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IMPLEMENTATION OF FLOW MICROEXTRACTION TECHNIQUES FOR MONITORING OF PARAMETERS OF ENVIRONMENTAL INTEREST

Ruth Suárez Sánchez

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Doctoral Programme of Chemical Science and Technology

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Universitat de les Illes Balears

Dr. Víctor Cerdà Martín and Dr. Jessica Avivar Cerezo, both from the Universitat de les Illes Balears

WE DECLARE:

That the thesis entitled *Implementation of flow microextraction techniques for monitoring of parameters of environmental interest,* presented by Ruth Suárez Sánchez to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for the mention as European Doctorate.

For all intents and purposes, we hereby sign this document.

Signature

Dr. Víctor Cerdà Martín

Dr. Jessica Avivar Cerezo

Palma de Mallorca, 14 September 2015

Universitat de les Illes Balears

Dr. Víctor Cerdà Martín y Dra. Jessica Avivar Cerezo, ambos de la Universitat de les Illes Balears

DECLARAN

Que la tesis doctoral que lleva por título *Implementation of flow microextraction techniques for monitoring of parameters of environmental interest*, presentada por Ruth Suárez Sánchez para la obtención del título de doctor, ha sido dirigida bajo nuestra supervisión y que cumple con los requisitos necesarios para optar a la mención de Doctor Europeo.

Y para que quede constancia de ello firmamos este documento

Firmas

Dr. Víctor Cerdà Martín

Dr. Jessica Avivar Cerezo

Palma de Mallorca, 14 de septiembre de 2015

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To my parents and to Emilio

(A mis padres y a Emilio)

Abbreviations

$[C_4MIM][PF_6]$	1-butyl-3-methylimidazolium hexafluorophosphate
$[C_6MIM][PF_6]$	1-hexyl-3-methylimidazolium hexafluorophosphate
$[C_8MIM][PF_6]$	1-octyl-3-methylimidazolium hexafluorophosphate
3,4-HPO	3-hidroxy-4-pyridinone
AAS	Atomic absorption spectrometry
Al	Aluminium
ANOVA	Application of analysis of variation
AS	Anionic surfactants
BI	Bead injection
BZ3	Benzophenone-3
CCD	Central composite design
CE	Capillary electrophoresis
CPE	Cloud point extraction
CS	Cationic surfactants
DBAS	Disulfine blue active substances
DLL	Dynamic link libraries
DLLME	Dispersive liquid-liquid microextraction
EDB	Ethylhexyl dimethyl p-aminobenzoate
EHS	Ethylhexyl salicylate
EU	European Union
FAAS	Flame atomic absorption spectrometry
FIA	Flow injection analysis
GC	Gas chromatography
GF-AAS	Graphite furnace - atomic absorption spectrometry
HBL	Hydrophilic/lipophilic balance
HC	Holding coil
HF-LPME	Hollow fiber - liquid phase microextraction
HMS	Homosalate
ICP-AES	Inductively coupled plasma - atomic emission spectrometry
ICP-MS	Inductively coupled plasma - mass spectrometry
IF	Impact factor
IL	Ionic liquid
IN	Inner diameter
IV	Injection valve

LC	Liquid chromatography
LED	Light emitting diode
LLE	Liquid-liquid extraction
LMG	Lumogallion
LOD	Limit of detection
LOF	Lack of fit
LOV	Lab-on-valve
LPME	Liquid phase microextraction
MAF-8HQ	8-hydroxyquiniline
MALLE	Membrane assisted liquid-liquid extraction
MBC	4-Methylbenzylidene camphor
MBSA	Methylene blue active substances
MCFIA	Multicommutated flow injection analysis
MPFS	Multipumping flow systems
MPV	Multiposition valve
MSA	Magnetic stirring assisted
MSC	Multisyringe chromatography
MSFIA	Multisyringe flow injection analysis
NTA	Nitrilotriacetic acid
OCR	2-ethylhexyl 2-cyano-3,3-diphenylacrylate
OD	Outside diameter
PEEK	Polyetheretherketone
PMMA	Polymethylmethacrylate
PMT	Photomultiplier
PP	Peristaltic pump
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
RC	Reaction coil
RSD	Relative standard deviation
SAA	Surface active agents
SDME	Single drop microextraction
SFA	Segmented flow analysis
SIA	Sequential injection analysis
SI-LOV	Sequential injection lab-on-valve
SP	Syringe pump
SPE	Solid-phase extraction

SPME	Solid phase microextraction
SPS	Solid phase spectrometry
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
UV-VIS	Ultraviolet-visible
VIS	Visible
WHO	World health organization
WWTP	Waste water treatment plant

Abstract

Water monitoring has become essential owing to the increasing income of contaminants into the aquatic environment. In this sense, governments have increased water control through tighter regulations. Hence, it is important to develop efficient analytical methods in terms of cost, precision, throughput and environmental impact to control pollutant releases and verify compliance with respect to defined regulations.

However, environmental analysis poses some difficulties due to matrix complexity, since the analytes of interest are usually at trace levels and there is a wide variety of potential interferences, making almost mandatory the sample pretreatment prior detection. New trends are focused in the use of microextraction techniques, such as solid phase microextraction (SPME) and dispersive liquid-liquid microextraction (DLLME). Nonetheless, pretreatment steps are time consuming and involve a large consumption of reagents and sample when carried out in a manual approach. Thus, automation of sample pretreatment plays a major role in order to achieve efficient and fast analytical methods.

In this thesis, different automatic analytical flow-based methodologies were developed for the determination of parameters of environmental interest. They are based on the implementation of microextraction techniques, i.e. SPME and DLLME, in flow based systems, in particular exploiting sequential injection analysis, multisyringe flow injection analysis and lab-on-valve. Spectrophotometric and fluorimetric detection techniques were used allowing the development of fully automated analyzers. Thus, in this thesis the potential of flow analysis techniques to accommodate a variety of pretreatment techniques is proven by the development of seven automated analytical systems to determine iron, aluminium, anionic and cationic surfactants and UV filters, applied to environmental water samples. All the developed analyzers provide a significant reduction of the reagents and sample consumption, a great reproducibility and sensitivity and an improved sample throughput in comparison to classical methods. In addition, benefits of hyphenating flow and chromatographic techniques and coupling strategies are presented in a comprehensive review.

The works included in this thesis are listed below:

1. A study of the applicability of bidentate 3-hydroxy-4-pyridinone ligands as nontoxic reagents for the determination of iron in natural waters in a sequential injection approach with spectrophotometric detection.
2. A fully automated LOV system for iron speciation by microsequential injection solid phase spectrometry using 3-hydroxy-1(H)-2-methyl-4-pyridinone as chromogenic reagent and a nitrilotriacetic acid Superflow resin, which could be replaced in a fully automated way, that expanded the applicability of the method to inland and coastal bathing waters.
3. A fully automated in-syringe dispersive liquid−liquid microextraction system for the fluorimetric determination of aluminium in seawater using lumogallion as a fluorescence reagent.
4. A fully automated in-syringe magnetic stirring-assisted (MSA) dispersive liquid-liquid microextraction system applied to the fluorimetric determination of aluminium in seawater samples using lumogallion. The implementation of the MSA system permitted the reduction of organic solvents consumption and a faster and improved mixing efficiency.
5. A simplification of the methylene blue active substance index method using in-syringe magnetic stirring-assisted dispersive liquid-liquid microextraction with the novelty of setting the syringe up-side down in order to use chloroform as extraction solvent to achieve comparability toward the standard procedure for methylene blue active substances determination.
6. An automated method for the determination of cationic surfactants in water samples using in-syringe magnetic stirring-assisted dispersive liquid-liquid microextraction with solvent washing method.
7. A fully automated, fast, simple, cost-effective and environmental friendly method based on in-syringe magnetic stirring-assisted dispersive liquid-liquid microextraction coupled to HPLC allowing the on-line extraction, preconcentration, separation and detection of six UV filters from surface seawater samples, exploiting ionic liquids as alternative environmental friendly extractants.
8. A comprehensive review dealing with the state of art and future trends in coupling separation techniques, such as chromatographic and capillary electrophoresis, and flow analysis techniques for environmental and biological samples analysis. In addition, different coupling strategies are discussed, highlighting their benefits and disadvantages.

Resumen

Debido al aumento de la entrada de contaminantes en el medio acuático, el control del agua es esencial. En este sentido, los gobiernos han aumentado el control del agua a través de regulaciones más estrictas. Por lo que es importante desarrollar métodos analíticos eficientes en términos de coste, precisión, frecuencia de análisis e impacto ambiental para controlar las emisiones de contaminantes y así verificar el cumplimiento de las normas definidas.

Sin embargo, los análisis medioambientales plantean algunas dificultades debido a la complejidad de las matrices de las muestras, ya que por lo general los analitos de interés están a nivel de trazas y hay una amplia variedad de posibles interferentes, haciendo casi obligatoria una etapa de pretratamiento de la muestra antes de la detección. Las nuevas tendencias se centran en el uso de técnicas de microextracción, tales como la microextracción en fase sólida (SPME) y la microextracción líquido-líquido dispersiva (DLLME). No obstante, las etapas de pretratamiento son largas e implican un gran consumo de reactivos y muestra cuando se llevan a cabo de forma manual. Así, la automatización del pretratamiento de la muestra juega un papel importante para lograr métodos analíticos eficientes y rápidos.

En esta tesis, se han desarrollado diferentes metodologías basadas en técnicas de análisis en flujo para la determinación de parámetros de interés ambiental. Estas metodologías se basan en la implementación de técnicas de microextracción, es decir SPME y DLLME, en sistemas en flujo, en particular utilizando sistemas de análisis por inyección secuencial, sistemas de análisis por inyección en flujo multijeringa y lab-on-valve. El uso de técnicas de detección espectrofotométricas y fluorimétricas ha permitido el desarrollo de analizadores totalmente automáticos. Así, el potencial de las técnicas de análisis en flujo para implementar diferentes técnicas de pretratamiento de la muestra queda demostrado en esta tesis con el desarrollo de siete sistemas analíticos automáticos para determinar hierro, aluminio, tensioactivos aniónicos y catiónicos, y filtros UV, aplicados a muestras de agua ambiental. Todos los analizadores desarrollados proporcionan una reducción significativa del consumo de los reactivos y de la muestra, una gran reproducibilidad, sensibilidad y una mejora en frecuencia de análisis en comparación con los métodos clásicos. En esta tesis, también se presenta una revisión bibliográfica mostrando los beneficios del acoplamiento entre las técnicas de análisis en flujo y las técnicas cromatográficas así

como estrategias de acoplamiento.

