buffer (0.6 mol L^{-1}). Both buffer solutions were tested at two different pH values: 10 or 11. No significant differences were observed (< 5% sensitivity) between boric acid and carbonate buffers at pH 10. However, with the use of carbonate buffer, air bubbles were formed inside the tubing of the flow system. When the carbonate buffer with pH 11 was used, poor repeatability of the signal was obtained. Hence, the chosen buffer solution was the boric acid solution at pH 11.

The influence of the PAR reagent concentration was also evaluated and the concentration of 25 μmol L^{-1} was chosen from the tested values (10, 25, 50 and 100 μmol L^{-1}). When the concentration was increased above 25 μmol L^{-1}, no significant variation (< 10%) of the calibration curves slopes was observed; the chosen concentration also produced a lower intercept value.

3.3.2.2. Study of the Retention of Copper(II) and Zinc(II)

Since PAR is sensitive to both copper(II) and zinc(II), a dual-extraction approach was adopted in order to be able to determine both metal ions individually and sequentially. The strategy chosen for the selective copper(II) determination consisted of the use of a PVC-based PIM containing D2EHPA as the extractant to retain zinc(II). Some studies were conducted to maximize the on-line retention of zinc(II) in natural water samples into the PIM and consequently determine copper(II). As the PIM column was linked to one of the peripheral ports of the selection valve, direct aspiration of a standard, containing both copper(II) and zinc(II), through the column was attempted first; however, the retention was not efficient. Alternatively, with the aim to enhance the interaction between the solution and the PIM, the standard was aspirated from another port of the selection valve to the holding coil of the SIA system, and subsequently propelled through the column, and aspirated back to the holding coil. Using this approach, two experiments were conducted. In the first one, the standard was propelled through the PIM column, followed by the sequential aspiration of PAR reagent, buffer solution and Zn(II)-free standard into the holding coil. After flow reversal the stack of solution zones in the holding coil were propelled towards the detector. In the second experiment, a similar procedure was adopted except that the flow was stopped when the standard zone was in the PIM column for 5 s. No significant difference in the maximum absorbance signals was observed between the two experiments, thus to further improve the retention of zinc(II) the procedure involving the propelling and aspirating the standard zone through the PIM column was conducted twice (ESI Fig. 3.2A). This retention procedure was thus the chosen approach for further optimization.

According to Paluch et al (9), copper(II) can be retained in a column packed with Chelex 100 resin at pH of 2. Hence, for the selective zinc(II) determination, this was the strategy chosen to eliminate the interference of copper(II). A packed column was linked to one of the peripheral ports of the selection valve, through which the standard/sample was directly aspirated towards the holding coil, thus retaining any copper(II) present in the original standard/sample (ESI Fig 3.2B).

3.3.3. Interferences Studies

Being a non-specific chromogenic reagent, PAR forms an orange complex with a variety of different metal ions and so, the potential interference from other metal ions was assessed. The ions that can be present in natural water samples and thus interfere with the proposed analytical method are described in Table 3.2. The selected concentrations for each ion corresponded to the maximum concentration that can be expected in environmental waters (20). The obtained absorbance of a standard with and without the possible interfering ion was measured and the interference percentage calculated (Table 3.2).

Table 3.2. Interference study of metal ions ($[M^{n+}]$) commonly present in environmental waters at their maximum expected concentrations ($[M^{n+}]_{max}$) (20). SD – Standard deviation (n=3).

Tested ion	$[M^{n+}]_{max}$ in streams, µg L^{-1}	Tested $[M^{n+}]$, µg L^{-1}	Interference in Cu(II) determination, %	SD	Interference in Zn(II) determination, %	SD
Al^{3+}	400	400	-1.0	1.0	-1.3	0.9
Ca^{2+}	15000	15000	1.0	1.0	-3.6	2.0
Co^{2+}	0.2	10	1.6	0.1	1.5	0.1
Cu^{2+}	< 12	40	-	-	2.5	0.7
Fe^{3+}	700	400	14.9	2.8	8.5	0.3
		200	8.2	1.3	-	-
Mg^{2+}	4000	5000	3.1	2.6	35.8	5.8
		2500	-	-	-4.3	3.0
Mn^{2+}	7	50	4.3	3.0	1.4	0.3
Ni^{2+}	1	50	4.5	1.8	1.2	0.4
Zn^{2+}	20	40	5.1	2.1	-	-

The only interferences above 10% were from iron(III) and magnesium(II); however, values above 400 µg L^{-1} Fe(III) are not usually found in environmental waters; and the tested magnesium concentration was above the expected values. The interference from iron(III) had been previous reported and could be eliminated by precipitation with phosphate prior to the analysis (12).

3.3.4. Features

The features of the newly developed SIA method for the biparametric determination of copper(II) and zinc(II) are summarized in Table 3.3.

Table 3.3. Calibration curves and dynamic concentration ranges for copper(II) and zinc(II), and respective limits of detection (LOD). A – absorbance; SD - standard deviation; M^{2+} – metal ion; RSD – relative standard deviation.

Metal ion	Dynamic range ($\mu g\ L^{-1}$)	Calibration curve[a] $A = slope \pm SD\ [Me^{2+}] + intercept \pm SD$	LOD ($\mu g\ L^{-1}$)	RSD (%)
Copper(II)	10.0 – 40.0	$A = 9.00 \times 10^{-4} \pm 1 \times 10^{-4}\ [Cu^{2+}] + 0.112 \pm 0.007$	3.1	2.0
Zinc(II)	10.0– 40.0	$A = 1.80 \times 10^{-3} \pm 1 \times 10^{-4}\ [Zn^{2+}] + 0.099 \pm 0.003$	5.6	1.3

[a] n = 3

The limits of detection were calculated according to the IUPAC recommendations as the concentration corresponding to the sum of three times the standard deviation to the mean value of ten consecutive blank solution measurements (21,22).

The relative standard deviation (RSD) for Cu(II) and Zn(II) determination was calculated with twelve replicate analysis (four consecutive cycles) of a standard with 20 $\mu g\ L^{-1}$ of each metal ion.

A complete cycle, which includes three replicas for each determination and the washing of the PIM column at the end, has the duration of 10 min. The corresponding PAR, sodium hydroxide and boric acid consumption per cycle is 8.1 µg, 1.4 mg and 5.6 mg, respectively.

3.3.5. Application to Natural Water and Soil Leachate Samples – Validation of the Method

The newly developed SIA system for the determination of copper(II) and zinc(II) was applied to river water samples (S1-S9) and soil leachates (S10-S14). The validation was attained by comparison of the results obtained with the newly developed SIA method with those obtained by the reference procedure (ICP-OES) (Table 3.4).

Table 3.4. Comparison of the results obtained with the newly developed SIA system for copper(II) and zinc(II) determination (three replicates) to those obtained with ICP-OES (two replicates). S1-S9 – river water samples; S10-S14 – soil leachate samples; SD – standard deviation; RD – Relative deviation.

Sample ID	Copper(II)					Zinc(II)				
	SIA		ICP			SIA		ICP		
	$[Cu^{2+}]$ µg/L	SD	$[Cu^{2+}]$ µg/L	SD	RD %	$[Zn^{2+}]$ µg/L	SD	$[Zn^{2+}]$ µg/L	SD	RD %
S1*	14.9	1.2	14.3	0.1	+3.7	22.8	2.4	21.6	0.3	+5.4
S2*	19.5	2.6	20.6	0.3	-5.8	20.1	0.9	19.3	0.3	+3.9
S3*	22.8	1.8	20.8	0.3	+6.4	31.4	3.0	33.5	0.4	-6.5
S4	197	8	183	3	+7.6	<LOD	-	<LOD	-	-
S5	140	8	143	2	-2.3	<LOD	-	<LOD	-	-
S6	107	5	114	2	-6.4	<LOD	-	<LOD	-	-
S7	72.2	4.1	74	3	-2.4	<LOD	-	<LOD	-	-
S8	156	10	143	5	+9.3	<LOD	-	<LOD	-	-
S9	<LOD	-	<LOD	-	-	<LOD	-	<LOD	-	-
S10	<LOD	-	2.08	0.1	-	15.2	2.0	14.9	0.2	+2.0-
S11	<LOD	-	2.71	0.1	-	14.3	1.6	14.6	0.3	-2.0
S12	<LOD	-	<LOD	-	-	340	12	332	3	+2.5-
S13	<LOD	-	<LOD	-	-	32.9	3.5	33.2	0.3	-0.9
S14	<LOD	-	0.58	0.1	-	203	3	193	3	+4.9-

*samples spiked with zinc (II)

A linear relationship was established between the copper(II) and zinc(II) concentrations determined by the newly developed SIA system (C_{SIA} (µg L^{-1})) and the reference procedure (C_{ICP} (µg L^{-1})) (ESI Fig 3.3). The linear regression for the copper(II) determination was C_{SIA} = 1.05 (± 0.10) C_{ICP} – 2.63 (± 10.99), where the values in brackets represent the 95% confidence interval. The linear regression for the zinc(II) determination was C_{SIA} = 1.03(± 0.02) C_{ICP} – 0.46(± 2.53), where the values in brackets represent the 95% confidence interval. These data show that the estimated slope and intercept do not differ statistically from 1 and 0, respectively (23). In addition, the relative deviation between the two sets of results proved that there were no significant

differences between the newly developed SIA method and the reference procedure, RD ≤ 10% (Table 3.4).

The accuracy of the newly developed SIA method was evaluated by analysing a certified reference water sample (ERM CA011 – Hard drinking water - Metals) with 1963 ± 62 and 605 ±17 μg L^{-1} of copper(II) and zinc(II), respectively. The concentration values obtained with the newly developed SIA system were 1789 ± 61 and 609 ± 24 μg L^{-1} for copper(II) and zinc(II), respectively, corresponding to relative deviations of -8.9% and +0.7%. These results indicated that the newly developed SIA system offered acceptable accuracy.

3.4. Conclusions

With the aim of directly and individually quantify copper(II) and zinc(II) with the same colour reagent, two polymeric materials, namely a PIM and Chelex 100, packed in columns, were efficiently used. To the authors' best knowledge, this was the first time that a PIM was used with this objective of on-line retaining and eliminating interferences from a sample. PVC-based PIMs containing D2EHPA proved to be efficient in retaining zinc(II), allowing for the quantification of copper(II). Chelex 100 was the polymeric material used to retain copper(II) at pH 2.0, as has already been reported in previous studies (9). Zinc(II) was not retained at this pH, thus allowing for its quantification to be performed free of copper(II) interference.

Some flow-based methodologies have been previously developed (9,13–18,24,25) for the determination of these two metals in different kinds of samples using various chromogenic reagents (Table 5). Unlike these methods, the newly developed SIA system can determine copper(II) and zinc(II) individually and directly using a single manifold with the use of a single colorimetric reaction (PAR-Metal), thus reducing the time required per analysis while offering similar and in some cases better sensitivity. For example, the Zincon-Metal approach does not allow individual determination (14,16–18).

Table 3.5. Analytical features of flow-based systems developed for copper and zinc spectrophotometric determination in water samples (presented in descending chronological order).

System	Sample	Sample volume (µL)	SPE	Reagent	Sample throughput (h^{-1})	LOD (µg L^{-1})	Ref.
SIA	Natural waters	550	PIM and Chelex 100	PAR	6	Cu - 3.1 Zn- 5.6	This work
SIA	Water and soil leachates	413	Chelex 100	PAN	3	Cu – 3.0 Zn – 1.4	(9)
µSI-LOV	Freshwaters	600	NTA	Dithizone	Cu – 15 Zn - 13	Cu – 0.11 Zn – 2.39	(13)
SIC	Water	90	-	PAR	9	Cu – 13 Zn - 13	(24)
MSFIA	Waters	400	-	Zincon	43	Cu – 0.1 Zn – 2	(14)
SIA	Water samples	150	-	Zincon	36	Cu – 48 Zn - 13	(17)
BIS-FIA	Waters, pharmaceuticals and soils	1000	Sephadex QAE A-25	Zincon	15	Cu – 29 Zn - 40	(16)
FIA	Water and brass		Chelex 100	Zincon	70	Cu – 800 Zn – 350	(18)

SPE – solid phase extraction; LOD – limit of detection; Ref. – Reference; SIA – sequential injection analysis; PIM – polymer inclusion membrane; PAR - 4-(2-pyridylazo)resorcinol; PAN – 1-(2-pyridilazo)-2-naphtol; µSI-LOV – micro sequential injection – lab-on-valve; SIC – sequential injection chromatography; MSFIA – multi-syringe injection analysis; BIS-FIA – bead injection spectrometry – flow injection analysis; FIA – flow injection analysis.

The use of PAR as chromogenic reagent, instead of other reagents used by other authors (e.g., dithizone and PAN) (9,13), displays some advantages: PAR is considered a non-hazardous substance, unlike dithizone that is considered eye and skin irritant according to European regulations (EC) (26); PAR is also a water soluble reagent and so, does not need the use of organic solvents in its preparation. Additionally, the newly developed SIA system presents also the advantage of displaying near real-time results for copper(II) and zinc(II) content in natural waters and soil leachate samples (10 minutes per each sample analysis), involving relatively low-cost and portable equipment.

Acknowledgements

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Electronic Supplementary Information

Use of a polymer inclusion membrane and a chelating resin for the flow-based sequential determination of copper(II) and zinc(II) in natural waters and soil leachates

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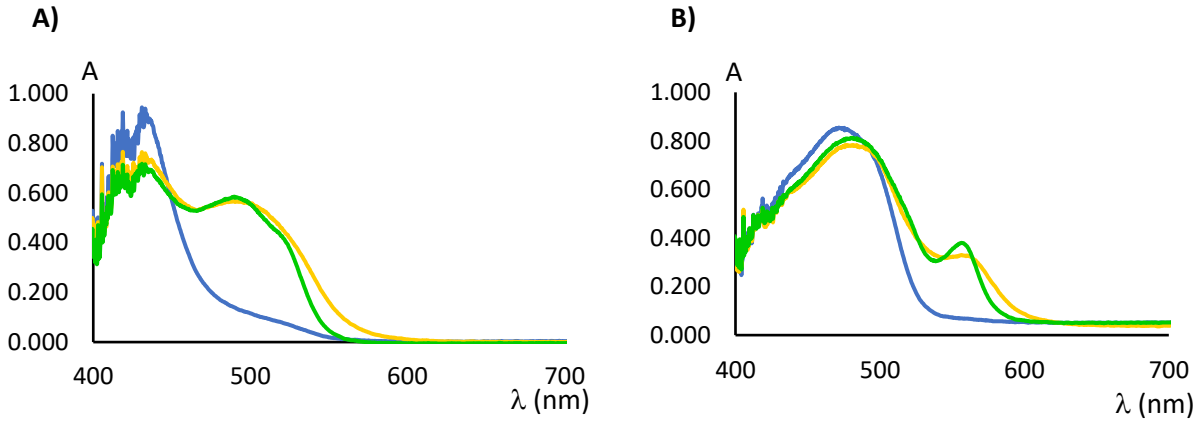

Fig 3.S1. Spectra of the colour metal complexes with PAR (A) and PAN (B); spectra of the blank (reagent in milliQ water) (blue lines), Cu(II)-PAR/PAN complex (yellow lines) and Zn(II)-PAR/PAN complex (green lines); PAR/PAN concentration of 0.1 mmol L^{-1}; metal ion concentration of 0.5 µg L^{-1}; carbonate buffer (0.6 mmol L^{-1}) solution at pH = 10.