Los trabajos incluidos en esta tesis son los siguientes:

1. Un estudio de la aplicabilidad de ligandos bidentados 3-hidroxi-4-piridinona como reactivos no tóxicos para la determinación de hierro en aguas naturales utilizando un sistema de inyección secuencial con detección espectrofotométrica.
2. Un sistema totalmente automático mediante lab-on-valve para la especiación de hierro por inyección microsecuencial y espectrometría en fase sólida utilizando 3-hidroxi-1 (H)-2-metil-4-piridinona como reactivo cromogénico y una resina de ácido nitrilotriacético Superflow, que puede ser renovada de forma totalmente automática y que ha permitido ampliar la aplicabilidad del método a muestras de agua de baño, es decir de río y de mar.
3. Un sistema totalmente automático utilizando la microextracción líquido-líquido dispersiva en jeringa para la determinación fluorimétrica de aluminio en agua de mar usando lumogallion como reactivo de fluorescencia.
4. Un sistema totalmente automático utilizando la microextracción líquido-líquido dispersiva en jeringa con agitación magnética asistida aplicado a la determinación fluorimétrica de aluminio en muestras de agua de mar usando lumogallion. La implementación del dispositivo de agitación magnética permitió la reducción del consumo de disolventes orgánicos, la mejora de la eficiencia de mezcla y una mayor rapidez.
5. Una simplificación del método para la determinación del índice de sustancias activas frente al azul de metileno utilizando un método de microextracción líquido-líquido dispersiva en jeringa con agitación magnética asistida con la novedad de colocar la jeringa hacia abajo con el fin de utilizar cloroformo como extractante y así lograr la comparabilidad con el procedimiento estándar para la determinación de sustancias activas de azul de metileno.
6. Un método automático para la determinación de tensioactivos catiónicos en muestras de agua utilizando la microextracción líquido-líquido dispersiva en jeringa con agitación magnética asistida incluyendo una etapa de lavado del disolvente.
7. Un método totalmente automático, rápido, simple, rentable y respetuoso con el medioambiente basado en un sistema de microextracción líquido-líquido dispersiva en jeringa con agitación magnética asistida acoplado a HPLC. Esto

ha permitido la extracción, preconcentración, separación y detección en línea de seis filtros UV en muestras superficiales de agua de mar, utilizando líquidos iónicos como extractantes.

8. Una revisión del estado actual y futuras tendencias en el acoplamiento de técnicas de separación, como las cromatográficas y la electroforesis capilar, a las técnicas de análisis en flujo para el análisis tanto de muestras ambientales como biológicas. Además, en esta revisión se discuten diferentes estrategias de acoplamiento, destacando sus ventajas y limitaciones.

List of publications

This thesis is presented as a thesis by publication. The publications included in this thesis are:

[1] Ruth Suárez, Burkhard Horstkotte, Carlos M. Duarte, Víctor Cerdà, *Fully-Automated Fluorimetric Determination of Aluminum in Seawater by In-Syringe Dispersive Liquid-Liquid Microextraction Using Lumogallion*, Analytical Chemistry, 84 (2012) 9462-9469. DOI: 10.1021/ac302083d. IF: 5.695.

[2] Burkhard Horstkotte, Ruth Suárez, Petr Solich, Víctor Cerdà, *In-syringe-stirring: A novel approach for magnetic stirring-assisted dispersive liquid-liquid microextraction*, Analytica Chimica Acta, 788 (2013) 52-60. DOI:10.1016/j.aca.2013.05.049. IF: 4.517.

[3] Raquel B. R. Mesquita, Ruth Suárez, Víctor Cerdà, Maria Rangel, António O. S. S. Rangel, *Exploiting the use of 3,4-HPO ligands as nontoxic reagents for the determination of iron in natural waters with a sequential injection approach*, Talanta, 108 (2013) 38-45. DOI: 10.1016/j.talanta.2013.02.058. IF: 3.511

[4] Ruth Suárez, Burkhard Horstkotte, Victor Cerdà, *In-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction for automation and downscaling of methylene blue active substances assay*, Talanta, 130 (2014) 555-560. DOI: 10.1016/j.talanta.2014.06.063. IF: 3.545

[5] Burkhard Horstkotte, Ruth Suárez, Petr Solich, Victor Cerdà, *In-syringe magnetic stirring assisted dispersive liquid-liquid micro-extraction with solvent washing for fully automated determination of cationic surfactants*, Analytical Methods, 6 (2014) 9601-9609. DOI: 10.1039/v4ay01695e. IF: 1.821

[6] Ruth Suárez, Raquel B. R. Mesquita, Maria Rangel, Víctor Cerdà, António O. S. S. Rangel, *Iron speciation by microsequential injection solid phase spectrometry using 3-hydroxy-1(H)-2-methyl-4-pyridinone as chromogenic reagent*, Talanta, 133 (2015) 15-20. DOI: 10.1016/j.talanta.2014.03.059. IF: 3.545

[7] Sabrina Clavijo, Jessica Avivar, Ruth Suárez, Víctor Cerdà, *Analytical strategies for coupling separation and flow-injection techniques*, TrAC Trends in Analytical Chemistry, 67 (2015) 26-33. DOI: 10.1016/j.trac.2014.11.019. IF: 6612

[8] Ruth Suárez, Sabrina Clavijo, Jessica Avivar, Víctor Cerdà, *On-line in-syringe magnetic stirring assisted dispersive liquid-liquid microextraction HPLC - UV method for UV filters determination using 1-hexyl-3-methylimidazolium hexafluorophosphate as extractant*, Talanta (2015) accepted. DOI:10.1016/j.talanta.2015.10.031. IF: 3.545

CHAPTER 1
INTRODUCTION

In this chapter, a general overview of environmental analysis is presented, together with a detailed description of flow analysis techniques and their evolution. In addition, most relevant separation and preconcentration techniques, such as solid phase extraction, liquid-liquid extraction and their implementation in flow based systems is described. Also benefits and strategies of coupling chromatographic and flow analysis techniques are discussed.

1. INTRODUCTION

All freshwater bodies are interconnected, from the atmosphere to the sea, via the hydrological cycle. Thus, water constitutes a continuum, with different stages ranging from rainwater to sea waters, being a vital resource to mankind. Moreover with the exponential increase in population, water is currently one of the biggest concerns worldwide. Several contaminants of different kinds are being introduced into the aquatic environment by anthropogenic activities, e.g. air pollutants from industry and power plants, emissions from transportation vehicles, radionuclides from nuclear weapons tests and pesticides, sewage, detergents and other chemicals. Precisely, for all this, monitoring and control of the exposure to dangerous chemical substances in water samples has become essential [1].

Furthermore, since many diseases are related to the exposure to hazardous substances, most countries have increased the water control through tighter regulations. For instance, the European Union created the Water Framework Directive [2] in order to improve, protect and prevent further deterioration of water quality across Europe. This fact has lead to a high increase in healthcare costs but also to a decrease of around 70% of the impact of pollution in European surface waters caused by industrial discharges of toxic substances over the past 30 years [3].

Hence, it is important to control pollutant releases, determine emission patterns and verify compliance with respect to defined standards and regulations. Environmental studies are focused on the determination of the presence of target analytes, concentration levels and distribution in particular areas or ecosystems in different kind of matrices. These species may be found at different levels of concentration at the different environmental compartments. Furthermore, environmental analysis poses some difficulties due to sample matrices complexity, the low concentration of analytes of interest which are usually at trace levels and the wide variety of potential interferences present. Moreover, field sampling programmes often generate a large number of samples to be processed, which makes necessary the development of efficient analytical methods in terms of cost, precision, throughput and environmental impact. Automation of analytical methods can fulfil these requirements.

Flow analysis techniques are well-established and powerful tools for automation, providing several benefits, such as: (a) increased sample throughputs, (b) versatile methods, (c) on-line sample pretreatment, (d) minimization of sample handling, (e)

more environmental friendly procedures and (f) enabling the coupling to a variety of detection systems [4]. The main drawback of flow analysis techniques is their lack of selectivity. However, flow analysis techniques can implement pretreatments or be coupled to selective detectors or separative techniques, e.g. high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), to achieve high selective analytical methods. The benefits of combining these techniques are noteworthy. Moreover, sensitivity can be improved by preconcentration with e.g. solid phase extraction (SPE) and liquid-liquid extraction (LLE).

Below is given a brief description of the evolution of flow analysis techniques. In addition, special attention is paid to the implementation of sample treatment, e.g. SPE and dispersive liquid-liquid microextraction (DLLME) and the coupling between flow and chromatographic techniques.

1.1. Evolution of flow analysis techniques

In the 1950's clinical tests started to be increasingly demanded for diagnostic purposes in medicine, leading to a huge number of samples to be processed. Thus, the need for efficient analytical methods in terms of analysis frequency, precision and costs promoted the development of automatic methods of analysis [5]. Since then, flow-based techniques have played a major role in automation [4], providing advantages that should be highlighted, such as minimization of both reagent and sample consumption, minimization of sample contamination risk since these techniques are closed systems, as well as enhanced sampling throughput, implying less personal and consumable costs.

Additionally, in automated flow systems human error is avoided and the analytical system is isolated from the environment, which leads to improved repeatability and reproducibility. Furthermore, analyst safety is also improved by automation, when dealing with samples or chemicals which are harmful, e.g. radioactive samples or carcinogenic chemicals. For instance, automation of liquid-phase extraction methods is of high interest since this reduces analyst exposure to organic solvents. Moreover, the minimization of reagents consumption not only improves cost efficiency but also provides lower waste generation making analytical methodologies greener.

Flow analytical methods rely on three principles: (a) reproducible sample injection, (b) controlled dispersion of the sample zone and (c) reproducible timing of its movement from the injection point to the detection. In contrast to other methods of analysis, chemical reactions take place while the sample material is being dispersed within the reagent. This is why it is important to control dispersion in flow methods. The reproducible timing is also vital since no physical or chemical equilibrium is achieved. Therefore, all injected samples have to be processed exactly the same way [3].

1.1.1. Segmented flow analysis

Segmented flow analysis (SFA) arose in 1957 as a mechanical tool for automating a number of analytical methods [5], affording not only substantial increased throughput, but also substantial savings in samples and reagents. SFA laid the foundations for modern flow analysis techniques. SFA is an automatic continuous methodology, which generally comprises a peristaltic pump (PP) for continuous aspiration of the sample and reagents, a series of plastic tubes (the manifold) intended to carry liquid streams and a detector, as it is shown Figure 1.1.