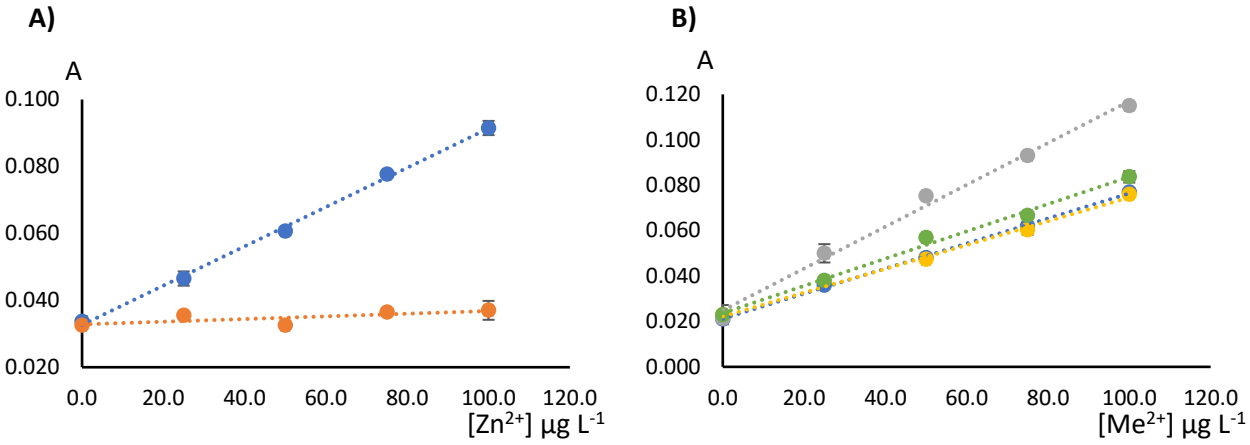

Fig 3.S2. Study of the effect of the PIM column (A) and the Chelex column (B) on the calibration curves of zinc(II) and copper(II); A) direct zinc(II) calibration curve without (blue) and with (orange) using a PIM; B) calibration curve with mixed standards of copper(II) and zinc(II) aspirated through the Chelex column (green) and without going through the Chelex column (grey); calibration curve with zinc(II) standards with (yellow) and without (blue) using a Chelex column.

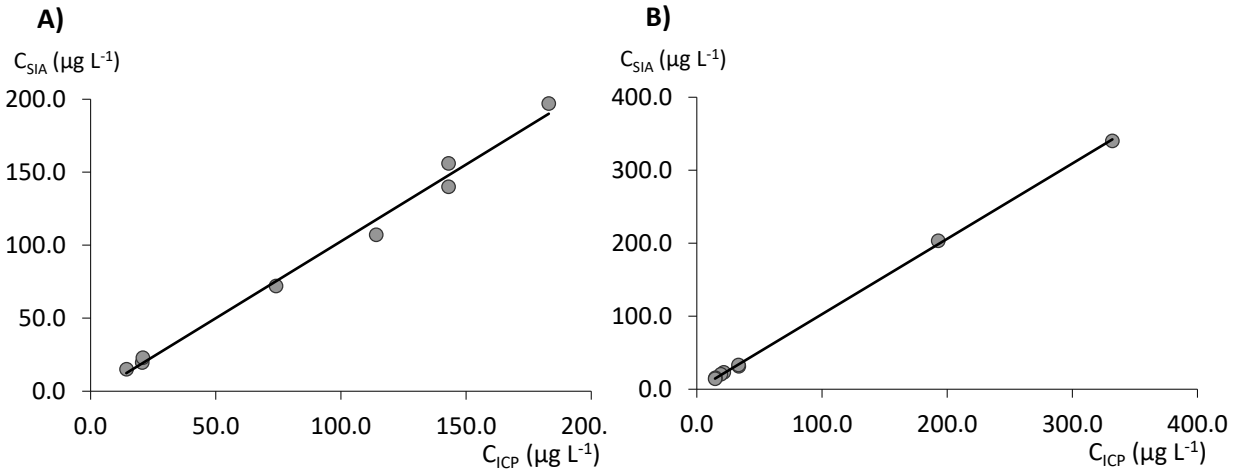

Fig 3.S3. Comparison of the results obtained with the newly developed SIA system and those obtained with a reference method (ICP-OES); A) copper(II) determination; B) zinc(II) determination; the lines represent the linear relationship between the two methods.

CHAPTER 4

Greener and wide applicability range flow-based spectrophotometric method for iron determination in fresh and marine water

Greener and wide applicability range flow-based spectrophotometric method for iron determination in fresh and marine water

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Talanta

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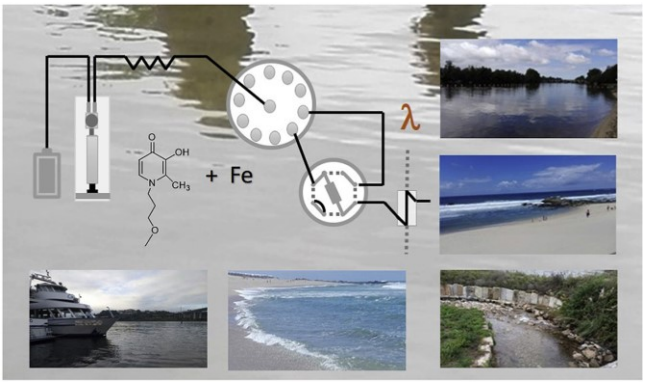
Graphical Abstract



Greener and wide applicability range flow-based spectrophotometric method for iron determination in fresh and marine water

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Abstract

A flow-based method for the spectrophotometric determination of iron in recreational waters, both fresh and marine (variable salinity content), was developed. For that purpose, 3-hydroxy-4-pyrydinone ligand functionalized with an ether function was synthetized and used as chromogenic chelator (1-(3'-methoxypropyl)-2-methyl-3-benzyloxy-4-(1H)pyridinone - MRB13) for iron quantification. This water-soluble reagent was previously reported as a greener alternative to quantify iron, due to its low toxicity and a more environmental friendly synthesis. Furthermore, it also displayed a high affinity and specificity for iron. With the main objective of quantifying iron in a variety of water types (different matrices and iron content), two strategies were developed, one of them including on-line solid-phase extraction (SPE), and the other without resorting to a SPE process. Water matrix clean-up and iron enrichment was achieved using a nitrilotriacetic acid resin column. The potential interference of metal ions usually present in water samples was assessed and no significant interference (<10%) was observed. The limits of detection were 11 and 2.9 µg L-1 without and with SPE, respectively. For one determination (three replicates), the corresponding consumption of MRB13 is 90 µg, sodium hydroxide is 1.4 mg, and boric acid is 5.6 mg. The method was applied to certified water samples and the results were in agreement with certified values. The developed method was also applied to fresh and marine water, and recovery ratios of 103 ± 4 and 101 ± 7 without and with SPE, respectively, were achieved.

Keywords: Flow analysis, 3-hydroxy-4-pyridinone, solid-phase extraction, NTA

4.1. Introduction

Iron is a micronutrient essential for living organisms; however, as it can be introduced in the environment by human activity, it is important to monitor its content, namely in aquatic systems (1–3). At low concentrations, iron is vital for almost all living organisms, participating in a wide variety of biological processes; nevertheless, in excess, iron becomes dangerous, producing also aesthetic effects, thus affecting the colour, taste and odour of the water (1,2,4). In this context, iron quantification has been of particularly interest to environmental analytical chemists, aiming for new methods to improve the limit of detection, use low toxicity reagents, and reduce reagents consumption and effluents production. As flow analysis techniques can be quite useful for this purpose, several works were described (Table 4.1).

The recommended methods for iron quantification (5–7) are based on atomic absorption spectrometry, inductively coupled plasma spectrometry, molecular absorption (like the phenanthroline colorimetric procedure) and chemiluminescence. However, atomic absorption and emission methods present some limitations like its high cost and non-portability of the corresponding equipment, low tolerance for high salinity samples, together with the need for a skilled operator. Due to their inherent simplicity of operation and lower lost, colorimetric methods have been extensively studied, but the use of toxic reagents (phenantroline, thiocyanate, bathophenantroline, 2,2-bipyridyl, eriochrome R and cetyltrimethylammonium) (8) is nowadays a concern, being the green chemistry principles a priority. The chemiluminescence method, using a luminol-hydrogen peroxide system, is highly sensitive for iron determination; however, this reaction is not specific for iron and other ions such as manganese(II), chromium(III), cobalt(II) and copper need to be separated prior to detection (6).

In the present work, a low toxicity iron chelator was employed for the development of a spectrophotometric flow-based method. The chosen reagent is a 3-hydroxy-4-pyridinone, (3,4-HPO) ligand functionalized with an ether function to increase solubility in water (9). These iron chelators with chromogenic properties display significant advantages as a low toxicity reagent with high affinity and specificity for iron(III) (9–11), making them an attractive choice for the quantification of iron from a green chemistry perspective. In the last few years, some 3,4-HPOs with different substituents in different positions have been developed, including the latest 1-(3'-methoxypropyl)-2-methyl-3-benzyloxy-4-(1H)pyridinone (MRB13), the chelator used in the present work. The way the synthesis of MRB13 is achieved is also important to remark, as its route of synthesis is more efficient and sustainable if compared to the one for other ligands (9).

Table 4.1. Analytical characteristics of developed spectrophotometric flow system for iron determination in water samples (presented in descending chronological order).

System	Type of water	Sample volume (µL)	SPE	Reagent	Sample throughput (h^{-1})	LOD (µg L^{-1})	Ref.
SI	Fresh and marine	650 800	- NTA	MRB13	20 8	10.9 2.9	This work
µSI-LOV	River, ground, tap, sea and estuarine	40-700	NTA	CP256		7.3	(12)
pFI	Sea	300	-	Ferrozine	90/40*	3.1/0.57*	(13)
rFIA	Bottled, tap and lake	-	-	Ferrozine	20	2	(14)
µSI-LOV	River	150	-	MRB12	50	15	(4)
SI	Sea	15 mL	-	Sulfosalicylic acid and EDTA	30	90	(15)
SI	River and sea	903	- NTA	CP256	58/42** 28/24**	33/3** 27/3**	(16)
SI	Natural	5000	-	PEG-HPO	24	48	(17)
SI	River	700	-	1.10-phenantroline	-	10	(18)
µSI-LOV	Fresh and marine	400	NTA	Hmpp	14	9	(11)
FA	Fresh	420	-	SCN^-	64	60	(1)
SI	Waste and environmental	100	-	SCN^-	-	200	(19)
SI µSI-LOV	Natural	300 50	-	3,4-HPO	102 90	83 7	(10)
SIA	River, well, ground, potable and sea	250	-	Ferrozine	41	0.15	(20)

MSFIA	Drinking	5000	Modified Amberlite XAD-4	Chrome azurol S	6	5.6	(3)
MSFIA	Treatment unit	3200	Modified Amberlite XAD-4	Chrome azurol S	-	2.3	(2)
SI-LOV	Industrial waste	45	-	5-Br-PSAA	18	25	(21)
FIA	River	500	-	DPD	20	0.02	(22)
FIA	Tap and bottled	500	-	DPD	25	0.01	(23)
SI	Waste, tap and river	150	-	1.10-phenantroline	-	12	(24)

Ref. – reference; SPE – solid phase extraction; SI – Sequential injection analysis; pFI – programmable flow; μSI-LOV – micro sequential injection lab-on-valve; CP256 – hexadentate 3-hydroxy-4-pyridinone ligand; rFIA – reverse flow injection analysis; FA – Flow analysis, HMPP – 3-hydroxy-1(H)-2methyl-4-pyridinone; MSFIA – multisyringe flow injection analysis; PEG-HPO - 3-hydroxy-4-pyridinone ligand functionalized with an hydrophilic ethylene glycol chain; 5-Br-PSAA – 2-(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfoptopyl)amino]aniline; FIA – flow injection analysis; DPD – *N,N*-dimethyl-*p*-phenylenediamine.
*different values correspond to different flow strategies-stop in holding coil/stop in flow cell.
**different values correspond to the use of different flow cells.

Besides aiming to use a low toxicity colorimetric agent, the objective of this work was to design a flow method that could be applied to recreational water samples with different salinities. In fact, most of previously reported methods do not cope with salinity interference, including those involving atomic absorption or emission spectrometry. To accomplish this objective, in this work a versatile method involving two different strategies, one including a solid phase extraction (SPE) process, is proposed. The SPE was used both to remove the sample matrix interference and/or the enrichment of the analyte. This in-line SPE process has been increasingly used as sample pre-treatment due to some advantages over other extraction techniques, namely liquid-liquid, as little or no organic reagents are employed (25,26). With this purpose, a column with nitrilotriacetic acid (NTA) was employed, as it was previously reported that in certain conditions (pH = 2), NTA has the capability of specifically retain Fe(III) (12). This retention occurs because NTA in the fully deprotonated form acts as a sequestering agent and this property is pH dependent (27).

By combining the capabilities of in-line SPE, it was possible to devise a flow method to measure iron in fresh and marine water, with favourable analytical features over previously reported ones.

4.2. Experimental

4.2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and MilliQ water, MQW (resistivity > 18 MΩ cm, Millipore, USA).

A stock solution of 50.0 mg L^{-1} of Fe(III) was prepared by dilution of the respective 1000 mg L^{-1} atomic absorption standard solution (Fluka, Germany). An intermediate solution of 4.00 and 1.00 mg L^{-1} of Fe(III) solution was prepared by dilution of the 50.0 mg L^{-1} stock solution. Working standards from 5.00 to 80.0 µg L^{-1} with 0.01 mol L^{-1} of nitric acid were weekly prepared by dilution of the 1.00 mg L^{-1} solution. Working standards from 50.0 to 600 µg L^{-1} with 0.01 mol L^{-1} of nitric acid were weekly prepared by dilution of the 4.00 mg L^{-1} solution.

A 0.01 mol L^{-1} nitric acid solution was prepared by dilution of the concentrated solution (d = 1.39; 65%, Merck; Germany).

A 0.50 mol L^{-1} borate buffer solution was prepared by dissolution of the solid (H_3BO_3, Aldrich, Germany) in a solution of 0.2 mol L^{-1} NaOH (Panreac, USA), with the final pH adjusted to 10.0 with sodium hydroxide.

A stock solution of 40.0 mmol L^{-1} of 3-hydroxy-4-pyridinone (MRB13, molar mass = 233.1 g mol^{-1}) was prepared by dissolution of the corresponding quantity of the reagent in water. A 0.6 mmol L^{-1} of MRB13 (9), reagent solution, was daily prepared by dilution of the stock solution in water.

Artificial seawater was prepared according to Kester et al (1967). This seawater solution was composed by: 23.926 g kg^{-1} NaCl (Merck; Germany), 4.008 g kg^{-1} Na_2SO_4 (Merck; Germany), 0.677 g kg^{-1} KCl (Merck; Germany), 0.196 g kg^{-1} $NaHCO_3$ (Merck; Germany), 0.098 g kg^{-1} KBr (Merck; Germany), 0.026 g kg^{-1} H_3BO_3 (Aldrich; Germany), 0.003 g kg^{-1} NaF (Merck; Germany), 0.05327 mol kg^{-1} $MgCl_2 \cdot 6H_2O$ (Merck; Germany), 0.01033 mol kg^{-1} $CaCl_2$ $2H_2O$ (Merck; Germany), and 0.00009 mol kg^{-1} $SrCl_2 \cdot 6H_2O$ (Fluka; Germany). Standards of Fe(III) were prepared with artificial seawater and acidified with nitric acid 0.01 mol L^{-1}.

All solutions used for the interferences assessment (Al, Ca, Co, Cu, Mg, Mn, Ni and Zn) were prepared by diluting commercial atomic absorption standard solution (1000 mg L^{-1}, Spectrosol, England).