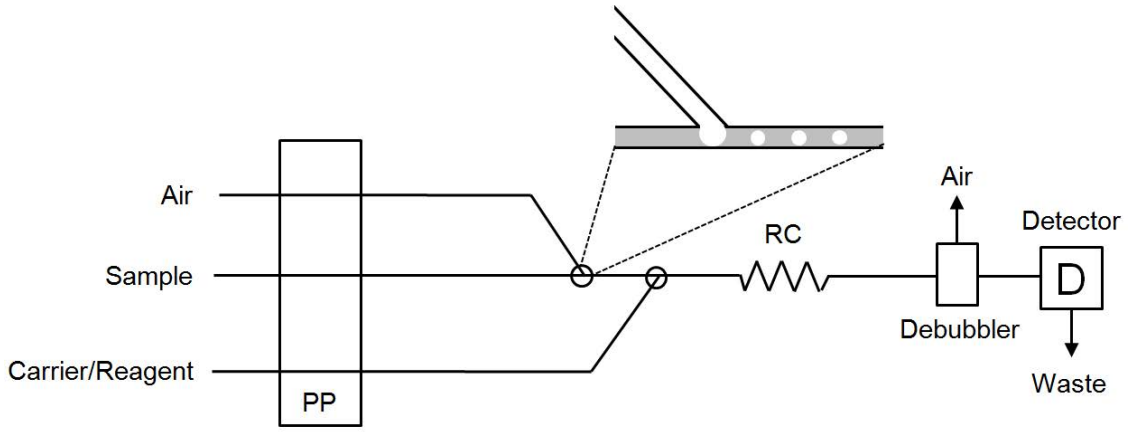

Figure 1.1 Scheme of a segmented flow analysis system. RC: reaction coil, PP: peristaltic pump.

SFA systems are characterized by sample segmentation by air bubbles, followed by a cleaning cycle to avoid cross contamination between samples. Air bubbles prevent dispersion of the sample plug and facilitate the formation of a turbulent flow what helps homogenizing the mix of the sample and the reagent between each pair of bubbles. Nevertheless, the use of bubbles has some disadvantages, such as pulsation due to their high compressibility, moreover their injection and subsequent removal complicates the system design; and they reduce the efficiency of separation techniques

(dialysis, LLE), hinder the implementation of stopped-flow methods and preclude miniaturization in many cases. The typical signal of SFA has a rectangular shape given that each individual segment is isolated from the neighboring segments of flushing water, being the height of the rectangle proportional to the analyte concentration.

On balance, the major drawback of this technique is the presence of air bubbles that may affect the reproducibility of the system, the speed flow rate and the signal shape. Also, since reagents and sample are continuously pumped to the system, it has a high consume in comparison to more evolved flow analysis techniques. Nonetheless, the reagents and sample consumption is greatly reduced compared to batch methodologies of analysis. Therefore, SFA methodologies have been gradually replaced by subsequent flow analysis techniques.

1.1.2. Flow injection analysis

Flow injection analysis (FIA) is an unsegmented flow technique and belongs to the first generation of flow-based methods. It was proposed by Ruzicka and Hansen in 1975 [6]. Since then almost 20000 papers using this technique have been published in international scientific journals. Basic components of FIA are virtually the same as those of SFA including a PP to propel the sample and reagents, the manifold carrying the liquids and the detector (Figure 1.2).

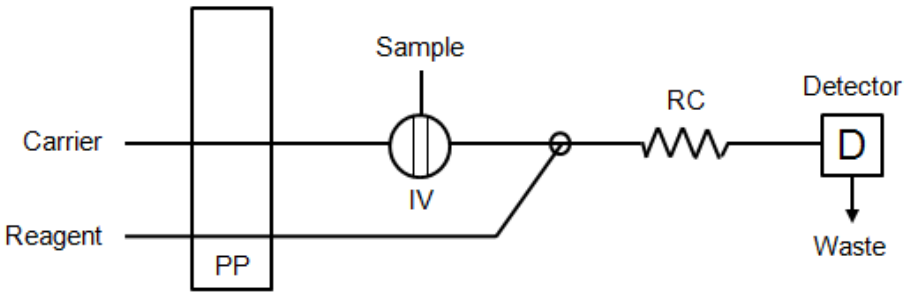

Figure 1.2 Schematic representation of a generic manifold of a flow injection analysis manifold. IV: injection valve, PP: peristaltic pump, RC: reaction coil.

Unlike in SFA, the sample is not inserted by continuous aspiration; rather, a constant volume of sample is inserted into the carrier stream via an injection valve (IV) for later merging with the reagents and detection of the reaction product. In comparison with SFA technique, in FIA the injected sample volume is lower and reagents consumption

decreases substantially [7]. Furthermore, FIA works in laminar flow, what reduces the carry-over between samples, not requiring the cleaning step. In addition, it should be noted that FIA systems are more versatile than SFA allowing the application of stopped flow or kinetic analytical methods.

1.1.3. Sequential injection analysis

Ruzicka and Marshall developed a new flow based technique termed sequential injection analysis (SIA) in 1990 [8], which is considered the second generation of flow analysis techniques. SIA was developed as an alternative to FIA, based on the same principles as FIA but offering more versatility to the developed systems. One of the essential features of SIA is its computerized control. FIA instrumental novelty was the use of the injection valve, which reduced the sample consumption. Thus, SIA is based in the use of multiposition valves (MPV) connected to piston pumps instead of PPs. In addition, SIA systems can be controlled by computer selecting how the central port of the valve is connected to its peripheral ports, starting and stopping the pump in order to aspirate or propel liquids sequentially avoiding continuous pumping. Volume and flow-rates can be automatically adjusted. In addition, the computerized control permits data acquisition and processing using specific software.

The main advantage of SIA over classical FIA is the strong reduction of reagent consumption and waste generation. However, the main drawback of this technique is its lower sampling rate. Figure 1.3 shows a schematic depiction of a SIA system. Thus, this technique involves a high precision bidirectional piston pump usually a syringe pump (SP), a holding coil (HC), a MPV, and a flow through detector. Sample and reagents are sequentially aspirated into the HC. The MPV is then switched to the detector position, and the flow direction is reversed, propelling the reaction plug to the detector.

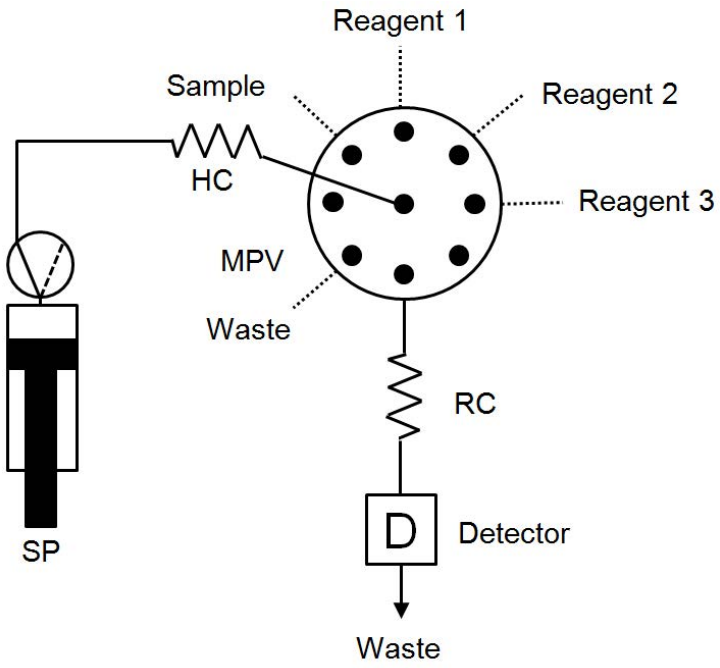

Figure 1.3 Scheme of a generic manifold of a sequential injection analysis system. HC: holding coil, MPV: multiposition valve, RC: reaction coil, SP: syringe pump.

SIA is much closer to the original SFA system than FIA, affording the determination of up to twenty parameters per sample. In SIA the number of parameters to determine can be implemented simply by using a switching valve with an appropriate number of channels to hold the different reagents, delivery of which can be precisely programmed via a computer. Thus, SIA matches multiparametric capabilities of SFA, but operating in a much simpler and, efficient manner.

1.1.4. Multicommutated flow injection analysis

Multicommutated flow injection analysis (MCFIA) [9], introduced by B.F. Reis et al., was developed in 1994. MCFIA is based in the use of fast-switching three-way solenoid valves and PPs. These solenoid valves can be actuated independently and are controlled by a computer in a fast switching mode, ON or OFF according to the path used by the solution. MCFIA systems permit time-based injections and the return of the solutions to their reservoirs when they are not required, e.g. reverse FIA technique, allowing reagents saving and so waste reduction. The number of valves used varies depending on the system. Figure 1.4 shows a schematic depiction of a typical MCFIA system.

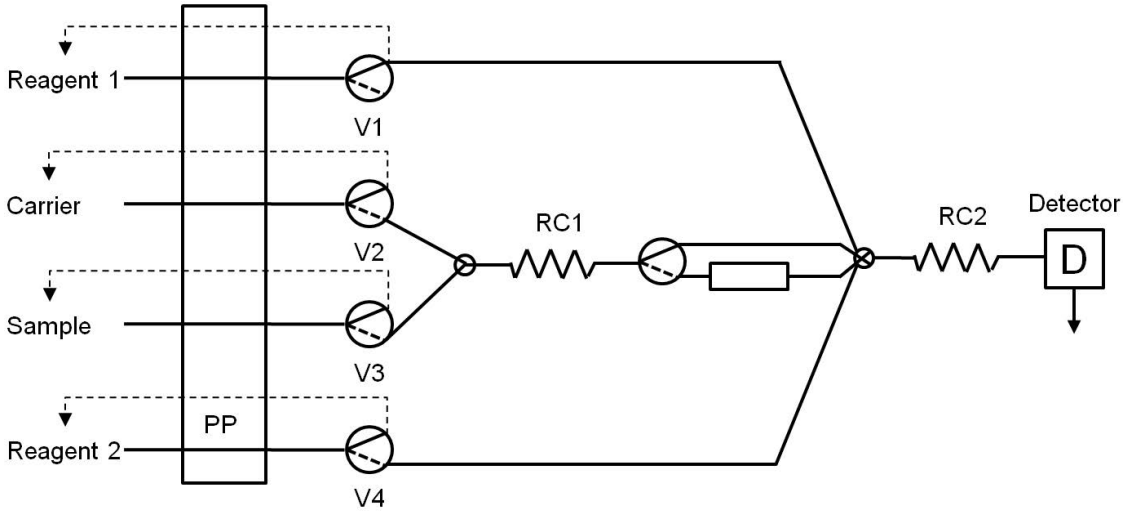

Figure 1.4 Scheme of a generic manifold of a multicommutated flow injection analysis system. PP: peristaltic pump, RC: reaction coil, V: three-way solenoid valve.