A stock solution of 1000 mg L^{-1} of Fe(II) was prepared by dissolution of the corresponding quantity of ammonium iron (II) sulphate hexahidrate (Merck, Germany) in 0.5 mol L^{-1} nitric acid. An intermediate stock solution of 50.0 mg L^{-1} of Fe(II) was prepared by dilution of the 1000 mg L^{-1} standard solution. An intermediate solution of 1.00 mg L^{-1} of Fe(II) solution was prepared by dilution of the 50.0 mg L^{-1} intermediate stock solution. Working standards from 5.0 to 80.0 µg L^{-1} with 0.01 mol L^{-1} of nitric acid were prepared by dilution of the 1.00 mg L^{-1} solution. Work standards prepared similarly as Fe(III) working standard solutions.

4.2.2. Preparation of the NTA column

NTA resin (60 – 160 µm, NTA Superflow, Qiagen, Netherlands) was used as sorbent for SPE of Fe(III) and packed in a laboratory-made column with 25 mm length of Tygon tube (Gilson, France), 1.85 mm i.d. and 67 µL inner volume. Approximately 100 mg of NTA resin was suspended in water and introduced as a slurry in the column between two pieces of dishwashing foam.

4.2.3. Apparatus

Solutions were propelled by a syringe pump of 5 mL (Crison, Spain) controlled by computer software. The pump was connected to the central channel of a ten-port electrically actuated selection valve (Valco VICI Cheminert C25-3180D 06B – 0699C, USA) with a PTFE tubing. An injection valve (Valco VICI Cheminert 60736-E45 230, USA) was connected to the selection valve by the port 7 and 8. All the components of the flow system were connected by PTFE tubing from Omnifit (0.8 mm i.d., UK). The syringe pump, the selection valve and the injection valve were controlled by AutoAnalysis Station 5.0 computer software (Sciware, Spain).

As detection system, an Ocean Optics USB 4000 charged coupled device (CCD) detector spectrophotometer (USA) equipped with a pair of 600 mm optic cable and a Mikropack DH-2000-BAL deuterium halogen light source was used. An Ultem® flow cell (SMA-Z-50 cell, Ocean Optics, USA) with 50 mm optical path and silica windows and 130 µL inner volume was used.

4.2.4. Flow manifold and procedure

The flow manifold for the developed method for spectrophotometric determination of Fe(III) in waters is depicted in Fig. 4.1.

The sequence of steps for iron determination and respective volumes are shown in Table 4.2. The method is subdivided in two different parts, corresponding to the different strategies for Fe(III) determination.

The sequence of steps described from A to D corresponds to the determination of iron without SPE (FA). This strategy can be chosen for samples that do not need matrix cleaning and/or pre-concentration of the analyte. Reagent, buffer solution, and sample were sequentially aspirated to the holding coil (steps A to C), and the mixture sent to the detector for absorbance measurement (step D).

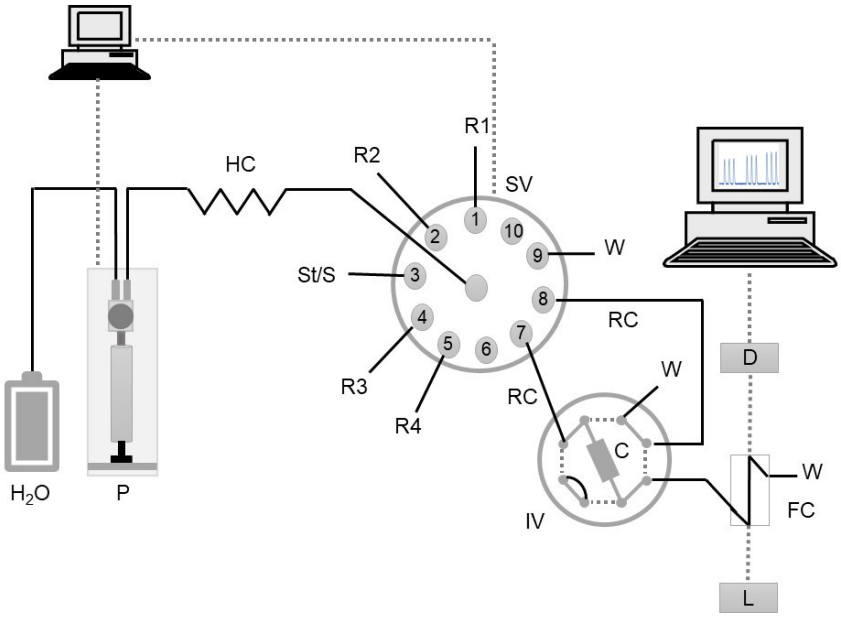

Fig 4.1. Flow manifold for Fe(III) determination in waters. St/S – standard solution or sample; R1 – MRB13 solution (0.6 mmol L^{-1}); R2 – borate buffer solution (pH 11); R3 – nitric acid solution 0.01 mol L^{-1}; R4 – nitric acid solution 0.5 mol L^{-1}; C – NTA resin column; P – syringe pump; SV – selection valve; IV – injection valve; HC – holding coil (300 cm); RC – reaction coils (10 cm); D – Ocean Optics USB 4000 CCD; L – light source; FC – Z flow cell (50 mm path length); W – waste.

The steps from E to I describe the sequence for the determination of iron with SPE (SPE-FA), providing the sample matrix (high salinity) clean-up and/or preconcentration of iron. In this case, the sample was aspirated to the holding coil and then sent through the column towards the waste (steps E and F). Subsequently, the plugs of reagent and buffer were aspirated to the holding coil (steps G and H), the injection valve was switched and the plugs sent through the column to the detector (step I). At the end of each sample analysis (one cycle that corresponds to three replicas) the NTA column was washed and reconditioned with nitric acid 0.5 and 0.01 mol L^{-1} respectively (steps J – M).

Table 4.2. Protocol sequence for the iron determination in waters by (i) Strategy without solid phase extraction, FA: A – D; (ii) Strategy with in-line solid phase extraction SPE-FA: E – M.

Strategy	Step	SV position	IV position	Volume (mL)	Flow-rate (mL/min)	Description
Preliminary steps before starting consecutive cycles				5.000	-	Syringe reset position - syringe fill with carrier
				1.000	5.000	Propel carrier to waste
FA	A	1		0.250	3.529	Aspirate MRB13 solution
	B	2		0.020	3.529	Aspirate borate buffer solution
	C	3		0.650	3.529	Aspirate standard/sample solution
	D	8	ON	2.100	3.529	Propel through the CCD detector for Fe quantification
SPE-FA	E	3		0.800	3.529	Aspirate standard/sample solution
	F	7	ON	1.400	1.500	Propel through the NTA column
	G	1		0.250	3.529	Aspirate MRB13 solution
	H	2		0.020	3.529	Aspirate borate buffer solution
	I	7	OFF	1.500	2.000	Propel through the CCD detector for Fe quantification
SPE-FA - washing & conditioning	J	5		0.200	3.529	Aspirate HNO_3 solution 0.5 mol L^{-1}
	K	7	OFF	0.500	3.529	Propel through the NTA column – cleaning step
	L	4		0.250	3.529	Aspirate HNO_3 solution 0.01 mol L^{-1}
	M	7	ON	0.500	3.529	Propel through the NTA column – reconditioning step

4.2.5. Water sample collection and preparation

Water samples from various recreational locations from Porto district (Portugal) were collected 20 cm below the surface. The samples were filtered with Acrodisc 25 mm syringe filters 0.45 µm (Pall, USA) and acidified with nitric acid (0.01 mol L^{-1}) according the reference sampling procedure (5). Samples were kept refrigerated until analysis.

4.2.6. Reference procedure

For comparison purposes, the determination of iron in waters was carried out by the reference procedure with inductively coupled plasma – optical emission spectrometry (ICP-OES) (5), in a Perkin Elmer Optima 7000 dv (USA) equipment. Results were compared with those obtained with the developed flow method.

Additionally, the developed flow method was applied to certified water samples available for the determination of trace elements: ERM-CA615 (ground water), ERM-CA011 (hard drinking water), SLRS4 (river water, CRM, Canada) and TM27.3 (lake water, Canada). The certified water samples were diluted in order to fit the linear range of the calibration of the developed method.

4.3. Results and discussion

Regarding the flow system configuration (Fig. 4.1) an option was made to use a selection valve coupled to an injection valve. The selection valve was used to select, in a versatile way, the different solutions and samples with the respective volumes. The injection valve was used to incorporate of the NTA resin column in a loop assembly. This configuration enabled the analyte retention in the NTA to be carried out in one direction, and the elution in the opposite direction, thus minimizing the compaction of the column, without aspirating through the column. Additionally, when the SPE process was not necessary, the injection valve only acts as a flow path to the detector.

The development of the two strategies of the flow system involved several studies to assess the influence of some variables. Those parameters were optimized in order to use low volumes of reagents and standards, increase the determination rate and also increase the sensitivity for the iron determination.

4.3.1. Development of the FA strategy – iron determination without SPE

The use of this newly reported chelator, MRB13, for the spectrophotometric determination of iron was studied. In a recent work (9), the features of this ligand and some parent ligands were compared in a flow mode for iron measurement. However, no application for iron quantification with the use of this chelator (MRB13) was described.

In the present work, the performance of MRB13 was compared with a parent ligand (MRB12) already referred by Mesquita *et al* as a chromogenic chelator for iron determination (17) in waters. For that purpose, the selected volumes for reagent, buffer and standard were those state in the same reported work. There were no significant differences in the slope or intercept in the calibration curve (<10%), using the two different ligands. In the reported work (17), a carbonate buffer was used. However, in flow systems, carbonate buffers may generate bubble formation. So, in this work an alternative borate buffer was tested. A 0.6 mmol L^{-1} carbonate buffer and 0.5 mmol L^{-1} borate buffer, both with pH≈10 were compared; no significant differences in the slope (<10%) of the calibration curves were observed. Therefore, borate buffer was the selected solution.

The effect of MRB13 solution concentration (1.2, 0.6 and 0.3 mmol L^{-1}) on the calibration curve (slope and intercept) was also assessed. Using 1.2 and 0.60 mmol L^{-1}, no significant differences were observed in the slope (<10%) of the calibration curve; however, the intercept decreased about 50%. When using the 0.30 mmol L^{-1}, the slope decreased 12%. Then, the concentration of the MRB13 reagent solution was set to 0.60 mmol L^{-1}. The volumes of aspiration of standard, buffer solution and MRB13 solution were also evaluated. First, the volume of 500 µL of sample/standard (17) was set, and then the results compared with those obtained with 550, 650 and 750 µL. No significant differences were observed in the slope of the calibration curve; however, a decrease of 16% in the intercept was observed when using the volumes of 650 and 750 µL. So, the volume of sample/standard was set to 650 µL. The influence of the volume of buffer was also evaluated: volumes of 34 and 20 µL were tested. As the results for the two volumes did not displayed significant differences (<10%), 20 µL was the chosen volume.

4.3.1.1. Interference studies

The potential interference of some metal ions that can be present in water samples was tested. As evidenced on Table 4.3, no significant interference in the iron determination was observed.

Table 4.3. Interference study of some metal ions, commonly present in natural waters, in iron determination. Values for the concentration of ions that can be present in water streams (5).

Tested ion	Water streams μg L^{-1}	Tested μg L^{-1}	Interference in Fe determination %
Al^{3+}	400	400	-0.9
Ca^{2+}	15000	15000	-5.5
Co^{2+}	0.2	10	-1.7
Cu^{2+}	< 12	100	+0.6
Mg^{2+}	4000	5000	-3.8
Mn^{2+}	7	100	+0.6
Ni^{2+}	1	100	0.0
Zn^{2+}	20	100	-1.2

As the main goal of this work was to propose a method that could cope with different salinities, the influence of this parameter was studied. For that purpose, standards were prepared in MQW, artificial sea water, and seawater diluted 1:2 (to mimic the composition of an estuarine water). Using iron standards prepared in artificial seawater, the signals were erratic, possibly due to the high refraction signal produced during the detection. This phenomenon is usually called schlieren effect, caused by refractive index gradients between the saline solution and the other flowing aqueous solutions. With the standards prepared in ultrapure water and lower salinity content water, no significant differences were observed (< 10%) in the slope and intercept of a typical calibration curve (n=3).

4.3.2. Development of the SPE-FA method

In order to overcome the above-mentioned problem associated with salinity and also to carry out analyte enrichment, targeting a better detection limit, an on-line solid-phase extraction process was implemented (Fig. 4.1).

Then, some variables were evaluated. The first one was the influence of the sample loading volume. By increasing the volume, an increasing enrichment factor should be obtained. The use of 650, 800 and 900 µL volumes were compared. An increase of 25% of the slope was observed when the volume was increased from 650 to 800. On the contrary, for a 900 µL loading volume, the slope decreased, and the stability of the repeatability of the absorbance signals also decreased. This could be due to physical processes associated with some alteration of the positioning of the sorbent inside the column. So, the sample volume of 800 µL was the one chosen.

The flow rate for the sample loading (0.5, 1.0, 1.5, 2.0 mL min^{-1}), and the one for the iron elution (1.0, 1.5, 2.0 mL min^{-1}) from the NTA column was also assessed. The chosen flow rates were 1.5 mL min^{-1} for the loading step and 2.0 mL min^{-1} for the extraction step. These flow rates were chosen as a compromise between sensitivity and determination rate.

4.3.2.1. Interference studies

The influence of salinity was a parameter of study for the determination of iron with SPE, as this water property can interfere in the quantification. For that purpose, standards were prepared in ultrapure water, artificial sea water (28), and seawater diluted 1:2 (this last one to reproduce approximately the composition of an estuarine water). The calibration curve for these different standards (prepared with different matrix) were compared, and no significant differences for the slope and intercept were observed (< 10%). Therefore, the salinity of the standards did not influence the absorbance signal and so, the developed flow procedure can be applied to different water samples. The NTA resin acts as a matrix clean-up process; this occurs at the loading step, being the iron retained at the NTA resin, while the water sample matrix is transported away from the resin and discarded.

The interference of metal ions was not assessed at this strategy, because the use of MRB13 reagent showed to be specific as iron chelating agent (see section 3.1.1). As the eluting agent is MRB13, no interferences are expected in this strategy for iron detection.

Some previous studies pointed out that iron at ferrous state (Fe^{2+}) is not retained by the NTA resin (11,16) and, because of that, by using this SPE strategy the detected ion would be the iron in ferric state (Fe^{3+}). This issue was revisited: standards of Fe^{2+} were prepared between 5.0 and 80.0 µg L^{-1} and analyzed with SPE system; the absorbance signal of the different standards did not statistically differ from the absorbance of the blank solution (< 5%). This confirms that, by using the NTA, only the determination of Fe(III) is performed. However, this is not a problem for this study, as it is not expected to find significant ferrous iron concentrations in superficial waters, if compared with ferric iron.

4.3.2.2. NTA column breakthrough

The breakthrough of the NTA packed column was evaluated. This value would correspond to the maximum quantity of Fe(III) that could be retained by the column. This was estimated by increasing the quantity of the analyte that perfuses the column (increasing the standard concentration) and calculating the recovered quantity of iron (in mass). The absorbance was plotted against the mass of iron; the signal increased until 1.6 µg of iron, that corresponds to a 2.00 mg L^{-1} standard. For higher concentration values, the absorbance signal maintained constant, possibly having reached the breakthrough of the NTA column.

However, using the 2.00 mg L^{-1} iron standard, the stoichiometric ratio (1Fe:3MRB13), between iron and MRB13 reagent is almost reached; so, this value could not be the breakthrough of the NTA column, but merely a lack of reagent. Actually, this is not a problem because the 2.00 mg L^{-1} standard is twenty-five times higher than the highest standard concentration of the calibration curve.

4.3.3. Application to water samples – accuracy assessment

4.3.3.1. Certified water samples

For accuracy assessment, the developed flow system was applied to determination of iron in certified water samples. For that objective, the two strategies for iron determination were tested (Table 4.4). The relative deviation between the certified value and the one obtained with the developed system were below 10%, validating the developed method for iron determination.

Table 4.4. Comparison of the results obtained with the developed flow system for iron determination in certified water samples with the certified value for iron; direct determination (FA) and with on-line SPE (SPE - FA). RD – Relative deviation.

Sample ID	$[Fe^{3+}]_{certified}$ µg L^{-1}	$[Fe^{3+}]_{FA}$ µg L^{-1}	RD %	$[Fe^{3+}]_{SPE-FA}$ µg L^{-1}	RD %
Ca011	198 ± 5	196 ± 7	-1.0	-	-
SLRS4	103 ± 5	98 ± 3	-5.0	110 ± 5	+6.6
TM27.3	10.9 ± 0.3	11 ± 3	0.0	11 ± 1	+5.0
Ca615	5.1 ± 0.3 mg L^{-1}	5.19 ± 0 mg L^{-1}	+1.6	4.9 ± 0.3 mg L^{-1}	-4.3
				5.0 ± 0.6 mg L^{-1}*	-1.6

*sample diluted 1:100 in artificial sea water (28).

4.3.3.2. Recovery studies

Since the concentration of a number of the analyzed samples were below the limit of detection of the developed system and/or the reference procedure (ICP-OES), recovery tests were performed for both strategies. The recovery percentages calculations were made according to the IUPAC recommendations (29) and the results are depicted in Table 4.5. The developed flow methodology for the determination of iron in fresh and marine water provided recovery ratios of 103 ± 4 without SPE and 101 ± 7 with SPE (average ± standard deviation). The statistical t-test for a 95% significance level was calculated for the two strategies of iron quantification.

For the direct determination, t-value was 0.184 and the correspondent critical value was 3.163. For the determination of iron with SPE, the calculated t-value was 0.410 and the correspondent critical value was 3.163. The statistical t-test for both strategies of iron determination indicates there is no evidence of systematic errors or the presence of some matrix interference (30). Therefore, the developed system can be applicable for the quantification of iron in a variety of waters samples with different salt content.

Table 4.5. Recovery percentages obtained with the developed flow-based system in FA mode (samples F1, F2 and F3) and SPE – FA mode (samples M1, M2 and M3).

Type of water	Sample ID	$[Fe^{3+}]_{initial}$ μg L^{-1}	$[Fe^{3+}]_{added}$ μg L^{-1}	$[Fe^{3+}]_{found}$ μg L^{-1}	Recovery (%)
Fresh waters	F1	26.3 ± 5.1	50.0	74.2 ± 3.0	95.8
			200	233 ± 11	103
	F2	34.4 ± 3.6	50.0	85.3 ± 3.6	102
			200	245 ± 5	104
	F3	16.0 ± 0.0	50.0	70.8 ± 3.0	109
			200	219 ± 8	102
Marine waters	M1	< LOD	30.0	28.7 ± 1.1	95.7
			60.0	62.2 ± 8.8	104
	M2	< LOD	30.0	32.5 ± 5.5	108
			60.0	57.4 ± 1.4	95.7
	M3	11.3 ± 1.5	30.0	43.9 ± 1.0	109
			60.0	67.9 ± 3.0	94.3

4.3.4. Features

The dynamic ranges of both strategies as well as the calibration curves and the limit of detection and quantification (LOD and LOQ, respectively) for the determination of Fe were summarized in Table 4.6.

The LOD and LOQ values were calculated according to IUPAC recommendations as the concentration corresponding to the sum of three and ten times (for limit of detection and quantification respectively) the standard deviation to the mean value of ten consecutive blank solution measurements (31,32).

Table 4.6. Features of the developed flow-based system for iron quantification, FA, flow analysis system without SPE; SPE-FA, flow analysis system with solid phase extraction; LOD, limit of detection; LOQ, limit of quantification. SD - standard deviation.

Strategy	Dynamic range ($\mu g\ L^{-1}$)	Typical calibration curve[a] A = (slope ± SD) $\mu g\ L^{-1}\ Fe^{3+}$ + intercept ± SD	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)
FA	25.0 - 800	A = ($2.21 \times 10^{-4} \pm 1 \times 10^{-6}$) Fe^{3+} + 0.045 ± 0.001	10.9	32.4
SPE-FA	5.0 – 80.0	A = ($6.62 \times 10^{-4} \pm 2 \times 10^{-5}$) Fe^{3+} + 0.043 ± 0.002	2.9	12.1

[a] n=3

The calibrations curves presented in Table 4.6 correspond to the mean slope and intercept of three curves with the respective standard deviation. The repeatability was assessed by calculation of the relative standard deviation (RSD) of twelve replicate analysis of a standard (four consecutive cycles); the RSD for Fe determination with FA strategy was 2.5% (200 $\mu g\ L^{-1}$) and with the SPE-FA strategy was 3.8% (40 $\mu g\ L^{-1}$).

A complete analytical cycle (three replicas) for the determination of iron with the FA strategy takes 3 min, and for SPE-FA strategy takes 8 min (including the NTA column washing). The corresponding consumption values for a complete analytical cycle (three replicas) were: 90 µg of MRB13, 1.4 mg of sodium hydroxide and 5.6 mg of boric acid.

4.4. Conclusions

The developed flow-based methods for iron quantification in surface recreational water proved to be an efficient tool for water monitoring and applicable to the determination of iron in different salinity content waters (fresh and marine). The described method enables iron(III) determination with two possible strategies: a direct approach (FA) and using solid phase extraction (SPE-FA); within the same manifold. Water samples with a relatively high iron content and low salinity concentration, were assessed without resorting to the SPE strategy. If the water samples presented high salinity levels and/or a low concentration of iron, it was possible to resort to the SPE strategy with a NTA resin column incorporated in the system, enabling to clean-up the sample matrix and/or pre-concentration of iron(III).

The choice of a newly reported iron chelator (9) as a colorimetric reagent proved to be successful as both the limit of detection and quantification were better than the previously reported with similar chelators (11,12,16), except when resorting to a long pathlength flow cell. Additionally,

MRB13 has a simpler and cheaper synthesis meeting the requirements of green chemistry guidelines (9). MRB13 reagent proved to have high affinity and specificity for iron (9,17), similar to other parent ligands, as no significant interferences were observed in the presence of other ions commonly present in natural waters (< 10%).

The incorporation of the NTA column, as a SPE strategy, in the flow-based system proved to be an effective choice to quantify low concentration of iron and to apply to high salinity waters. With SPE-FA strategy, there was the discarding of the matrix, resulting a matrix clean-up, and analyte enrichment, thus improving the detection limit from 10.9 μg L^{-1} to 2.9 μg L^{-1}.

Overall, combining the new chelator MRB13 with an in-line SPE process, an efficient method was devised for iron determination in recreational waters, displaying a low reagent consumption, low effluent production using low toxicity reagents.

The developed method was applied to certified water samples (ground, river, lake and drinking water) and the results were in agreement with the expected results.

The portability of the system makes it appropriate for the in-situ monitoring of iron in water bodies.

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CHAPTER 5

A Sequential Injection Fluorimetric Methodology with In-Line Solid Phase Extraction for Biogenic Amines Screening in Water

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Abstract

A method for the screening of biogenic amines in waters, whose presence at some concentration levels potentially cause adverse effects on humans, was developed for the first time. A suitable and easy system to operate, with low reagent consumption was devised. The proposed flow-based system was divided into two analytical parts, pre-concentration and derivatization of the biogenic amines. Solid phase extraction, using a Chelex 100 resin, was the newly chosen strategy for preconcentration of the analyte and also removal of possible matrix interferences. Fluorescamine was used as derivatization reagent for biogenic amines followed by fluorimetric detection. The influence of different sorbent materials for preconcentration and flow system parameters such as pH of standards and buffer, composition of the eluent solution, flow-rates, standard/sample volume, were studied. The interference of ammonia was assessed, and no interference was observed. The limits of detection and quantification were 1.7 and 5.6 μmol L^{-1}, respectively. The developed system was applied to water samples and the recovery results were about 98 ± 7%.

Keywords: Primary amines; sequential injection analysis; preconcentration; Chelex 100; fluorescamine; fluorescence

5.1. Introduction

Biogenic amines (BAs) are nitrogenous organic basic compounds with low molecular weight (1–8). According to their structure, these organic molecules can have aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic structure (1,2); according to the number of amino groups present in the molecule, BAs can be classified as monoamines (like histamine and tryptamine), diamines (like putrescine and cadaverine) and poliamines (like spermine and spermidine) (Fig. 5.1). These amines are synthetized or degraded by living cells and participate in biological pathways, as in the neurotransmission and regulation of blood pressure, body temperature or even in the synthesis of DNA, RNA and proteins (2,3). So, they have important physiological functions; however, the continuous intake can influence the human health, affecting nervous and cardiovascular systems (4,5). BAs can also react with nitrite and form nitrosamines, which are carcinogenic compounds (6).

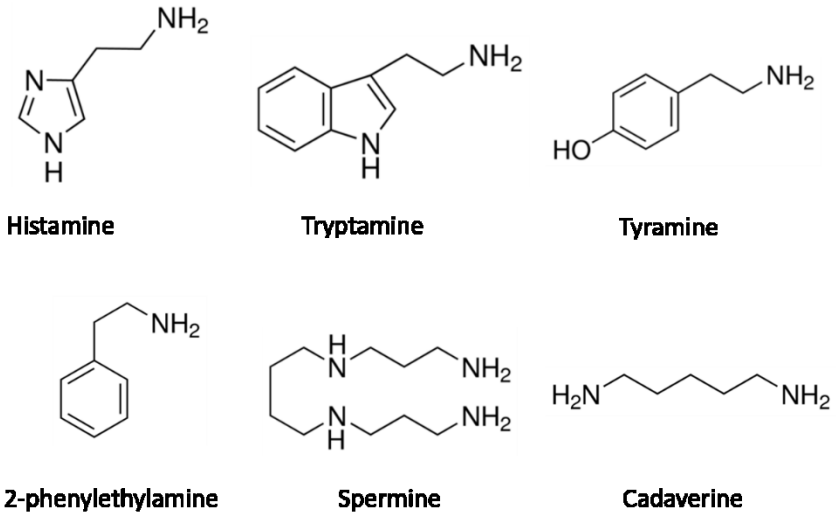


Fig. 5.1. Chemical structure of some biogenic amines.

Essentially, BAs are formed by the decarboxylation of amino acids, and can be present in biological or in environmental samples. The level of biogenic amines can be related to microbial contamination, as can be generated by microorganisms capable of producing decarboxylases (4), bacterial decomposition after a death of an organism can produce BAs, and so pollute the environment.

The analytical determination of BAs is not an easy task because these compounds are present in very low concentrations, and usually in complex matrices. The most frequently used methods for the determination of BAs involve separation and derivatization processes prior to ultraviolet (UV) spectrometric or fluorescent detection.

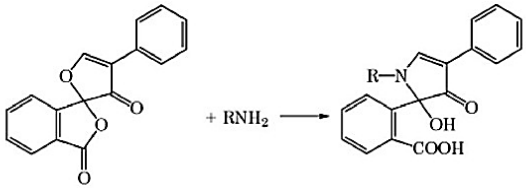
Most of the documented separation procedures are performed by one of the following techniques: liquid chromatography (LC) (5,9–17), gas chromatography (GC) (18,19), thin layer chromatography (TLC) (20) and capillary electrophoresis (CE) (21,22). BAs determination becomes even more difficult because they do not have any specific chromophore or fluorophore in their molecule (23,24). As BAs do not show good intrinsic radiation absorption properties, derivatization prior to detection is required when spectrophotometric detection is used. Ideally, a derivatization produces a stable and absorbent/fluorescent species that can easily be measured. The most commonly used technique is liquid chromatography coupled with various detection systems like mass spectrometry (MS) (5,9–11), UV detection (12–14) or fluorescence (15–17). In the last few years, MS has become a popular detection system because of the intrinsic high sensitivity and low limits of detection; however, the equipment high cost and the need of a specialized operator may be limiting aspect for its use (25,26).

In the last few years, BAs sensors have also been developed, which usually are based on different enzymes for the detection of the amines (27,28).

Most of the developed methods for amines determination were directed for the analysis of BAs in food and beverages and only a few applied to environmental samples, including water (1,2,5,6,29). As far as we know, until now, no regulation is available for BAs contamination in the environment (29). In this scenario, it would be interesting to develop a screening methodology to assess the total amount of these compounds in waters.

With the present work, a sequential injection (SI) method with on-line solid phase extraction (SPE) for the fluorescence detection of biogenic amines in water, using fluorescamine as derivatization agent, is proposed. With the main goal of preconcentrating BAs and eliminating possible interferences, SPE was the chosen strategy; for this purpose, a lab-made and reusable column of Chelex 100 was employed. Chelex 100 resin is a styrene divinylbenzene copolymer, classified as weakly acidic cation exchange resin due to its carboxylic acid groups, which act as chelating resin and is usually used to bind metal ions. However, in the anionic form, can potentially be used to bind cationic species like amines; for the first time, advantage was taken from this property to

retain the target analyte. Fluorescamine was used as derivatization agent for biogenic amines fluorimetric determination and it was firstly synthetized in 1972 by Udenfriend *et al* (30). Fluorescamine and its hydrolysis products do not present fluorescent properties, however reacts instantaneously with primary amines producing highly fluorescent derivative compounds (Fig 5.2).



Fluorescamine (non-fluorescent) Derivative compound (fluorescent)

Fig. 5.2. Derivatization reaction between fluorescamine and primary amines.

5.2. Experimental

5.2.1. Reagents and Solutions

All solutions were prepared with analytical grade chemicals and MilliQ water (resistivity >18 MΩ cm, Millipore, Bedford, MA, USA). Cadaverine dihydrochloride (Cad) – $C_5H_{14}N_2 \cdot 2HCl$, spermine tetrahydrochloride (Spe) – $C_{10}H_{26}N_4 \cdot 4HCl$, tyramine hydrochloride (Tyr) – $C_8H_{11}NO \cdot HCl$, tryptamine hydrochloride (Try) – $C_{10}H_{12}N_2 \cdot HCl$ and 2-phenyilethylamine hydrochloride (2-PEA) – $C_8H_{11}N \cdot HCl$ were purchased from Sigma (Germany). Histamine dihydrochloride (His) – $C_5H_9N_3 \cdot 2HCl$ was purchased from Merck (Germany). Stock solutions of 1.00 mmol L^{-1} of BAs standards were prepared by dissolution of the correspondent quantity of the solid. Working standards, in the range 5.0-60.0 µmol L^{-1}, were weekly prepared by dilution of the stock solution.

Chelex 100 sodium form resin, mesh 200-400 and mesh 50-100 (Bio-Rad, USA), Amberlite IR120 sodium form (Dow, USA) and MCX cartridges (mixed-mode polymeric sorbent, Waters, USA), were used to perform SPE studies in batch and in flow mode.

A 1.0 mol L^{-1} sodium chloride solution was prepared by dissolution of the solid (Panreac, USA) in a solution of 0.1 mol L^{-1} sodium hydroxide (Panreac, USA) and was used as elution solution.

Hydrochloric acid 1 mol L^{-1} was prepared from the concentrated solution (d = 1.2; 37%, Merck, Germany) and was used as column cleaning solution.

A 0.50 mol L^{-1} boric acid buffer solution was prepared by dissolution of the solid (Aldrich, Germany) in a solution of 0.2 mol L^{-1} NaOH (Panreac, USA), with the final pH adjusted to 9.0 with sodium hydroxide or nitric acid.

A solution of fluorescamine (Sigma, Germany) 0.3 mg mL^{-1} was weekly prepared by dissolving the solid in acetone (Merck, Germany).