Thus, main benefits of this technique are: (a) the small size of the switching valves and electronic interfaces, which enables the development of compact systems that can be applied to field work; (b) reduction of reagents consumption, since the sample and reagents can be dispensed at the required time and returned to their reservoirs when not; and (c) increased reproducibility is achieved because three-way solenoid valves do not require intervention by the operator since these can be computer controlled.

1.1.5. Multisyringe flow injection analysis

Multisyringe flow injection analysis (MSFIA) was developed in 1999 by our research group in cooperation with the firm Crison (Alella, Barcelona, Spain) [10] with the aim of combining the advantages of previous flow analysis techniques while avoiding their disadvantages [11, 12]. A typical MSFIA manifold is shown in Figure 1.5.

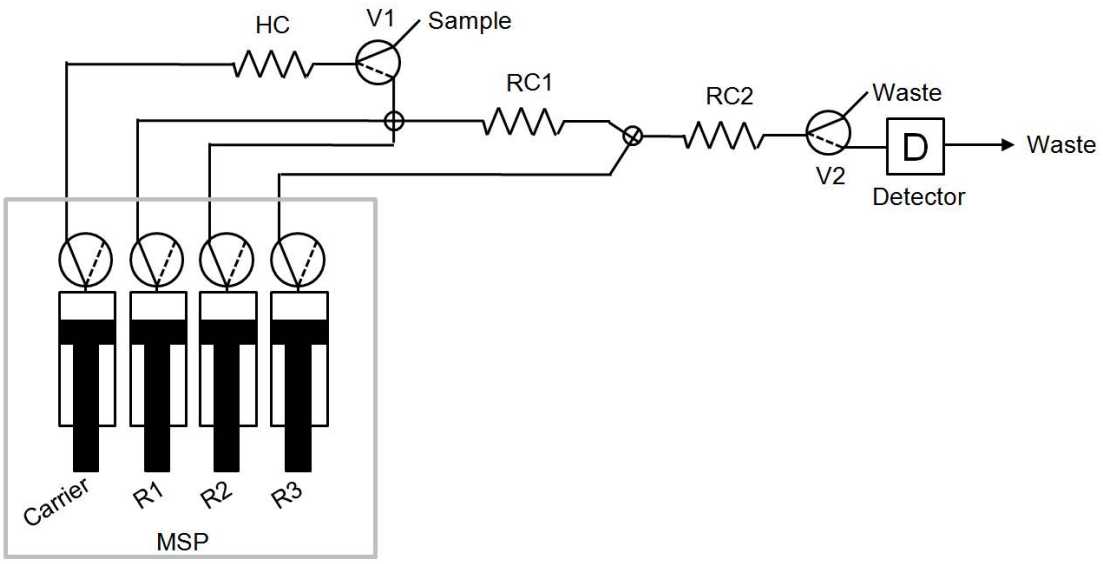

Figure 1.5 Scheme of a generic manifold of a multisyringe flow injection analysis system. HC: holding coil, MSP: multisyringe pump, R: reagent, RC: reaction coil, V: three-way solenoid valve.

The multisyringe burette consists of a conventional automatic titration burette that can be equipped with up to four syringes that the motor can move simultaneously. This is equivalent to use a multichannel PP in FIA but avoids the disadvantages of its fragile tubing. The ratio of flow-rates between channels can be modified by using syringes with different cross-sectional dimensions similarly to tubing diameters in FIA. Each syringe has a three-way solenoid valve at the head that permits multicommutation operation.

Thus, MSFIA combines some of the advantages of the above described flow analysis techniques, such as:

- The high throughput of FIA is a result of sample and reagents being incorporated in confluence which leads to improved mixing efficiency in comparison to SIA.
- The robustness of SIA. In fact, liquids only come in contact with the walls of the glass syringes and Teflon tubing as no PP tubes are used.
- The low sample and reagent consumption of SIA since reagents are used in the amounts strictly required and when needed.
- The high flexibility of SIA manifolds. Residence times are not determined by tubing dimensions, but rather by commands of the computer used to govern the whole system, which sets the times and flow-rates to be employed.

- The possibility of using MCFIA solenoid valves, which can be actuated without the need to stop the pump.

1.1.6. Multipumping flow system

Multipumping flow system (MPFS) [13] was developed by two research groups at the Pharmacy Faculty of the University of Porto (Portugal) and the Piracicaba CENA (Brazil) in 2002. MPFSs are based on the use of micropumps for sample/reagent introduction and as commutation units. The flow-rate is determined by the stroke frequency. Principal advantages of these systems are their high flexibility, ease of configuration, robustness, miniaturized size, low cost and dual function since micropumps can operate as both liquid propeller and valve. MPFSs also use samples and reagents sparingly.

Furthermore, mixing efficiency is better in MPFS due to pump piston strokes causing turbulences and providing higher peaks than those obtained with other flow analysis techniques. Typical MPFS systems are shown in Figure 1.6. Two configurations are represented, following either a FIA manifold by the parallel operation of the micropumps (Figure 1.6 A) or following a SIA manifold by two oppositely operating micropumps, forming one bi-directional pumping unit (Figure 1.6 B) [14]. As can be seen in Figure 1.6, MPFS systems are similar to MCFIA systems (Figure 1.4). In fact, the MPFS controller can control both micropumps and solenoid valves, being possible to implement systems exploiting both techniques, i.e MCFIA and MPFS, or one of them. The primary difference among these systems is that MPFSs require controlling not only valve switching, but also the stroke frequency, in order to ensure reproducible flow-rates. The simplicity and economy of MPFS facilitate the development of portable equipment for field measurements.

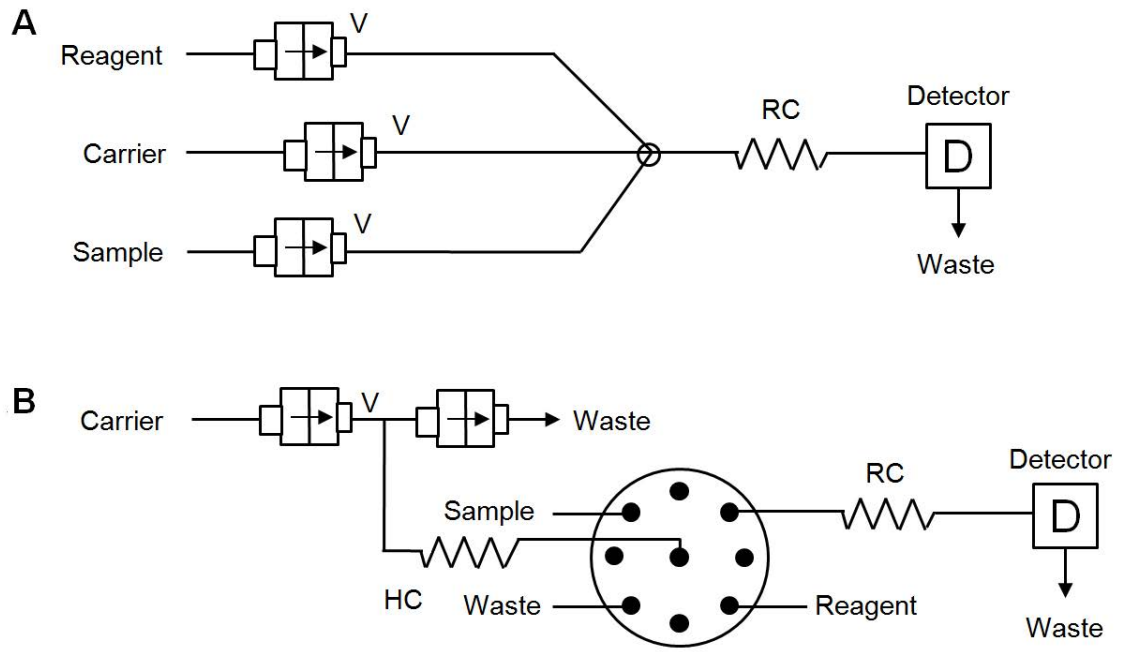

Figure 1.6 Scheme of a generic multipumping flow system. A: FIA configuration, parallel operating micropumps or B: SIA configuration, two micropumps form one bi-directional pumping unit. HC: holding coil, RC: reaction coil, V: three-way solenoid valve.

1.1.7. Lab-on-valve

A step forward in miniaturization and automation of the sequential injection (SI) operation mode was achieved with lab-on-valve (LOV). LOV was developed by Ruzicka in 2000 [15]. This system has been referred as the third generation of flow analysis techniques [16] working at the microliter level, and significantly facilitating integration of various analytical units in the valve, providing great potential for miniaturization of the entire instrumentation.

LOV was designed with the aim to integrate all necessary laboratory operations in a microconduit device. Different designs have been proposed, but the classical one integrated the sample processing channels with a multipurpose flow cell (Figure 1.7). Main components of a LOV system are a SP, a MPV whose commercial tap has been replaced by a LOV platform integrating the above mentioned utilities and a PP.

The SP is used to propel the solutions in the manifold. The MPV is responsible for the connection of the several ports to the central port of the LOV platform. The LOV

platform is usually made of polymethylmethacrylate (PMMA) or Ultem for improved chemical resistance [17].

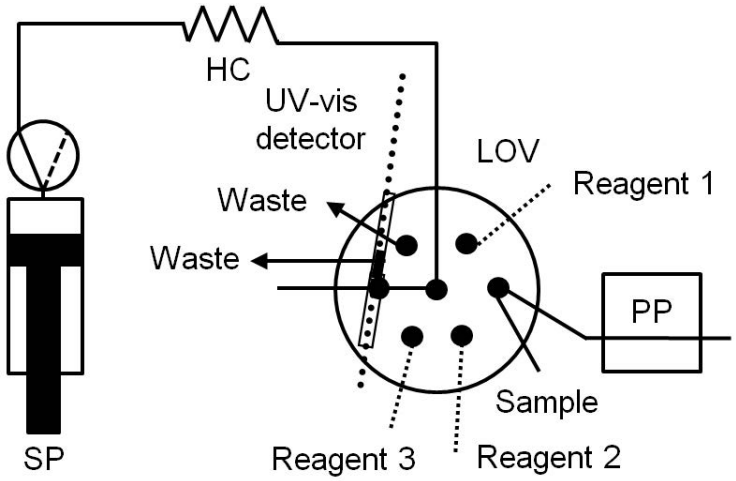

Figure 1.7 Scheme of a generic manifold of a lab-on-valve system. HC: holding coil, PP: peristaltic pump, SP: syringe pump.