For the interference study, a 100 µmol L^{-1} stock solution of ammonium sulfate (Merck, Germany) was prepared by dissolution of the solid in water.

5.2.2. Sample Collection and Preparation

Water samples from various locations of recreational parks with inland lakes with animals in Porto district, Portugal were sampled. The samples were firstly filtered with Acrodisc 25 mm syringe filters 0.45 µm (Pall, USA), the samples were kept refrigerated until analysis.

5.2.3. Preparation of the Solid Phase Extraction Column

To incorporate SPE strategy in the flow system solid phases were used as suspension for packing the column for BAs retention. A laboratory made column with 25 mm length of Tygon tube (Gilson, Villiers-le-Bel, France), 1.85 mm i.d. and 67 µL inner volume was used to pack the solid phase. Approximately 75 mg of each solid phase was introduced in the column between two pieces of dishwashing foam. The column was subsequently placed in a side port of the selection valve.

5.2.4. Apparatus

Solutions were propelled by a syringe pump of 5.000 mL (Crison, Barcelona, Spain) controlled by computer software. The pump was connected to the central channel of a ten-port electrically actuated selection valve (Valco VICI Cheminert C25-3180D 06B – 0699C, Houston, USA) with a PTFE tubing. All tubing that connected all the components of the flow system were of PTFE from

Omnifit (0.8 mm i.d., Cambridge, UK). The syringe pump and the selection valve were controlled by AutoAnalysis Station 5.0 computer software (Sciware, Spain).

As detection system, a fluorescence spectrometer (LS 55, Perkin Elmer, USA), equipped with a flow cell made of quartz (100 µL inner volume, Hellma, Germany) for fluorescence measurement of the BAs derivatives, was used. Analytical signal was recorded by BioLight Studio Software version 1.03.01.

5.2.5. Flow Manifold and Procedure

The flow manifold for the fluorimetric determination of BAs in waters is depicted in Fig. 5.3. The sequence of the steps and respective volume is shown in Table 5.1. The system is divided in two different parts: the first one for pre-concentration of BAs (steps A-D) and the second for the BAs derivatization and fluorimetric measurement (steps E-J). For the column cleaning, hydrochloric acid was propelled through the Chelex 100 resin column (steps K and L).

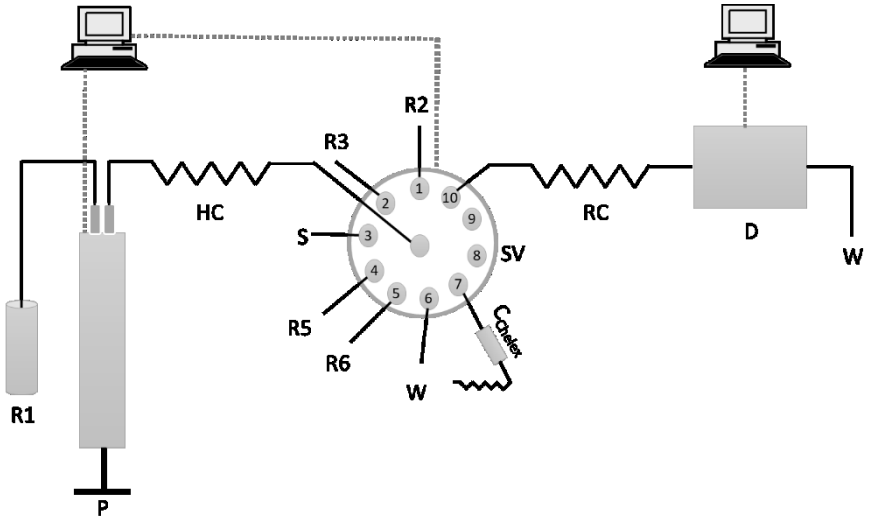


Fig. 5.3. Flow manifold for biogenic amines determination in waters using fluorescamine as a fluorescence reagent. S – sample or standard solution; R1 – ultrapure water; R2 – fluorescamine solution (0.3 mg mL-1); R3 – boric acid buffer solution (pH 9); R5 – NaCl:NaOH solution (1 mol L^{-1}:0.1 mol L^{-1}); R6 – HCl solution (1 mol L^{-1}); C_{Chelex} – Chelex 100 resin column; P – syringe pump; SV – selection valve; HC – holding coil (300 cm); RC – reaction coil (60 cm); D – fluorescence spectrometer (λex = 380 nm, λem = 490 nm); W – waste.

Table 5.1. Protocol sequence for BAs determination in waters.

Step	Selection valve position	Volume (mL)	Flow-rate (mL/min)	Description
Preliminary steps before starting consecutive cycles		5.000	-	Syringe reset position – syringe fill with carrier
		1.000	5.000	Propel carrier to waste
A	3	1.000	5.000	Aspirate standard/sample
B	7	1.500	0.500	Propel through the Chelex column for pre-concentration of the BAs
C	4	0.200	5.000	Aspirate elution solution
D	7	0.200	0.500	Propel the elution solution through the Chelex 100 column
E	7	0.200	0.500	Aspirate the eluate
F	2	0.050	5.000	Aspirate buffer
G	1	0.200	5.000	Aspirate fluorescamine solution
H	10	0.100	5.000	Propel through the reaction coil
I	10	0.050	5.000	Aspirate through the reaction coil to promote mixture of the solutions
J	10	1.600	5.000	Propel through the spectrometer
K	5	0.500	5.000	Aspirate HCl
L	7	1.000	1.000	Propel through the Chelex 100 column

5.2.6. Recovery Procedure

Known BAs concentrations of 10, 30 and 40 μmol L^{-1} were added to the samples. For that, the proper amount of a 2.00 mmol L^{-1} cadaverine standard solution was added to 10.0 mL of water samples and analyzed.

5.3. Results and Discussion

The developed system was divided in two analytical parts, being the first one the preconcentration of the amines and the second the derivatization with fluorescamine followed by fluorescence detection (λex = 380 nm; λem = 490 nm) (Fig 5.4).

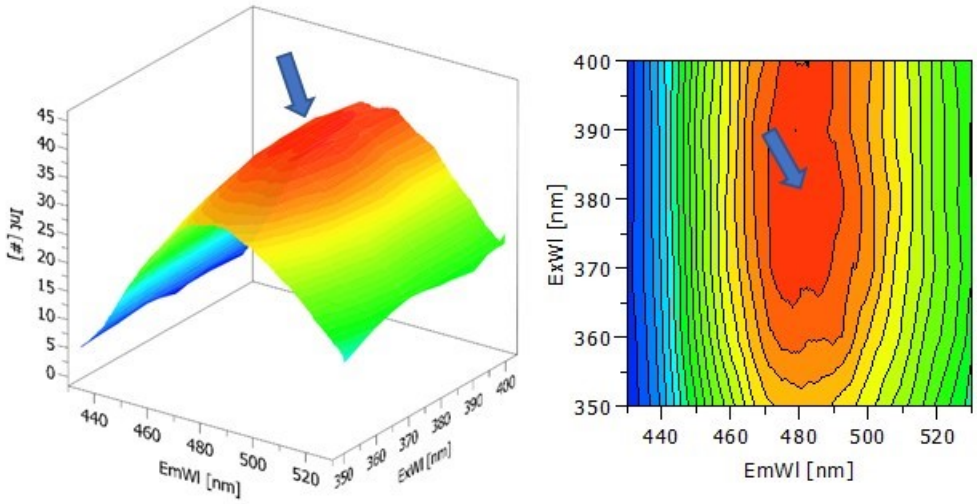


Fig. 5.4. 3D scan of the intensity of the fluorescent derivative compound. The arrow indicates the combined wavelength of excitation and emission where the higher intensity is obtained for this reaction. Ex Wl - excitation wavelength; Em Wl - emission wavelength; Int [#] – fluorescence intensity.

The fluorescamine solution stability was assessed for seven consecutive days, and there were no significant differences in the fluorescence intensity during that period (n=7; RSD = 3%). This study was performed in both batch and in flow mode.

Some preliminary calibration curves (20-80 μmol L^{-1}) in batch mode were performed with all the BAs in the study (cadaverine, spermine, spermidine, histamine, tyramine, tryptamine and 2-phenylethylamine). Comparing the sensitivities and intercept of every calibration curve (ESI Fig. 5.S1), a higher sensitivity and lower interception point was achieved for cadaverine. So, this BA was chosen as model to conduct all the subsequent sequential injection system optimization.

5.3.1. Development of the Sequential Injection System

5.3.1.1. On-Line Derivatization

The flow procedure for the on-line derivatization of BAs with fluorescamine for fluorescence detection was optimized based on some parameters (Table 5.2), namely volumes needed of each solution (fluorescamine, buffer and standard), the order of aspiration of the solutions, flow-rate, reaction coil length, different cells with different inner volumes, and buffer pH. The order of aspiration of the solutions influences the repeatability of the signal. The fluorescamine is not a stable reagent when in contact with water and so, when it is aspirated right before the detection, the intensity signal is more repeatable. The chosen conditions were those provide the highest slope and lowest interception for the cadaverine calibration curve.

To attain a reproducible intensity signal, different flow approaches were tested: aspiration and flow reversal towards detector; aspiration and flow reversal followed by stopping the flow before measurement; and aspiration and double flow reversal. The last approach was chosen as a more reproducible signal was obtained; this could be due to a better mixture of the different plugs. Under the optimized conditions, the typical calibration curve for cadaverine was $I = 0.0078 \pm 0.0007[Cad] + 0.2091 \pm 0.0177$, with limit of detection of 6.8 μmol L^{-1} and a limit of quantification of 22.7 μmol L^{-1}.

Table 5.2. Assessed parameters for the on-line derivatization optimization of biogenic amines with fluorescamine.

Parameter	Tested conditions	Selected condition
Fluorescamine volume	150, 200 and 250 µL	200 µL
Buffer volume	30 – 150 µL	50 µL
Standard/Sample volume	150 – 300 µL	200 µL
Flow-rate	2.5 – 6.0 mL min^{-1}	5.0 mL min^{-1}
Reaction coil length	40, 60 and 100 cm	60 cm
Buffer pH	8.5 – 10.0	9.0
Flow cell	100 and 500 µL inner volume	100 µL

5.3.1.2. On-Line Pre-Concentration

The flow procedure for the on-line preconcentration involved a number of studies to evaluate the influence of chemical and physical variables. Different solid phases were evaluated: MCX, Amberlite IR120, Chelex 100 (two different meshes, 50-100 and 200-400). In preliminary experiments, the study was conducted in a batch mode. MCX is a mixed-mode polymeric sorbent, commonly used for the extraction of basic compounds with cation-exchange groups, and often employed as pre-treatment step prior to chromatography. These cartridges showed to provide preconcentration capability of the solution for the different BAs (23). As preconcentration was achieved, an attempt was made to implement this process in the flow analysis system; thus, a lab-made column was prepared with MCX material and subsequently placed in a side port of the selection valve. However, the application under flow conditions was hindered by the excessive backpressure, as the sorbent material became too compact inside the column, and so the solution could not be propelled through it. Then, to find a possible alternative, the performances of some cation exchange resins were compared (Amberlite IR120 and Chelex 100). Chelex 100 mesh 200-400 was the one that showed to provide capability to preconcentrate the amines (Table 5.3), displaying an increase of the sensitivity and a decrease of both the limits of detection and quantification (ESI Fig. 5.S2), while displaying physical characteristics compatible with its use in a flow mode. In these conditions, this resin was used for the subsequent studies. Various sample loading volumes used for preconcentration, between 500 to 1000 µL, were evaluated. The intensity signal for a standard concentration of 60 µmol L^{-1} increased from 0.812 ± 0.044 (loading volume of 500 µL) to 1.254 ± 0.062 (loading volume of 1000 µL). The selected loading volume was 1000 µL to conduct the subsequent studies, as a further increase would compromise the determination rate.

Table 5.3. Cadaverine typical calibration curve with pre-concentration with Chelex 100 mesh 200-400 and without preconcentration. LOD – limit of detection; LOQ – limit of quantification. I – fluorescence intensity; [Cad] – cadaverine concentration.

	Dynamic range (μmol L^{-1})	Calibration curve	LOD (μmol L^{-1})	LOQ (μmol L^{-1})
With SPE	5.0 – 60.0	I = 0.021±0.001[Cad]+ 0.184±0.012	1.7	5.6
Without SPE	10.0 – 60.0	I = 0.008±0.001[Cad]+ 0.209±0.018	6.8	22.7

The preconcentration (loading and elution) flow-rate was also studied; lower flow-rates increase the analyte interaction with the sorbent, but increase the determination time. Flow rates between 0.333 and 1.000 mL min^{-1} were studied. The intensity signal increased up to a 0.500 mL min^{-1} and decreased after this value; so, 0.500 mL min^{-1} was chosen as loading flow-rate.

The influence of the pH of the standards in the preconcentration step was a parameter of study. So, standards with pH values of 4.5, 5.5 and 6.5 were tested. The pH did not influence the extent of preconcentration and so the pH of standard solutions was not adjusted in further experiments.

The composition of the elution solution was another important parameter of study. For the elution of BAs, different solutions were studied: NaOH:NaCl 0.1 mol L^{-1}: 1 mol L^{-1}; NaOH:NaCl 1 mol L^{-1}: 1 mol L^{-1} and boric acid buffer. With the boric acid buffer, no intensity signal was observed. With the highest concentration of NaOH, no reproducible signal was obtained. Using NaOH:NaCl 0.1 mol L^{-1}: 1 mol L^{-1} as elution solution, a reproducible fluorescence intensity signal was obtained, as it can be evidenced for a replicate analysis of a standard (n = 10; RSD = 4.3%); for this reason it was chosen as elution solution.

The Chelex 100 packed column for biogenic amines preconcentration was reused for a period of roughly 2 months. The daily maintenance of the packed column included a washing step with 1 mol L^{-1} hydrochloric acid (5 mL at 1 mL min^{-1}), followed by ultra-pure water (5 mL at 1 mL min^{-1}), before the end of each working day. The performance of the column remained constant, as shown in the interday repeatability of the method (Table 5.3).

The breakthrough of the column, corresponding to the maximum quantity of cadaverine retained by the packed column, was also evaluated. This quantity was estimated by increasing the amount of cadaverine that perfused the solid material, and calculating the quantity that was recovered. The correspondent intensity values were plotted against the mass of cadaverine. The signal increased until 6.1 μg of cadaverine and, for larger values, fluorescence intensity did not statistically increase (ESI Fig. 5.S3). Therefore, 6.1 μg was considered as the maximum amount of cadaverine retained in the lab-made Chelex 100 column used.

5.3.2. Interference Studies

After this optimization process, an interference study was carried out. Considering its structure, ammonium could be an interfering age nt that could react with fluorescamine or even to be retained in the preconcentration resin. And so, it was tested as a possible interfering agent in a batch approach. To a 40 μmol L^{-1} cadaverine solution, 10, 40 and 100 μmol L^{-1} of an ammonium solution were added. The intensity signal remained constant and so no significant interference was observed.

5.3.3. Method Performance with Other Biogenic Amines

Considering that only cadaverine was used as a model BA, the following study intend to assess if other BAs displayed similar analytical behaviour. Based on the studies already discussed, this would be expected, because fluorescamine reacts with all BAs in study. Then, the developed SI system was also applied to the determination of histamine, spermine, tyramine, tryptamine and 2-phenylethylemine. Tryptamine was not detected for concentrations lower than 60 μmol L^{-1}. For spermine and 2-phenylethyamine, no enhancement of the sensitivity (slope) was observed with SPE (ESI Fig 5.S5). As observed for cadaverine, an enhancement of the sensitivity was observed for histamine and tyramine with the use of the SPE (ESI Fig 5.S4). The dynamic concentration range for these three BAs was the same. Tyramine presented the highest sensitivity; however, it also displayed the highest standard deviation in the interday studies. For cadaverine, a lower limit of detection and quantification was achieved, as shown in Table 5.4.

Table 5.4. Typical calibration curves (n = 3) and dynamic concentration range of the calibration curve for cadaverine, histamine and tyramine and respective limits of detection (LOD) and limits of quantification (LOQ). I – Fluorescence intensity; [Cad] – cadaverine concentration; [His] – histamine concentration; [Tyr] – tyramine concentration.

Biogenic amine	Dynamic range (μmol L^{-1})	Calibration curve	LOD (μmol L^{-1})	LOQ (μmol L^{-1})
Cadaverine	5.0 – 60.0	I = 0.021±0.001[Cad]+0.184±0.012	1.7	5.6
Histamine	5.0 – 60.0	I = 0.016±0.001[His]+0.191±0.016	3.0	9.9
Tyramine	5.0 – 60.0	I = 0.027±0.002[Tyr]+0.215±0.030	3.3	11.1

5.3.4. Recovery Studies

Since the concentration of cadaverine in the analyzed samples was below the limit of detection, recovery tests were performed. The recovery percentages calculations were made according to the IUPAC (31) and the results are depicted in Table 5.5. The developed SI methodology provided recovery ratios of 98 ± 7% (average ± standard deviation) for cadaverine determination. The statistical test, t-test was calculated, and for a 95% significance level the calculated t-value was 0.149 and the correspondent critical value was 2.685, thus indicating no evidence of systematic errors or the presence of some matrix interference (32).

Table 5.5. Recovery percentages obtained with the developed SI system; the initial cadaverine concentration in the samples was above the LOD.

Sample ID	$[Cad]_{added}$ [µmol L^{-1}]	$[Cad]_{found}$ [µmol L^{-1}]	Recovery %
S1	10.0	9.4 ± 0.7	94.5
S1	30.0	28.1 ± 0.9	96.1
S1	40.0	38.3 ± 2.1	95.7
S2	10.0	9.7 ± 1.2	96.7
S3	10.0	10.1 ± 1.4	100.1
S4	10.0	9.01 ± 1.9	90.1
S4	40.0	44.2 ± 1.9	110.4
S6	10.0	10.9 ± 0.7	109.0
S6	30.0	27.0 ± 0.8	99.9
S6	40.0	36.7 ± 2.0	91.8

5.3.5. Figures of Merit

The proposed SI system displayed a limit of detection and quantification of 1.7 µmol L^{-1} and 5.6 µmol L^{-1}, respectively, referring to cadaverine standards. These values were calculated according to IUPAC recommendations, as the concentration corresponding to three and ten times, respectively, the standard deviation of the intercept of three consecutive calibration curves divided by the slope of the same calibration curves (33,34). The corresponding fluorescamine consumption is 60 µg, hydrochloric acid is 18 mg, sodium hydroxide is 0.8 mg, sodium chloride is 12 mg and boric acid is 2 mg per determination.

The typical calibration curve was presented in Table 5.4. The presented calibration curve corresponds to the mean slope and intercept of three curves with the respective standard deviation. After 10 replicate analyses of a standard, the relative standard deviation was estimated as 4.3%.

5.4. Conclusions

The developed sequential injection system for biogenic amines determination in waters proved to be an efficient tool for screening BAs (not alternative as accurate reference method) content in waters. As far as we know, only a few systems are described for biogenic amines determination in water and those ones are mainly based on separation methods, liquid chromatography (5) and capillary electrophoresis (1,2,6,29) giving individual amines results. The proposed flow system presents the advantage of displaying real-time results (6 minutes from loading until measurement) for the total content of BAs, involving relatively low-cost equipment, with portability capability.

As far as we know, in this paper, Chelex 100 was firstly used as sorbent for biogenic amines retention.

Recovery procedures pointed that there is no evidence of systematic errors or the presence of matrix interferences, being a reliable and simple option for screening BAs content in waters.

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Electronic Supplementary Information

Development of a flow-based scr eening method for biogenic amines in waters with on-line solid phase extraction and fluorimetric detection

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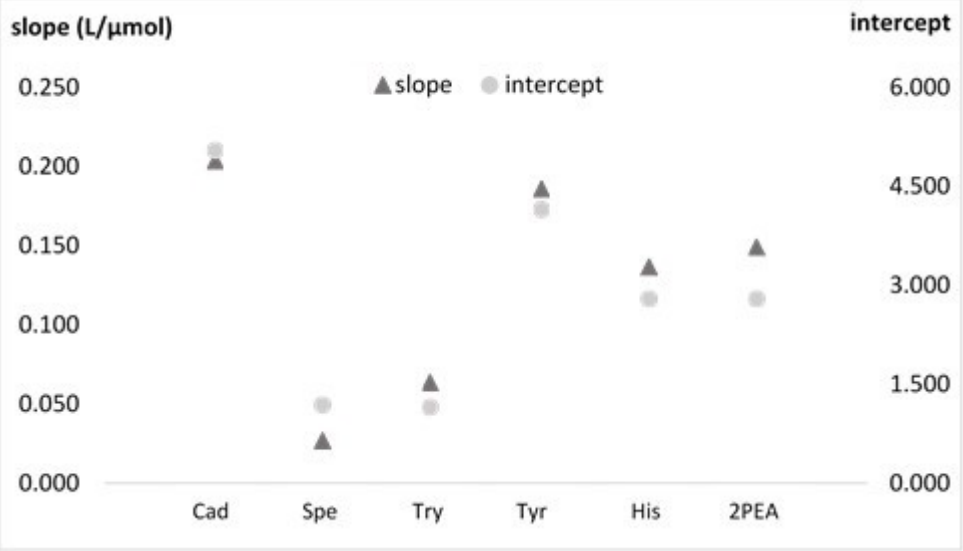

Fig. 5.S1. Slope and intercept of the calibration curves in batch mode for the different biogenic amines.

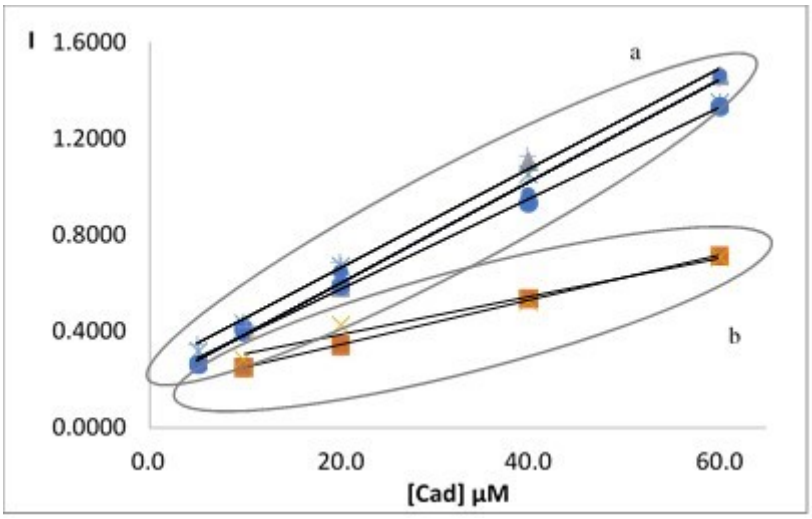

Fig. 5.S2. Cadaverine calibration curves with and without SPE. The group of curves (n=3) with higher slope represents SPE calibration curves (a) and the curves (n=3) with lower slope represents the cadaverine calibration curves without SPE (b).

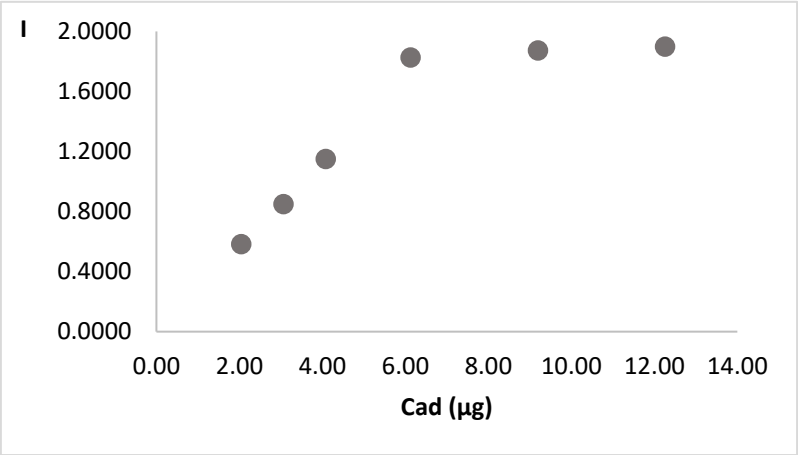

Fig. 5.S3. Study of the breakthrough of the Chelex 100 packed column; the maximum quantity of cadaverine retained by the resin is 6.1 µg.

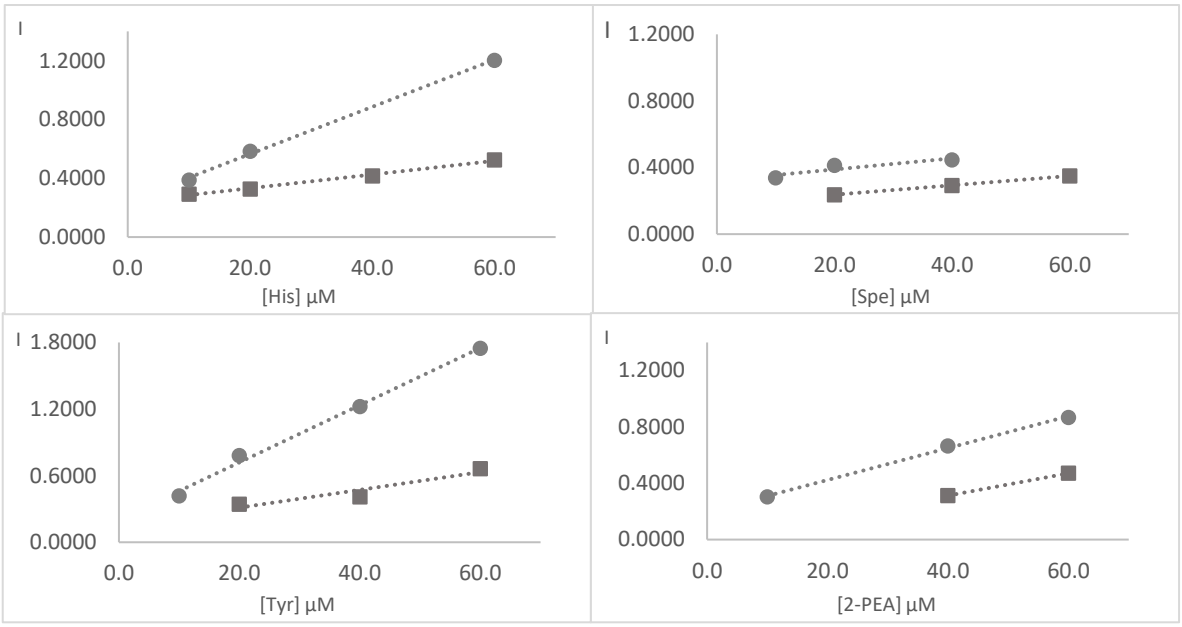

Fig. 5.S4. Calibration curves with and without pre-concentration with Chelex 100 column (■ - without SPE; ● - with SPE).

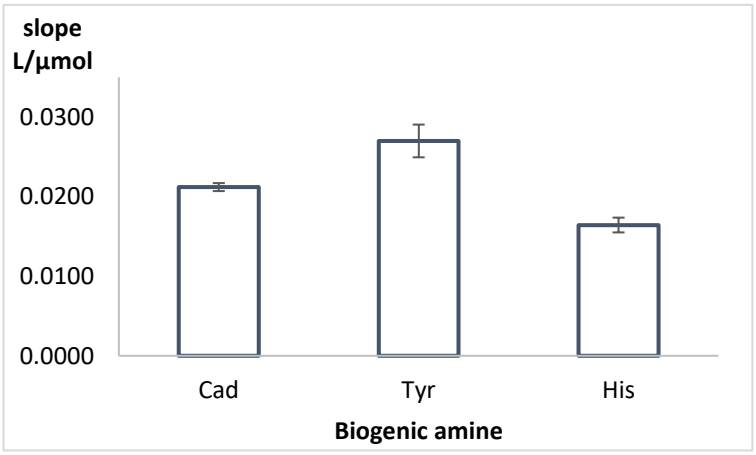

Fig. 5.S5. Slope and respective standard deviation of the typical calibration curves for cadaverine, tyramine and histamine.

CHAPTER 6

A Solid Phase Extraction Flow Injection Spectrophotometric Method for the Zinc Determination in Plants

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Abstract

A solid phase extraction flow injection system for the spectrophotometric determination of total zinc in plant digests was developed. Solid phase extraction was chosen as a strategy for zinc preconcentration and removal of some interferences. The determination of zinc was based on the colorimetric reaction with Zincon. As detection system, a multi-reflection flow cell coupled with a light emitting diode was used. The analytical characteristics of the methodology such as pH of standard/sample solution, nitric acid concentration, the placement of SPE column within the manifold and preconcentration flow rate were studied. The interference of some metals present in plants was also assessed. The limits of detection and quantification achieved were 0.04 mg L^{-1} and 0.12 mg L^{-1}, respectively. The corresponding Zincon consumption was 0.17 mg for each determination. The developed system was applied to plant digests and the results obtained were in agreement with those obtained with reference procedure.

Keywords: Zinc; flow injection analysis; Nitrilotriacetic Acid Superflow resin; multi-reflection flow cell; plant digests

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6.1. Introduction

Heavy metals play an important role in plants metabolism, and its concentration is strictly related with chemical composition of growth media. Some heavy metal such as zinc, one of the most common element in earth crust, also plays a very important role in metabolic processes in plants, the most significant is its activity as component of a variety of enzymes (1). However, zinc is very toxic at higher concentrations affecting plant growth. Zinc is widely used in many industries, namely in the fabrication of batteries and also in dental and medical applications, and this way introduced in the environment by anthropogenic activities.

Several methods are available for zinc determination on plant tissue or in plant digests. One of them is X-ray fluorescence, a non-destructive method and consequently rapid one, but it has the limitation of the lack of standards for plant tissue. The most common way to measure zinc in plants is to analyze the respective digest solution. Plant tissue can be digested by one of many procedures for organic matter decomposition. The plant tissue liquid digest that contain the analyte of interest is then analyzed by Atomic Absorption Spectrometry (AAS) or Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (2). These methods present high selectivity and low detection limits, but also present limitations like relatively high equipment cost, consumption of toxic gases. Simpler methods such as based on colorimetric or voltametric methods were also applied for zinc determination in a variety of samples, such as environmental or biological samples (Table 6.1) (3–22).

In this context, flow analysis systems are very suitable for automating wet chemistry methods, because good reproducibility, precision, low equipment cost, increased accuracy, simplified sample handling, reduced contamination risks, high degree of automation, and reduction in reagent/sample consumption (23). Although a number of flow systems for the determination of zinc in environmental samples, such as waters (9,18), were already developed, only a few have been devoted to plant analysis (7,10). This can be due to the need for a previous sample preparation involving digestion/extraction procedures, which are difficult to achieve in flow systems. Anyway, if the derivatization and subsequent measurement is automated, a significant improvement is achieved.

In this scenario, we propose a flow injection system for the spectrophotometric determination of total zinc content in plants digests. The method is based on the colorimetric determination involving Zincon as chromogenic reagent and a solid phase extraction (SPE) process for zinc pre-concentration and removal of some interferences. To implement this approach, an injector commutator device and a multi-reflection cell were used.