The original LOV manifold was developed to accommodate sample metering, dilution, reagent addition, mixing, incubation, separation and detection within a miniaturized device [15]. Moreover, LOV works in SIA mode, i.e. precise volumes of sample and reagents are aspirated sequentially into a HC where mixing, dilution and incubation take place. Then the mixture is propelled by reversed flow toward detection. Additional benefits of LOV systems are robustness and repeatability due to precise computer control and process downscaling [18]. In addition, LOV platforms can integrate a multipurpose flow cell that permits the implementation of optical detection by the use of optical fibers. The fibers can be placed in different positions, configured for absorption or fluorescence detection within the same set-up [19]. The length of the light path can also be adjusted by changing the position of the end of the optical fiber, for example in the LOV used in this thesis a light path between 2 mm and 10 mm can be obtained, as it is explained in more detail in chapter 3.

Furthermore, the LOV has not only extendedly proved to be a valuable tool for homogeneous solution-based assays, but also in heterogeneous assays because flexible fluid manipulation is also suitable for delivering beads in LOV being a powerful platform for bead injection (BI) [15]. Briefly, mini-columns are in situ generated by aspirating beads with particular surface characteristics and particle sizes [4]. The beads can even be automatically transported between different column positions within the LOV [20]. Thus, beads are trapped and perfused by analyte solutions, buffers and/

or auxiliary reagents. Reactions take place at the bead surface being possible to be monitored in real time, either directly on the solid phase or by monitoring the eluting liquid phase. More detailed information about the BI-LOV is given in section 1.2.1.

1.2. Automation of sample pretreatment

In recent years, the development of fast, precise, accurate and sensitive methodologies of analysis has become an issue of great concern. However, despite the advances in the development of highly efficient analytical instrumentation, sample pretreatment is usually necessary prior detection in order to extract, isolate and concentrate the analytes of interest from complex matrices, to have target compounds in a form and concentration suitable for analysis, avoiding interferences and protecting the instruments [21].

Extraction techniques are the most used for sample pretreatment, standing out those using small amounts of solvents, such as SPE and dispersive liquid-liquid microextraction (DLLME). Nowadays, SPE is used as routine methodology in laboratories. In recent years, new microextraction techniques have been developed as alternatives to SPE, e.g. liquid phase microextraction (LPME). Regarding miniaturization of traditional LLE, DLLME has been introduced with the advantage of using a negligible amount of organic solvent. Thus, the combination of microextraction techniques with flow systems provides green methodologies, since both benefit each other enhancing their individual advantages.

Thus, the ideal pretreatment technique should involve a minimum number of working steps, be easy to implement, be environmental friendly according to green chemical principles [22] and be economical [23]. In this section, on-line SPE and DLLME are described in more detail since these have been used in the development of this thesis.

1.2.1. Solid-phase extraction

SPE is the most widely used sample preparation technique for liquid samples due to its simplicity and limited usage of organic solvents. SPE belongs to the group of sorptive-based extraction techniques, in which the sample is placed in contact with a suitable material to extract the analytes of interest and for sample clean-up. Thus, the availability of different materials to carry out the extraction is essential. SPE is efficiently implemented in flow-based systems. However, it presents the major drawback of producing high back pressure, due to the progressively tighter packing or clogging of the column material when used in long term operation. Comprehensive reviews on mini-columns packed with solid materials have been published [24-26]. As a

way of example in Figure 1.8 it is shown a scheme of two flow systems exploiting a packed mini-column. Figure 1.8 A shows a MSFIA system with a mini-column to speciate iron involving the colorimetric reagents ferrozine and ammonium thiocyanate [27]. Figure 1.8 B shows a LOV with an integrated mini-column in the flow cell to speciate iron involving an environmental friendly chelant [28].

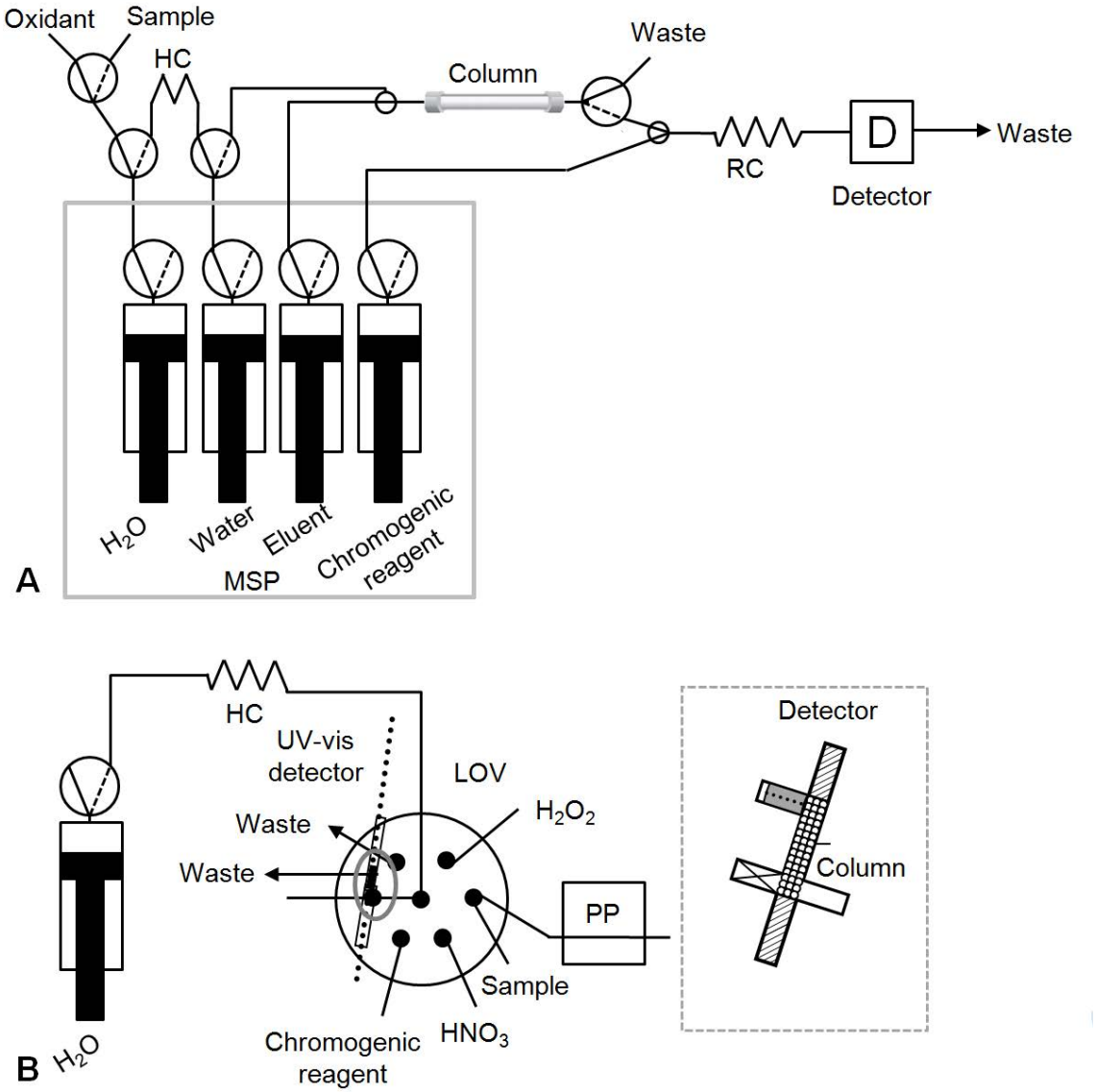

Figure 1.8 A) Multisyringe flow injection analysis system for iron determination. B) Lab-on-valve system for iron determination with a detailed scheme of the flow cell in which is placed the column. HC: holding coil, MSP: multisyringe pump, PP: peristaltic pump, RC: reaction coil.

Common steps in SPE are column conditioning to permit selective separations of either the species to be determined or the potential interferences present in the sample enhancing sensitivity and/or selectivity, sample loading and elution of the analyte of interest for detection or on-column detection. Thus, detection can be carried out on the eluted phase [29, 30] or on the solid phase, the latter denoted as solid phase

spectrometry (SPS) [28, 31-33]. As general rule, flow analyzers exploiting SPE are simple and robust, yielding reliable results.

SPE can be carried out using reusable analytical columns [34, 35] or renewable analytical columns. These SPE materials are expensive. Thus, their reuse is of great interest. However, when reusing a column some problems can occur including the build-up of pressure and cross contamination between successive assays. These limitations can be overcome by using a renewable analytical column. In some applications, after the measurement, the beads can be discharged, occurring physical regeneration, or in other cases the beads surface can be renewed, i.e. chemical regeneration.

Physical regeneration of the SPE material is an advantage since there is no need for the elution step and its applicability is not limited by the lifetime of the material. At the same time any possible contamination or carry-over is eliminated. The major benefit of using renewable columns is the increase in selectivity. Furthermore, if using SPS the sensitivity is enhanced since the analyte is retained in the detection zone avoiding elution and thus dilution, and the selectivity also increases given carry-over is also avoided.

Nevertheless, the implementation of SPE in flow systems can be tricky, since as cited above the solid phase can become packed and increase overpressure. Additionally, although it can be reconditioned for reuse, it has a lifetime and the extraction efficiency decreases with consecutive analysis. To overcome these problems, it is necessary to renew the column. This is facilitated by using LOV [15], since beads can be discharged and loaded automatically. LOV not only serves for reagent-based assays methodologies but also for BI. That is to say, functionalized beads can be manipulated in the flow conduits by LOV technique. Usually, a small amount of beads is injected into the flow channel where these are trapped and perfused by sample and reagents. Thus, the BI protocol usually is divided in five steps: (1) the beads are loaded in the flow channel and trapped in the flow cell building a bead column, (2) sample is injected and transported toward it, (3) the target analyte is captured on the bead surface, while the matrix components are washed away, after that a chromogenic reagent is injected and (4) some species are detected by spectroscopy or a eluent is injected for later detection in the same manifold or off-line. Finally, (5) beads are discharged or regenerated (Figure 1.9).

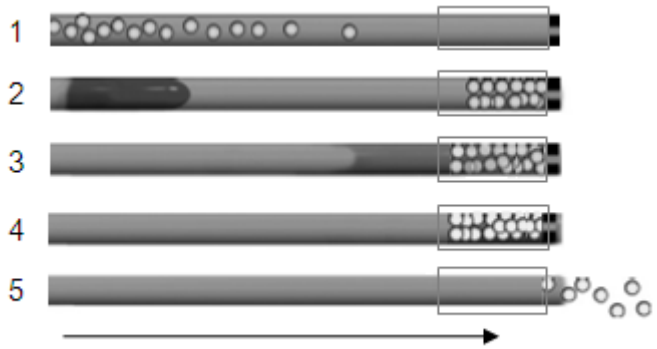

Figure 1.9 Schematic representation of the BI steps. (1) Load of bead suspension, (2) load of sample solution, (3) injection of the chromogenic reagent, (4) detection of target analytes, and (5) beads discharge.

1.2.2. Liquid-phase extraction

LLE is a classical and widely used technique for sample clean-up and preconcentration prior detection. It is based on the partition of compounds between the aqueous sample phase and an immiscible organic solvent, which is non- or slightly polar. Nevertheless, some shortcomings such as emulsion formation, use of large sample volumes and toxic organic solvents and hence, generation of large amounts of waste make LLE expensive, time-consuming and environmental unfriendly. In order to overcome these drawbacks and transform LLE into a more environmental friendly extraction technique [22], a plethora of LPME techniques have been recently introduced [36, 37]. The main objective of LPME is minimization of the use of organic solvents, thus not only achieving a lower environmental impact per analysis but also increased enrichment factors.

Among the various techniques exhibiting high analytical potential single-drop microextraction (SDME) and DLLME are being the most exploited ones. SDME is based on the utilization of a small droplet of organic solvent, formed and suspended at the tip of a syringe needle, which is immersed into the aqueous sample [38]. Up to date SDME has slightly been implemented for analyte enrichment in flow systems [39-41]. As a way of example, an automated in-syringe SDME system was developed for quantification of ethanol in wine, employing the well-known oxidation of ethanol by acidic dichromate solution [41].

DLLME was developed by Rezaee et al. in 2006 [42]. Since then, an impressive number of applications exploiting this microextraction technique have appeared in the

literature [43, 44]. In DLLME, a cloudy solution is formed when an appropriate mixture of extraction and dispersive solvents is injected into an aqueous sample, as a result of the formation of very fine droplets of extraction solvent which disperse into the aqueous sample. As a consequence a large surface area is accomplished between the extraction solvent and the aqueous phase, and the extraction equilibrium is reached very quickly. In batch approaches, the solution, while still in its cloudy state, is centrifuged in order to effectively separate the phases. After centrifugation, the analytes extracted from the initial solution and concentrated into a small volume of organic sedimented phase are determined by conventional analytical techniques. The extraction steps of batch DLLME are illustrated in Figure 1.10, including the use of solvents more (Figure 1.10 A) and less dense (Figure 1.10 B) than water.

DLLME is very useful due to its very short extraction time, simplicity of operation, low cost, and high recovery and enrichment factors offering potential for ultra-trace analysis. Despite the obvious advantages of DLLME and its contribution to the field of sample pretreatment, the recovery of the small amount of extract after phase separation is troublesome, particularly when solvents lighter than water are used. Manual handling of minute amounts of extractant solvent influences the reproducibility of the determination.

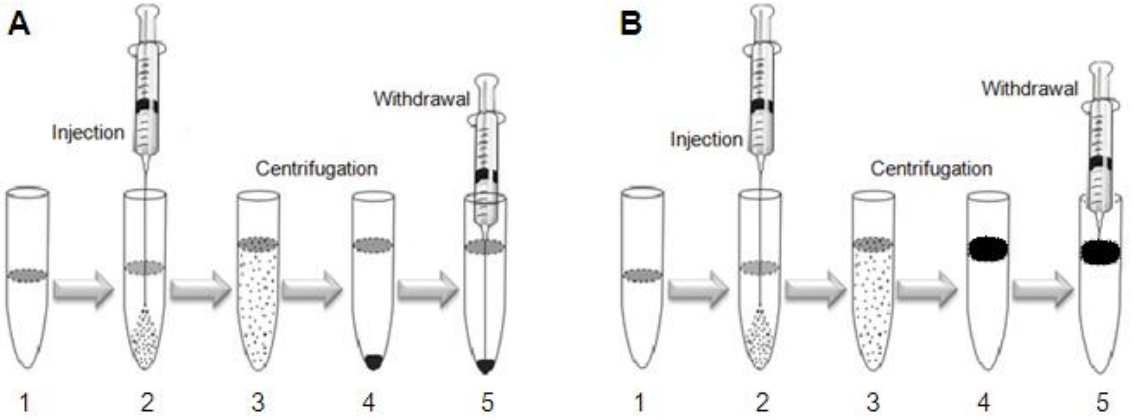

Figure 1.10 Schematic illustration of batch DLLME. A) For solvents more dense than water. (1) Sample solution, (2) injection of mixture of disperser solvent and extractant, (3) cloudy solution (dispersion), (4) sedimented phase after centrifugation and (5) collection of sedimented phase. B) Same steps for solvents less dense than water.

Different approaches have been developed for the automation of the DLLME exploiting flow analysis techniques offering several advantages, such as (a) a closed system for the handling of organic solvents, (b) a reduced risk of analyte loss and sample contamination, and (c) high reproducibility due to automatic handling of small volumes.

To the best of our knowledge, the first automation of DLLME was done by Anthemidis et al. in 2009 using SIA [45], in which the organic phase was retained in a microcolumn packed with polytetrafluoroethylene (PTFE)-turnings instead of using centrifugation, and analytes were subsequently eluted with 300 μL of isobutylmethylketone, which were then transported to an atomic spectrometer. The approach suggested by Anthemidis, in comparison with conventional DLLME, offers two important benefits: it is not necessary that the extraction solvent is denser than water, since the extraction takes place in a moving stream, and the separation of the organic phase is not based on centrifugation, but on retention, and most importantly, the process is fully automated. However, it also presents some disadvantages: the necessity of using a microcolumn for retention of the analyte, and the necessity of using several hundred μL of solvents for elution of the analyte which cause the dilution of it. For this reason, Andruch et al. [46] suggested a novel SI-DLLME approach based on the principle of the adjustment of solvents mixture density in which no centrifugation or microcolumn and consequently no elution solvent is needed. The main drawback of this variant is the complex system, using two MPVs and two SPs and requiring long cleaning procedures.

Later Maya et al. proposed the in-syringe DLLME [47]. The potential of this technique consists in using a SP as mixing chamber and phase separator. Since then, several applications of in-syringe DLLME have been developed [48-54]. For instance, this new variant was exploited for benzo(a)pyrene determination exploiting an integrated low-pressure LC system [47]. While one syringe is used for DLLME, a second syringe enables post-extraction addition of diluents to adjust viscosity, and a third delivers the mobile phase for chromatographic separation.

Another positive aspect of using SIA/MSFIA techniques for the automation of DLLME is the versatility provided to implement pre- and post-DLLME operations, such as the derivatization of metals with selective complexing agents, for subsequent DLLME and UV-vis spectrophotometric detection. For example, inorganic copper was determined based on its reduction to Cu (I) and the formation of an extractable complex with bathocuproine for further DLLME and quantification by long path-length spectrophotometry. Using an analogous system set-up, the efficient concentration and determination of total phenolic compounds as their reaction product with 4-aminoantipyrine was achieved [48]. The potential of this approach was also demonstrated using fluorimetric detection, enabling derivatization of aluminium with

lumogallion and the in-syringe DLLME of the reaction product prior detection [49]. This application is described in more detail in chapter 5.

A compact and fully automated system exploiting in-syringe DLLME and detection was proposed by Horstkotte et al. [50] placing two optical fibers at an angle of 180º, achieving an optical path-length of approximately 1 cm. Although the sensitivity reported was not very high, this approach can be useful for the fast screening of families of analytes, prior to a more accurate and expensive analysis [51, 52]. Likewise, it would be feasible to measure luminescence signals by simply connecting one optical fiber to a photomultiplier, or combined with a second optical fiber at 90º for fluorescence measurements.

Another advantage of implementing DLLME exploiting MSFIA is that solvents more and less dense than water can be used without difficulties. In Figure 1.11 A a schematic illustration of the steps of in-syringe DLLME using solvents less dense than water is shown. Firstly, the mixture of extractant and dispersant are loaded into the syringe, and secondly the aqueous sample, producing the dispersion. The flow rate of aspiration must be high in order to promote the mixing among phases. At this step, disruption of the organic solvent into small droplets in the aqueous sample is achieved. After a waiting time for phase separation by the flotation and aggregation of the extraction solvent droplets at the top of the syringe, the syringe is emptied through the detection flow cell to waste.

Further development of in-syringe LPME lead to magnetic-stirring-assisted DLLME (MSA-DLLME) placing a small magnetic stirrer within the syringe to enhance mixing between phases and speed extractions [53]. In this approach, graphically illustrated in Figure 1.11 B, sample and reagents are loaded within the syringe followed by a plug of air. The dispersion is obtained by stirring, allowing in-syringe DLLME without using disperser solvent [54]. First application of this variant was used to extract aluminium as its fluorescent complex with lumogallion from coastal seawater samples [53]. Analytical performance was improved with the stirring-assisted system in comparison with a similar in-syringe DLLME system using a disperser solvent [49]. Furthermore, increased analysis throughput was obtained and solvent consumption was reduced.

In addition, the use of a magnetic-stirring assisted system provides high versatility by the possibility of mixing sample and reagents for analyte derivatization prior to extraction. For instance, in-syringe MSA-DLLME was applied to determine chromate in

natural water samples. Chromate was first derivatized in-syringe reacting with diphenylcarbazide in acidic medium, followed by ion-pair extraction in neutral medium of the resulting chromium-diphenylcarbazone complex with acetate [54]. Moreover, in-syringe MSA-DLLME has been applied using solvents more and less dense than water by placing the syringe module up-side down or as it is usually placed to accumulate the organic droplet at the exit of the syringe. Up-side down in-syringe MSA-DLLME has been applied to determine anionic surfactants [55], cationic surfactants [56] and uranium [57], all of them in environmental samples. In Figure 1.11 C a schematic illustration of common in-syringe MSA-DLLME steps using solvents more dense than water are shown.