SPE is an emerging process to concentrate the analyte of interest and/or matrix removal (24), presenting some advantages over liquid-liquid extraction, namely to reduce the consumption of organic reagents. This economy of reagents is even larger if SPE is carried out in a flow analysis system. For this SPE step, a lab-made and reusable column of Nitrilotriacetic acid (NTA) was used. NTA is a simple aminocarboxylic acid, is a colorless solid which in the fully deprotonated form acts as a general chelating agent for all metal ions and this sequestering tendency is strongly dependent on the solution pH value (3,25).

The chromogenic reagent used for zinc determination was 2-carboxy-2′-hydroxy-5′-sulfoformazyl-benzene (Zincon). This reagent is a well-known and non-specific reagent for the photometric determination of metals (26) in a variety of environmental and biological samples. This reagent forms a stable blue complex with zinc at a pH of 9. The interference of other metals was minimized by including citrate in the chromogenic solution (9,18).

As a detection system, a multi-reflection flow cell coupled with a light emitting diode (MRC-LED) developed by Ellis P.S. *et al*. (27), was used. By using this system, an enhancement of sensitivity was expected, due to the physical characteristics of this flow cell, increasing the optical path length. A minimization of the physical dispersion of the sample, thus contributing to improve the detection limit, can also be achieved. Another important feature of this cell is the possibility to reduce the schlieren effect (27,28). In fact, different pH conditions are produced in the retention/elution steps and during the colorimetric measurement, so refractive index gradients are expected between adjacent solutions that could impair the analytical signal. The use of this especially designed flow cell proved to be an efficient solution to this problem.

A flow injection methodology with pre-concentration and spectrophotometric detection for zinc determination in plants digests is proposed. This method provides a simple, reliable and miniaturized methodology able to determine zinc content in plants, whose concentration normally lies between 27 and 150 mg kg^{-1} (1). The achieved quantification limit (15 mg kg^{-1}) allows to measure zinc in plants with lowest expected values.

Table 6.1. Analytical characteristics of flow systems developed for zinc determination in environmental samples (presented in descending chronological order).

System	Sample	Sample volume	Pre-concentration material	Reagent	Detection	Sample throughput (h^{-1})	LOD ($\mu g\ L^{-1}$)	Reference
FIA	Plant digests	200 µL	NTA beads	Zincon	Spectrophotometry	12	40	This work
µSI-LOV	Freshwaters	600 µL	NTA beads	Dithizone	Spectrophotometry	13	2.39	(3)
FIA	Water	5 mL	8-hydroxyquinolone funtionalized Amberlite XAD-2	-	FAAS	30	0.33	(4)
MIS	Wastewater	25-200 mL	Amberlite XAD-7	-	FAAS	-	0.027	(15)
MCSWIA	Wet aerosols	-	-	8-quinolazo-epsilon	Spectrophotometry	-	3	(16)
µSI-LOV	Seawater	75 µL	-	FluoZin-3	Fluorescence	60	0.02	(17)
MSFIA	Natural waters	1 mL	-	Zincon	Spectrophotometry	43	2	(18)
SIA	Natural waters	1.5 mL	-	-	Voltammetry	-	3.6	(19)
FIA	Seawater	10 mL[a] and 50 mL[b]	Serdolit Chelite Che	-	FAAS	8[a] and 4[b]	0.055[a] 0.013	(20)
SIA	Herbs	1440 µL	-	-	Voltammetry	10-15	11	(21)
FIA	Natural waters	4.5 mL	Polytetrafluoroethylene-turnings	-	FAAS	30	0.3	(22)
FIA	Environmental	7.6 mL	Bamboo charcoal	-	FAAS	45	0.36	(5)
FIA	Natural waters	7.5 mL	Spherical resin with PAR	PAN	Spectrophotometry	-	0.42	(6)
FIA	Plants digests	1.6 mL	Dowex 1-X8	Zincon	Spectrophotometry	30	100	(7)
FIA	Water	1.5 mL	-	PAN	Spectrophotometry	-	30	(8)
SIA	Water	150 µL	-	Zincon	Spectrophotometry	-	13	(9)
FIA	Plant digests	750 µL	-	NED	Spectrophotometry	65	200	(10)
FIA	Soil	-	C-18-bonded silica gel	-	FAAS	30	0.15	(11)
FIA	Water	30 µL	-	-	Voltammetry	20	17470	(12)
SIA		130 µL				-		
FIA	Water	-	-	-	Voltammetry	8-12	14.7	(13)
MFIA	Plant digests	300 µL	-	Zincon	Spectrophotometry	45	40	(14)

µSI-LOV-micro-Sequential Injection lab-on-valve, FIA-Flow Injection Analysis, FAAS-Flame atomic absorption spectrometry, MIS-microsample injection system, MCSWIA-Multicommutated Stepwise Injection Analysis, MSFIA-multi-syringe injection analysis, SIA-Sequential Injection Analysis, NED-1-naphthylethylenediamine, PAN-1-(2-Pyridylazo)-2-naphthol

[a]- Total dissolved concentration, [b]- dissolved labile metallic concentration

6.2. Experimental

6.2.1. Reagents and Solutions

All solutions were prepared with analytical grade chemicals and MilliQ water (resistivity >18 MΩ cm, Millipore, Bedford, MA, USA). A stock solution of 100 mg L^{-1} zinc (II) standard was prepared by dilution of the 1000 mg L^{-1} atomic absorption standard (Spectrosol, England). Working standards, 0.1–1.0 mg L^{-1}, were daily prepared by dilution of the stock solution.

Nitrilotriacetic Acid Superflow resin (Qiagen, Netherlands), highly cross-linked 6% agarose and bead diameter 60–160 µm, was used as bead suspension for packing the column for zinc (II) retention.

Nitric acid 5 mmol L^{-1} was prepared from the concentrated solution (d = 1.39; 65%, Merck, Germany) and used as elution solution.

A 0.50 mol L^{-1} boric acid buffer solution was prepared by dissolution of the solid (H_3BO_3, Aldrich, Germany) in a solution of 0.2 mol L^{-1} NaOH (Panreac, USA), with the final pH adjusted to 9.0 with sodium hydroxide or nitric acid. Sodium citrate, 0.015 mol L^{-1} was added to boric acid buffer solution in order to minimize some ions interference.

A solution of 2-carboxy-2'-hydroxy-5'-sulfoformacylbenzol (Zincon reagent - $C_{20}H_{15}N_4NaO_6S.H_2O$, Merck, Germany) 5.0 mmol L^{-1} was prepared by dissolving 0.12 g of the solid in the boric acid buffer solution. A solution of 100 µmol L^{-1} of Zincon reagent was prepared weekly by diluting the previous solution in boric acid buffer solution.

A certified reference spinach leaves sample (NIST - SRM 1570a) was analyzed in order to evaluate the accuracy of the developed method.

All solutions used in interference studies (Co, Cu, Ni, Mn) were prepared by diluting commercial atomic absorption standard solution (Spectrosol, England).

6.2.2. Sample Collection and Preparation

Plants were sampled in various locations of recreational parks in Porto district. Samples were cleaned with flowing tap water, deionized water, and oven dried (40 – 60ºC) until constant weight and ground.

Microwave-assisted digestion was performed in different dried samples and the certified reference spinach leaves as follows: two hundred milligrams were mixed with 5 mL of 65% HNO_3 in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Digestion procedure was conducted in five steps (29). The resulting clear solutions were transferred to 25 mL volumetric flasks and the volume made up with ultrapure water. Sample solutions were diluted in a multi-step approach in order to fit their composition to the linear range of the established calibration; the pH of the diluted solutions was between 3 and 5, adjusted with NaOH.

6.2.3. Preparation of the NTA Solid Phase Extraction Column

A laboratory made column with 25 mm length of Tygon tube (Gilson, Villiers-le-Bel, France), 1.85 mm i.d. and 67 µL inner volume was used to pack the NTA resin (60-160 µm, NTA Superflow, Qiagen, Netherlands). Approximately 35 mg of NTA resin was introduced in the column between two pieces of dishwashing foam and subsequently placed in the injector commutator.

6.2.4. Flow Injection Manifold and Procedure

As shown in Fig.6.1, the flow injection manifold consisted on two Minipuls 3 peristatic pumps (Gilson, Villiers-le-Bel, France) equipped with a Tygon pumping tubes (Gilson, Villiers-le-Bel, France) and a commutator injector (laboratory made) to inject defined volumes (200 µL) of samples or standard solutions. The tubing was made of PTFE (0.8 mm i.d., Cambridge, UK) and Gilson end fitting connectors and Y-shaped confluence to link the different parts of the manifold and a reaction coil of 40 cm of tubing.

The detection system consisted in an especially designed multi-reflection flow cell (MRC) (27), equipped with a red light emitting diode (LED -λ_{max} at 660 nm) as a light source connected to a 12 V power supply regulated to 5 V using a multimeter. Analytical signal was recorder by a Kipp and Zonen (Delft, Holland) BD chart recorder and the signal evaluation was made by peak height (cm).

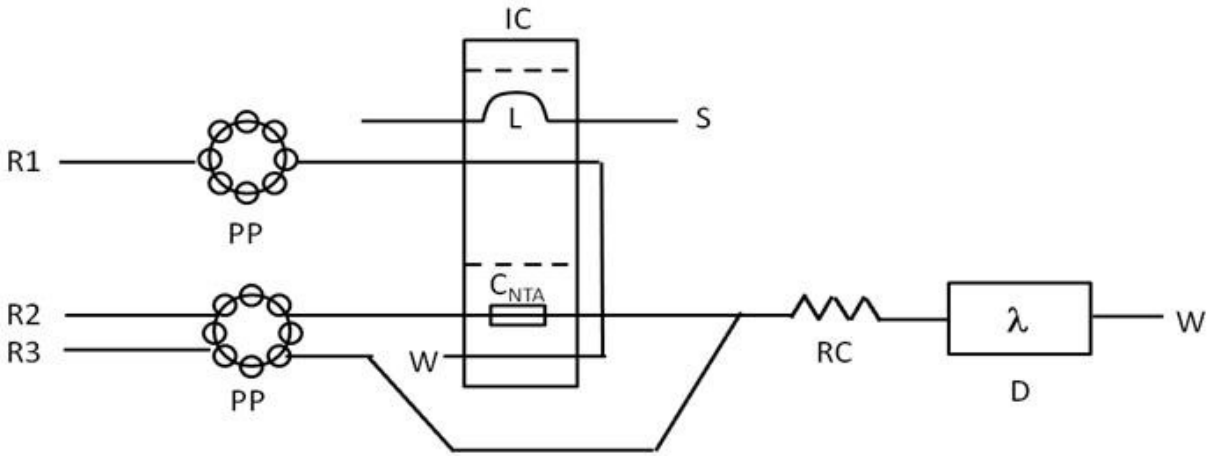

Fig.6.1. Flow injection manifold for Zn determination in plants digests. S – Sample or standard solution; R1 – ultrapure water (0.6 mL min^{-1}); R2 – HNO_3 5 mmol L^{-1} (1 mL min^{-1}); R3 - Zincon 100 µmol L^{-1} (0.7 mL min^{-1}); PP – Peristaltic pump; IC – Injector commutator (position corresponding to sample loading/zinc elution; dashed line corresponds to sample injection/zinc retention); L – sampling loop (V = 200 µL); C_{NTA} – NTA beads column; RC – Reaction coil (40 cm); D – detector multi-reflective flow cell coupled to a LED (660 nm); W – waste.

6.2.5. Reference Procedure

For comparison purposes, the determination of total Zn in plants was carried out on diluted digests of plant using the atomic absorption method as reference method (2). Sample solutions were diluted in a multi-step approach in order to fit their composition to the linear range of the established calibration. Results were compared to those obtained with the developed FIA-SPE method.

6.2.6. Certified Reference Sample

For further accuracy assessment, the developed system was applied to the quantification of zinc in a certified reference spinach sample (NIST - SRM 1570a), available for the determination of major, minor and trace elements in botanical material. The certified reference sample was prepared in the same way as the plant samples (as in 6.2.2), in order to obtain a final diluted solution to fit Zn content in the linear range of the established calibration.

6.3. Results and Discussion

The aim of this study was to develop a flow injection system for the determination of total zinc in plants. So, the system was divided in two analytical procedures, the first one being the injection/resin loading step followed by the elution step along with the spectrophotometric detection carried with MRC coupled with a red LED (λ_{max} 660 nm). Those two steps were carried out based on the use of a commutator injector.

For the first step, a SPE process involving a packed column of NTA beads for zinc pre-concentration and also interferences removal was used. The column was reusable for a period of roughly two weeks. The daily maintenance of the packed NTA column included a continuous wash with nitric acid (5 mM) for about ten minutes and with ultra pure water for the same time before the end of each working day. The performance of the column was maintained, as shown in the intraday repeatability of the method.

6.3.1. Development of the Flow Injection System

The flow procedure involved a number of studies to assess the influence of chemical and physical variables, namely the concentration of nitric acid, the placement of the SPE column within the manifold, and the pre-concentration flow-rate. Some parameters like the reactor length and inner diameter, and configuration of the confluence points were set based on preliminary tests.

The nitric acid concentration was important for the elution step; concentrations of 1.0, 2.5, 5.0 and 10 mM were tested. A concentration of 5 mM of acid was chosen for the elution solution. Below

this value, it took a longer time to elute Zn from the NTA resin and consequently the peak was smaller and wider. For 5.0 or 10 mM nitric acid concentration, equivalent results for zinc were registered; however, with a 10 mM conc., Cu interference was observed. So, 5.0 mM concentration for HNO_3 was chosen for the elution of Zn.

The preconcentration flow rate was also a parameter of study to maximize the interaction between zinc and the NTA resin and reduce the time for each determination. A flow rate of 0.7 mL min^{-1} was chosen as the best flow rate to accomplish those two conditions.

6.3.2. Interference Studies

Zincon is a non-specific chromogenic reagent that forms a blue complex with a variety of different metals. Sodium citrate is referred as a masking agent in order to minimize the interference of some metals such as copper, cobalt, manganese and nickel (9). The concentration of sodium citrate in the buffer solution was also studied because the presence of citrate also causes a decrease in the sensitivity of the zinc determination. Concentrations of 0.3, 0.1, 0.03 and 0.015 M were studied and the last one was chosen as a concentration that did not decrease the sensitivity and can also work as a masking agent for possible metals interferences.

The position of loading and consequently elution was also studied to minimize the interference of copper. If loading and the elution steps were carried out in the same direction, the influence of the presence of copper was minimized; this might occur as the stability constant of the NTA-Cu is higher than NTA-Zn, so it is more difficult to elute copper from the column (25). When loading and elution steps are carried out in the same direction, as the copper should be retained in the beginning of the sorbent material, on the elution step the time elapsed to elute the copper is larger, not significantly influencing the signal.

After this optimization process, several metal ions were tested as possible interference in the developed system for determination of zinc in plants digests. The ions studied in this experiment were Co, Cu, Mn and Ni. The selected concentrations for each ion corresponded to the maximum concentration that can be expected in plants (1). The obtained peak height of a standard with and without the possible interfering ion was analyzed and the interference percentage calculated (Table 6.2). No significant interferences were observed (< 5%), with the exception for Mn that showed an interference of 10%; however, this is in the worst case that could happen, when the concentration of this metal ion in plant is near 300 mg kg^{-1}.

Table 6.2. Interference study of some metal ions.

Tested metal ion	Normal concentration[a] (mg kg^{-1})	Tested concentration (mg kg^{-1})	Signal interference (%)
Co^{2+}	0.02 - 1	1	0
Cu^{2+}	5 - 30	30	0.4
Mn^{2+}	30 - 300	300	10
Ni^{2+}	0.1 - 5	5	0.4

[a] values referenced in Kabata-Pendias (1)

6.3.3. Figures of Merit

The developed FIA system has a limit of detection and quantification of 0.04 mg L^{-1} and 0.12 mg L^{-1}, respectively. These values were calculated according to IUPAC recommendations, as the concentration corresponding to three and ten times, respectively, the standard deviation of the intercept of five consecutive calibration curves divided by the slope of the same calibration curves(30,31). A cycle involving analyte retention, elution and spectrophotometric measurement takes around 5 minutes. The corresponding Zincon consumption is 0.17 mg, nitric acid is 1.6 mg, sodium citrate is 14 mg, sodium hydroxide is 28 mg and boric acid is 108 mg.

The typical calibration curve was $S = 3.7 \pm 0.2\ [Zn] + 0.18 \pm 0.03$, where S is the signal corresponding to the peak height and [Zn] is the zinc concentration in mg L^{-1}. The presented calibration curve corresponds to the mean slope and intercept of three curves with the respective standard deviation. After 10 replicate analysis of a plant digest, the relative standard deviation was estimated as 4.5%.

6.3.4. Application to Plant Digests

The developed system for the determination of total Zn in plants was then applied to plants digests. The accuracy validation was attained by comparing the obtained results with the developed FIA with the results obtained by a reference procedure for Zn determination, atomic absorption spectrometry (AAS) (Table 6.3).

A linear relationship was established between the developed FIA system (C_{FIA} (mg L^{-1})) and the reference procedure for Zinc determination (C_{AAS} (mg L^{-1}) (ES1). And the linear regression obtained was C_{FIA}= 1.01 C_{AAS} - 0.0269 (ESI Fig.6.S1). The intercept was 0.0269 with upper and lower limits of – 0.0376 and + 0.0914 and the slope is 1.01 with 95% confidence interval of 0.9708 – 1.0549. These figures show that the estimated slope and intercept do not differ statistically from 1 and 0 respectively, so, the two set of results do not show systematic differences (32). In addition, relative deviation, between the results obtained with the developed system and reference procedure were calculated and proved that there are no significant differences between the two set of results, RD ≤ 10 % (Table 6.3).

For further accuracy assessment, the developed system was applied to the quantification of zinc in a certified reference spinach sample (SRM 1570a), 82.3 ± 3.9 mg L^{-1}. The value obtained with the developed FIA system was 84.6 ± 1.2 mg L^{-1}, and so a relative deviation of 2.8 % was observed, that validates also the developed method for zinc determination.

Table 6.3. Comparison of the results obtained with the developed flow injection system (FIA) for zinc determination to those obtained with atomic absorption spectrometry (AAS) for accuracy validation. SD. Standard deviation, RD, Relative deviation.

	FIA		AAS		RD %
Sample ID	[Zn] mg L^{-1}	SD	[Zn] mg L^{-1}	SD	
P1	0.62	0.01	0.66	0.00	-6.1
P2	0.79	0.00	0.85	0.01	-7.1
P4	0.74	0.01	0.75	0.00	-1.3
P6	0.58	0.01	0.59	0.00	-1.7
P7	0.79	0.01	0.84	0.01	-6.0
P8	1.83	0.01	1.75	0.01	4.6
P9	0.80	0.00	0.79	0.01	1.3
P10	3.51	0.00	3.52	0.01	-0.3

6.4. Conclusion

The developed flow injection system for zinc determination in plant digests proved to be an efficient tool for plants analysis, displaying a low limit of detection (LOD=0.04 mg/L). As far as we know, only a few systems were described for the determination of zinc in plant digests using flow analysis (7,10,14). When compared with similar methodologies applied to plant digests the proposed method presents better features; the detection limit is about ten times lower than those displayed by Ribeiro *et al*. (7) and Dias *et al*. 2004 (10).

Although the limit of detection is similar to the one presented by Oliveira *et al*. 1996 (14), the system herein proposed presents some other advantages like the need for a lower quantity of dry sample for the digestion process and also a lower volume of this digest is needed for the analysis. The reagents used by Oliveira *et al*., like KCN or formaldehyde have very known toxicity, although they are employed with the objective of measuring two metal ions. The consumption of reagents and consequently production of effluents are also lower in our work. The proposed system involving the use of an ion-exchange resin also allows to measure Zn in digests displaying an intrinsic absorption, as the other matrix components are discarded during the pre-concentration step, and only the retained analyte is measured after elution.

Another novelty in the present study is the use of a multi-reflection cell as part of the detection system; it showed to be an advantageous set up for detection due to the physical characteristics of the flow cell. As described before (33), the physical configuration of the flow cell contributes to improve the limit of detection and minimizes refractive index gradients produced in the confluence of the nitric acid and the boric acid buffer. With the use of this cell coupled to a red-light emitting diode, an enhancement of sensitivity was also achieved. These advantages were confirmed in preliminary experiments in comparison with a conventional flow cell.

The results obtained with the developed system for zinc determination in plant digests were in agreement with those obtained with reference procedure. The result obtained with reference material was also in agreement with the expected result.

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Electronic Supplementary Information

A solid phase extraction flow injection spectrophotometric method for the zinc determination in plants

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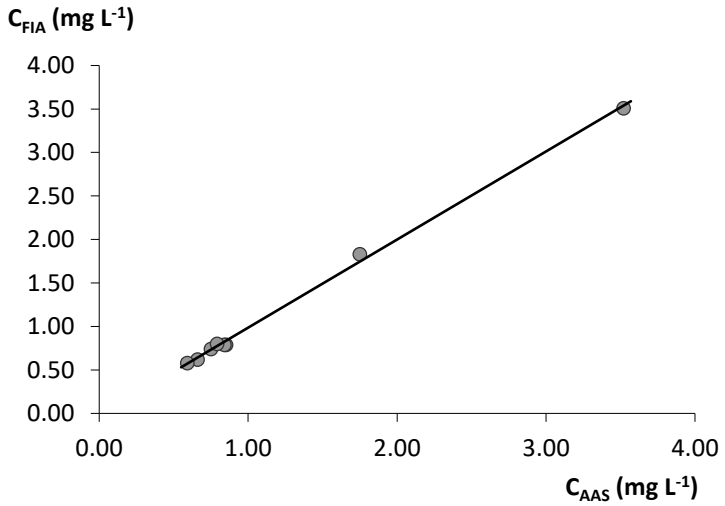

Fig. 6.S1. Comparison of the results obtained with the developed FIA system and those obtained with a reference method (AAS). The full line represents the linear relationship between the two methodologies.

CHAPTER 7

General Conclusions

7.1. General conclusions

The developed solid phase extraction flow-based methods proved to be valuable tools for environmental monitoring. By including the sample preparation step into the automatic method, the overall analysis set-up becomes simplified. When the two techniques are coupled (flow analysis and the solid phase extraction), the final features are more advantageous in comparison with the individual features. And so, a more favourable analytical method in terms of sensitivity, selectivity and throughput is devised. Additionally, it is also an advantageous method considering the Green Chemistry point of view.

The use of a PIM containing D2EHPA, and a Chelex 100 resin, showed to be an interesting choice to selectively retain zinc and copper, respectively (chapter 3). As far as we know, it was the first time that a PIM was used as a sorbent material in a flow-based method. This membrane provided the in-line retention of zinc (eliminating zinc from the matrix) and this way the copper determination was performed. Furthermore, a PIM is a membrane fairly easy to produce in the laboratory. The use of this lab-made sorbent did not impair the features of the method, when compared with similar methodologies already described by other authors for copper determination. By using, in the same manifold, two different extraction columns and a non-specific reagent, it was possible to perform the individual determination without resorting to extra calculations, thus reducing the associated errors. When comparing PAR with other non-specific reagents, it should be mentioned that is a water-soluble reagent and so the use of organic solvents on its preparation is not necessary. Furthermore, PAR is considered a non-hazardous substance.

The developed flow system for iron quantification in fresh and marine water (chapter 4) showed to be an efficient tool for water monitoring. In the same apparatus, two different strategies were developed for iron quantification, one of those resorting to a SPE process. In the proposed method, SPE could act as sample matrix clean-up and/or iron enrichment. For this purpose, a NTA resin column (chelating resin) was the chosen sorbent to perform iron extraction. The system can be applied for the determination of iron in waters with high salt content (like marine or estuarine) and/or low iron content. However, in the presence of fresh waters with high iron content and low salt content, there is the possibility of performing the quantification without resorting to the SPE strategy (direct determination), minimizing this way the time needed per analysis. The use of the newly reported low toxicity iron chelator, MRB13, provided similar features for iron spectrophotometric determination when compared with other similar parent ligands previously used.

A screening method for the total content of biogenic amines in natural waters was successfully developed (chapter 5). In this method, a SPE technique was incorporated in the flow system aiming for the pre-concentration of the analyte. This pre-concentration was successfully achieved by resorting to the Chelex 100 resin, a cation exchange resin. As far as we know, biogenic amines are mainly determined based on chromatography-like separation methods. These conventional methods are usually laborious, time-consuming and involve the use of organic solvents. With the developed flow-based system, an analytical method that employs relatively low-cost equipment and reagents was developed. Additionally, the method described in chapter 5 displays real-time results and has the possibility of portability for in-situ analysis.

A flow-based system for the zinc determination (chapter 6) was developed and efficiently applied to this metal quantification in plant digests. When compared to similar methods, the system described herein needed lower quantities of sample for digestion, lower volume of the digest for analysis, lower volume of reagents, and the colour reagent presents lower toxicity. By means of a column packed with NTA resin coupled to the flow system, possible interfering species of the sample matrix were discarded. The use of a multi-reflection flow cell equipped with a light emitting diode showed to be an advantageous detection system due to the intrinsic physical characteristics of the flow-cell; the use of this flow cell minimized refractive index signals and improved the sensitivity of the method (lowering the limit of detection) when compared with a conventional flow cell.

I would also like to point that flow-based systems are very versatile, being their apparatus fairly easy to adapt for different determinations. The associated versatility of the flow-based systems played also an important role when coupling different sample preparation strategies within the flow-based apparatus. Coupling solid phase extraction to flow systems for the miniaturized and automatic sample preparation and quantification, brought notable advantages to the chemical analysis field.

7.2. Some Suggestions for Future Work

The continuous need of improving sensitivity and selectivity of the analytical determinations leads to the continuous need of developing new analytical tools in the analytical chemistry field. Furthermore, the analytical chemistry development is indispensable due to the rising knowledge of the risk associated to chemicals and to the emerging contaminants.

As suggestions for future work, several other potentialities could be exploited by taking advantage of the improved features when coupling sample pre-treatment and flow-based methods. It would be of high value the development of new methods for the determination of toxic metals, usually present at trace level in the environment, including in-line SPE as the strategy for pre-concentration, aiming for reducing the quantification limits.

For the in-line SPE, the use of new sorbent materials to respond to different analytes could be of high interest, thus promoting the selectivity and sensitivity of the analysis. The new materials could be designed, for instance, for the quantification of cadmium, lead, nickel and other metals, to improve analytical features, specially lowering limits of detection.

Taking advantage of the intrinsic characteristics of the systems and conditions developed during this thesis, it could be interesting to adapt the methods to the new generation of flow-based systems (higher associated miniaturization) such as lab-on-valve or μSIA. Furthermore, these new generation of flow systems have the advantage of possible portability, making them suitable for real-time analysis.

List of Publications

List of Publications

Publications in International Scientific Periodicals with Referees

Tânia C. F. Ribas, Raquel B. R. Mesquita, Tânia Moniz, Maria Rangel, António O. S. S. Rangel, Greener and wide applicability range flow-based spectrophotometric method for iron determination in fresh and marine water, Talanta 216 (2020) 120925.

https://doi.org/10.1016/j.talanta.2020.120925

Tânia C. F. Ribas, Ildikó V. Toth, António O. S. S. Rangel, A sequential injection fluorimetric methodology with in-line solid phase extraction for biogenic amines screening in water, International Journal of Environmental Analytical Chemistry 99 (2019) 270-281.

https://doi.org/10.1080/03067319.2019.1591390

Tânia C. F. Ribas, Ildikó V. Toth, António O. S. S. Rangel, A solid phase extraction flow injection spectrophotometric method for the zinc determination in plants, Microchemical Journal 130 (2017) 366–370.

http://dx.doi.org/10.1016/j.microc.2016.10.016

Poster Presentations

Tânia C. F. Ribas, Charles F. Croft, Tânia Moniz, M. Inês G.S. Almeida, Raquel B. R. Mesquita, Maria Rangel, Spas D. Kolev, António O. S. S. Rangel, Biparametric sequential injection system with on-line solid phase extraction for the determination of copper and zinc in waters, 26th Encontro Nacional da SPQ, Porto, Portugal (July 2019).

Tânia C. F. Ribas, Charles F. Croft, Tânia Moniz, M. Inês G.S. Almeida, Raquel B. R. Mesquita, Maria Rangel, Spas D. Kolev, António O. S. S. Rangel, Use of solid-phase extraction in a

sequential injection mode for the determination of micronutrients, 14th International Conference on Flow Analysis, Bangkok, Thailand (December 2018), also presents as a pitch presentation.

Tânia C. F. Ribas, Charles F. Croft, Raquel B. R. Mesquita, M. Inês G.S. Almeida, Spas D. Kolev, António O. S. S. Rangel, Polymer Inclusion Membranes (PIMS) as an alternative for on-line solid phase extraction (SPE) in flow analysis, Symposium of the Analytical Division of SPQ, Porto, Portugal (March 2018).

Tânia C. F. Ribas, Charles F. Croft, Raquel B. R. Mesquita, M. Inês G. S. Almeida, Spas D. Kolev, António O. S. S. Rangel, Polymer inclusion membranes (PIMs) as an alternative solid phase extraction (SPE) material: comparison studies between batch and flow procedure, 21st International Conference of Flow Injection Analysis and related techniques (21st ICFIA), St Petersburg, Russia (September 2017).

Tânia C. F. Ribas, Ildikó V. Tóth, António O. S. S. Rangel, Use of on-line solid phase extraction and a multi-reflection flow cell coupled with a LED for the spectrophotometric determination of zinc: application to plant digests, 20th International Conference of Flow Injection Analysis and related techniques (20th ICFIA), Palma de Mallorca, Spain (October 2016)

Tânia C. F. Ribas, Ildikó V. Tóth, António O. S. S. Rangel, Primary amines in natural waters: sequential injection methodology for its fluorescent detection, 20th International Conference of Flow Injection Analysis and related techniques (20th ICFIA), Palma de Mallorca, Spain (October 2016).

Tânia C. F. Ribas, Ildikó V. Tóth, António O. S. S. Rangel, A solid phase extraction flow injection methodology with spectrophotometric detection for the zinc determination in plant digests, Symposium of the Analytical Division of SPQ, Lisboa, Portugal (June 2016).

Tânia C. F. Ribas, Ildikó V. Tóth, António O. S. S. Rangel, Microplate and flow injection strategies for the spectrophotometric detection of zinc (II) with two complexing agents: PAR and Zincon, XX Luso-Galego Conference of Chemistry, Porto, Portugal (November 2014).

Tânia C. F. Ribas, Ildikó V. Tóth, António O. S. S. Rangel, High throughput microplate assays for the spectrophotometric study of metal complexes involving Zincon and PAR, Symposium of the Analytica Division of SPQ, Coimbra, Portugal (April 2014).

Oral Communications

Tânia C. F. Ribas, Charles F. Croft, Tânia Moniz, M. Inês G.S. Almeida, Raquel B. R. Mesquita, Maria Rangel, Spas D. Kolev, António O. S. S. Rangel, Interferences minimization using solid phase extraction in a multiparametric sequential injection system, XXIV Encontro Luso-Galego de Química, Porto, Portugal (November 2018).