Despite of the several advantages of in-syringe DLLME, it has some drawbacks, as for example the limited volume of sample that can be used due to syringe size restrictions and so the lower enrichment factors achieved, and the lack of selectivity between similar compounds. The latter can be overcome by coupling the in-syringe DLLME to chromatographic techniques. Thus, in-syringe DLLME has been coupled with multisyringe chromatography (MSC), LC and GC. An in-syringe MSA-DLLME–MSC system was exploited for screening of phenolic pollutants in environmental samples [58]. In another application in-syringe DLLME was coupled to GC for phthalates [59] and PHAs [60] determination using one syringe for the extraction, and a second one containing air connected to an injection valve to inject the aliquot of the extractant into de the GC-MS instrument. Moreover, the analysis throughput can be improved, since while the analytes extracted from one sample are being analysed by the chromatograph, in-syringe DLLME of the next sample can be performed simultaneously.

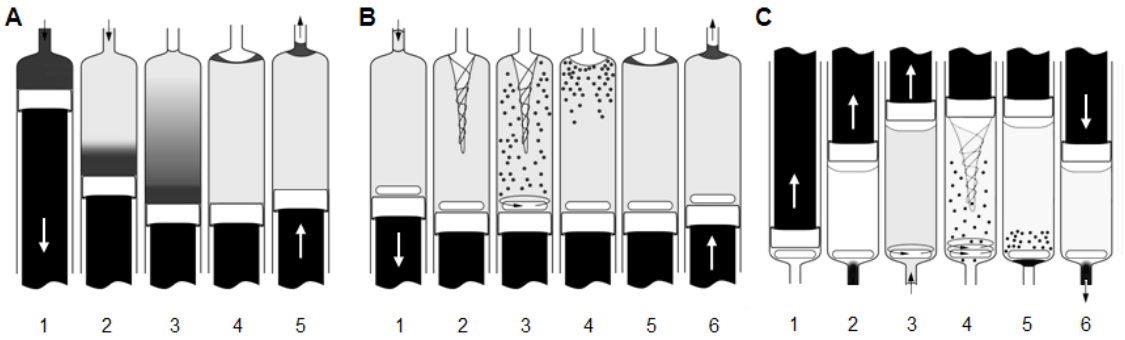

Figure 1.11 A) In-syringe DLLME: (1) aspiration of reagents and organic solvent, (2): aspiration of sample, (3): DLLME, (4): phase separation, (5): propulsion of enriched organic phase to detector. B) in-syringe MSA-DLLME for solvent lighter than water (up). (1): aspiration of reagent, (2): aspiration of sample, (3): aspiration of organic solvent, (4): DLLME, (5): phase separation, (6): propulsion of enriched organic phase to detector. C) in-syringe MSA-DLLME for solvent more dense than water (down). (1): aspiration of reagent for derivatization, (2): aspiration of sample, (3): aspiration of organic solvent, (4): DLLME, (5): phase separation, (6): propulsion of enriched organic phase to detector.

1.3. Coupling of flow analysis techniques to separation techniques

Despite of the advantages of flow analysis techniques, these are non-separative tools presenting lack of selectivity by themselves. As mentioned before, one of the main objectives of analytical chemists is to develop sensitive, fast, precise, affordable and selective analytical methods, to fulfil current society demands. In this scenario, coupling of separation and flow analysis techniques seems to be a powerful strategy in order to achieve the above mentioned objectives.

This hyphenation benefits both techniques resulting in improved analytical methods. For instance, some advantages are the development of fully automated methods with on-line sample pretreatment before injection into the chromatograph, the avoidance of contamination and sample loss, the improvement of reproducibility and ensuring the good performance of the chromatographic instrument. In addition, these automatic systems reduce the time required for the sample pretreatment and allow derivatization and preconcentration of the analytes.

Thus, flow analysis techniques have been coupled with separation techniques in different analytical approaches including SIA [61], MSFIA [62, 63] and LOV [20, 29, 60]. Also, the advent of monolithic columns allowed the development of low-pressure chromatographic techniques, as a result of the combination of SIA and MSFIA with monolithic columns, respectively [64]. However, it has to be noted that the similarities in the aggregation state of the phases and in the instrumentation involved of flow analysis techniques and liquid chromatography (LC) have resulted in more advances in the coupling of these techniques.

More detailed information can be found in a comprehensive review entitled "*Analytical strategies for coupling separation and flow injection techniques: state of the art and future trends",* attached at the end of this section. In this article, the benefits and limitations of coupling flow analysis techniques and separation techniques, i.e. LC, GC and CE are discussed. Furthermore, an updated revision of hyphenated systems applied to several types of environmental and biological samples is presented.

1.4. References

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1.5. Original paper

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Analytical strategies for coupling separation and flow-injection techniques

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ABSTRACT

Flow techniques (FTs) are non-separative tools and high-performance separation techniques that require sample pretreatment. On-line combinations of flow sample-pretreatment units and discrete sample-introduction devices are very attractive, as they allow the whole analytical sequence to be developed in a single instrumental assembly. In this review, we present the state of the art and future trends in coupling separation techniques, such as chromatography and capillary electrophoresis, and FTs. FTs can be coupled as a front end to separation techniques or by integrating the separation column in the flow system, generating new low-pressure chromatographic techniques. We discuss different coupling strategies, highlighting their benefits and disadvantages. We also present an updated review of coupled systems applied to environmental and biological samples.

Contents

1. Introduction

One of the main objectives of analytical chemists – whatever their field of work – has been to pursue techniques, protocols and devices to improve the sensitivity and the selectivity of analytical methods, without compromising the reliability of the results, the overall speed of the procedures and their cost. In this sense, analytical instrumentation has evolved exponentially, bringing as a result a wide gamut of instruments, capable of performing highly accurate determinations, based on a variety of physicochemical principles. A lot of emphasis has been focused on the development of separation techniques, such as chromatographic techniques and capillary electrophoresis (CE), with substantial improvements, tending to miniaturization [1].

These techniques possess certain inherent features that offer distinct advantages over conventional analytical techniques, e.g.:

- complex mixtures, including isomers and homologues, can be separated;
- the procedure is adaptable to macro and micro size sample volume; and,

Abbreviations: DLLME, Dispersive liquid-liquid microextraction; FIA, Flow-injection analysis; FT, Flow technique; LLE, Liquid-liquid extraction; LOV, Lab-on-valve; MISPE, Molecularly-imprinted solid-phase extraction; MSC, Multisyringe chromatography; MSFIA, Multisyringe flow-injection analysis; PTV, Programmable temperature vaporizer; SFE, Supercritical-fluid extraction; SIA, Sequential-injection analysis; SIC, Sequential-injection chromatography; SPE, Solid-phase extraction; SPME, Solid-phase microextraction.

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- both quantitative and qualitative analyses are possible due to the capacity for coupling with a wide range of selective, sensitive detectors.

However, the bottleneck of separation techniques is the sample pretreatment required prior to separation. The analytes to be determined are frequently in low concentrations and the presence of interferences can be difficult and even hinder the analysis. Sample-preparation steps typically account for most analysis time, and the quality of these steps largely determines the success of the analysis from complex matrixes. Improvement of sample preparation in terms of speed, reliability and sensitivity is therefore of the greatest interest [2].

Modern analytical strategies tend towards automation and integration of sample pretreatment in the chromatographic systems as far as possible. In this context, the use of flow-analysis techniques for automation has provided a number of enhanced analytical methods affording high throughput [1,3]. Flow techniques (FTs) are typically faster, more robust and more flexible than their batch counterparts. In addition, flow methods use samples and reagents sparingly – that reduces analytical costs and waste production – and minimize human intervention [3].

One of the most outstanding features of flow systems is their inherent ability to accommodate a plethora of unit operations {e.g., on-line liquid-liquid extraction (LLE), precipitation or coprecipitation in knotted reactors, or solid-phase extraction (SPE) in-column with hydrophilic or hydrophobic packing materials [1,3]. Although it is more common to use solid phases to carry out the clean-up of the sample and preconcentration of analytes of interest, recently there were interesting developments in liquid-liquid extraction (LLE) {e.g., the development of lab-in-syringe systems, which allow automatic LLE to be carried out inside the syringe, saving time, reagents and solvents, and consequently reducing generation of residues [4]}.

On-line combinations of flow-sample-pretreatment units and discrete sample-introduction devices are very attractive, as they allow the whole analytical sequence to be developed in a single instrumental assembly [1]. This approach, together with the benefits described above (e.g., increased analyst safety, sample, reagents and waste reduction) make these automated, integrated systems based on FT and separation techniques multi-parametric green tools that fulfil nine of the 12 principles of green analytical chemistry described elsewhere [5].

FTs have been coupled with separation techniques in a variety of analytical approaches including sequential-injection analysis (SIA) [6–8], multisyringe flow-injection analysis (MSFIA) [9,10] and lab-on-valve (LOV) [11–13]. Also, the advent of monolithic columns allowed the development of low-pressure-chromatography techniques called sequential-injection chromatography (SIC) and multisyringe chromatography (MSC), as a result of the combination of SIA and MSFIA with monolithic columns, respectively [14]. Fig. 1 shows typical couplings between FT and the main separation techniques, as well as the general features of coupled flow systems and the specific characteristics of each configuration. Also, Fig. 2 is a milestone diagram of FT coupling with separation techniques.

In this review, we discuss the benefits and the limitations of coupling FTs and separation techniques (i.e., LC, GC and CE). We also

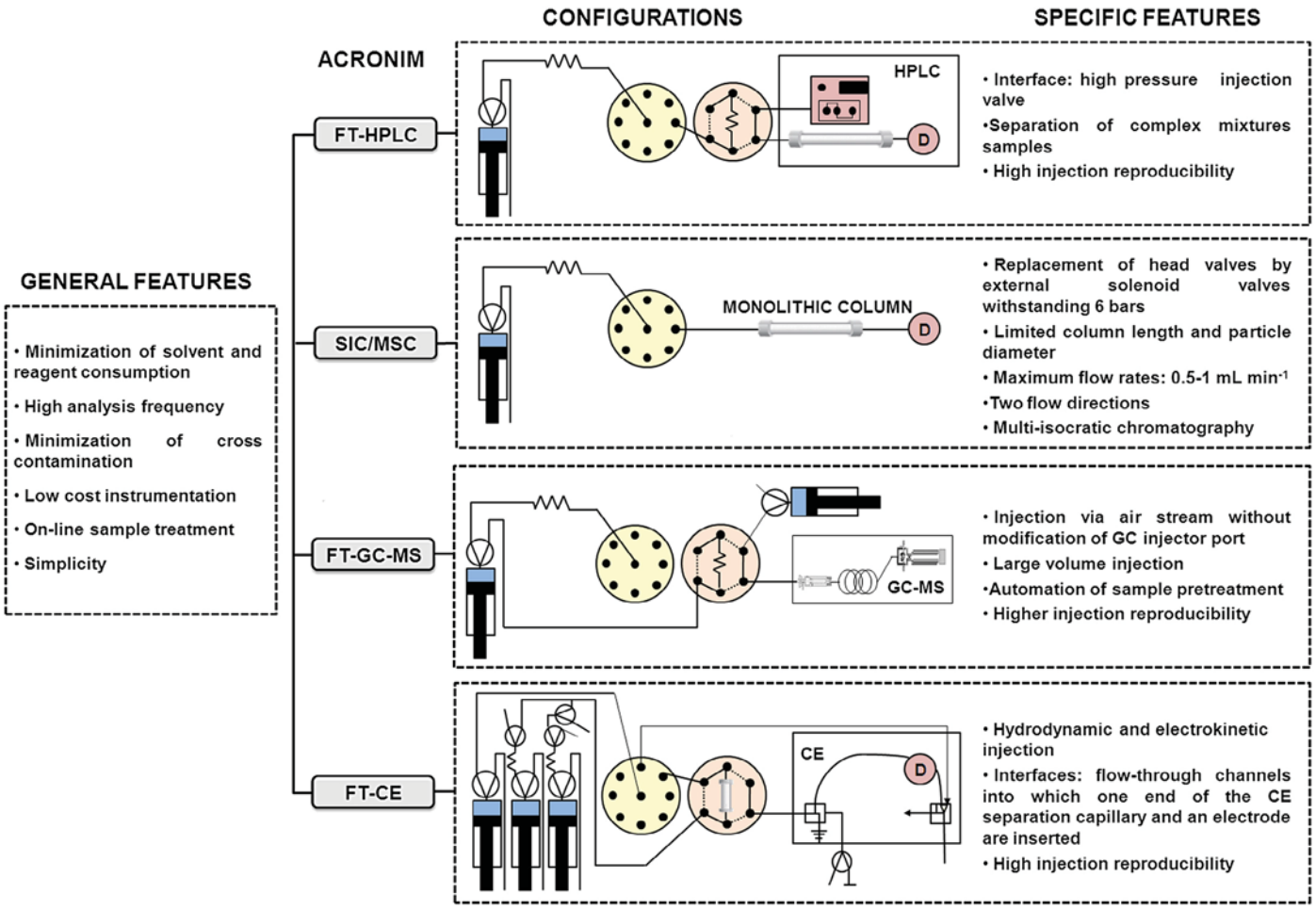

Fig. 1. Basic configurations of on-line coupling of flow-processing devices and different separation techniques. General and specific features.

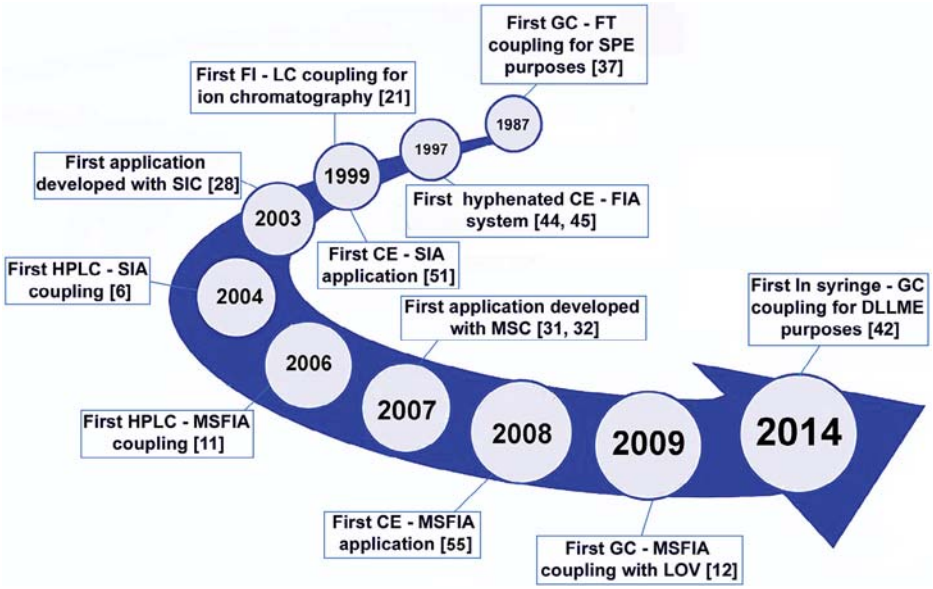

Fig. 2. Milestone diagram of coupling flow and separation techniques.

present an updated review of coupled systems applied to several types of environmental and biological samples.

2. Ways of coupling separation devices to flow techniques

FTs have been coupled in several ways as a front end to LC, GC or CE, and, depending on the degree of human participation and the hardware used, these can be classified as off-line, at-line, on-line or in-line.

Off-line coupling includes manual injection of the sample pre-processed into the chromatographic or electrophoretic equipment [1,15,16]. The advantages of this coupling rely on the minimization of LC band-broadening effects, overlapping peaks, co-elution with void volume and peak asymmetry, but off-line coupling can be considered as the lowest degree of automation, losing benefits of automation.

At-line procedures involve the use of a programmable robotic station to link the flow system with the separation or detection instrument. Usually, the pretreated sample is delivered from the flow system to a vial or into the injector of the analytical instrument [1].

On-line coupling implies direct contact between the flow system and chromatographic or electrophoretic components via a flow interface. The sample injected or processed in the flow system is transported to the interface and introduced into the separation system by pressure-driven flow in CE [17], mobile phase in LC [9–11] or gas stream in GC [12,13] (Fig. 1).

In contrast to at-line coupling, on-line methods foster repeated injections, while separation is still in progress.

Finally, in-line coupling involves complete, close integration of the flow system and separation or detection instrument [18]. Nonetheless, a significant drawback of in-line coupling is its inability to handle samples in parallel because the overall analytical process is confined in the integrated flow device.

3. Flow-injection systems coupled to liquid chromatography

The combination of liquid chromatography (LC) and FTs provides a powerful tool to resolve a significant number of analytical problems. The lack of selectivity of FTs is complemented by the selectivity and the sensitivity of LC, with an optimum efficiency/cost relationship. FTs can be coupled with high-performance LC (HPLC) with the aim of automating the sample treatment or generating new low-pressure chromatographic techniques with the introduction of the chromatographic column in the flow system (Fig. 1). This section focuses on these two arrangements, highlighting their advantages, versatility and limitations.

3.1. Automation of sample treatment prior to high-performance liquid chromatography

In HPLC, sample treatment is mainly used to solubilize the analytes in a suitable solvent and eliminate all potential interferences prior to injection. In practice, the most commonly used approach for on-line coupling of sample treatment and HPLC is the "column switching" technique using a high-pressure injection valve as interface. This technique provides clean-up, preconcentration and determination of analytes on-line, increasing precision and exactitude in the analysis, and reduced time and sample manipulation during sample-preparation steps. Despite showing great versatility to solve many analytical problems and to afford the automation of chromatographic equipment, FTs are straightforward, cost-effective alternatives to switching-column modules that foster the performance of clean-up and preconcentration schemes in the low-pressure mode [1].

FTs and HPLC have mainly been coupled following two configurations. The first implies pre-column arrangements where the flow system is used for preconcentration or sample clean-up [19]. The second implies post-column arrangements, which are intended to facilitate the detection of the target analytes by derivatization [20]. Furthermore, these couplings have been implemented in three modes – at-line, on-line and in-line.

An appropriate interface is necessary to carry out coupling between the pretreatment system and the chromatographic instrument (see Fig. 1). This usually consists of a high-pressure injection valve with a loop, whose size can be modified according to the requirements of each experiment. Moreover, the same mobile phase is used as the transfer phase if possible for the highest elution efficiency avoiding broad-shaped peaks. Treated sample aliquots are then driven to the high-pressure injection valve (interface) to introduce the plug into the column. Reproducible injection into the chromatograph relies on accurate synchronized operation of the whole system.

FIA was the first FT coupled to HPLC due to the similarities of these techniques in the aggregation state of the phases involved and in the instrumentation that facilitates the implementation. The first coupling between FTs and LC was developed in 1999 by Grudpan et al. exploiting a dialysis device coupled to ion chromatography in order to determinate anions in natural waters [21], as is shown in the milestone diagram of FT coupling (Fig. 2). As can be seen in Table 1, FIA-HPLC systems have been proposed for determination of different compounds in several matrices, such as chloramphenicol in water [22], reporting in general higher sensitivity and selectivity than their off-line counterparts.

SIA has also been coupled to HPLC providing advantages in front of FIA, in terms of manifold simplicity, robustness, and low consumption of reagents and sample. The ports of the multiposition valve can be used to couple various units (e.g., detectors, reactors, and pumps), allowing the performance of different sample treatments. For instance, an SIA-HPLC system has been used for the determination of gemfibrozilin in drug samples [8] (see Table 1).

The use of the LOV technique exploiting a bead-injection strategy solves some of the drawbacks of using SPE (e.g., backpressure problems, clogging and analyte carryover) being responsible for handling the beads (i.e., filling, conditioning and renewal of the column when required), sample loading and elution of the analytes of interest, which provides versatility to the system together with low consumption of sample and reagent, reduced amount of SPE material and analysis time, high reproducibility and minimal sample contamination [38].

The MSFIA technique has also been coupled to HPLC, being exploited as a front end of the chromatographic system. One of the first applications of this coupling was developed in our group for screening phenolic pollutants in water and environmental samples at ng mL^{-1} levels [9]. Another application was based on the use of dispersed carbon nanotubes (CNTs) as the sorptive material for selective analyte retention and clean-up of chlorotriazine herbicides in environmental waters and crude soil extracts with on-line injection of the eluate into HPLC [10].

A step forward in automation was achieved when coupling MSFIA with LOV. An MSFIA-LOV-HPLC approach was successfully applied for pharmaceutical-multiresidue determination in environmental and biological samples using a particulate LC column [11] (see Fig. 2). Multiclass determination of UV filters in environmental samples [24] has been automated, exploiting an MSFIA-LOV system using molecularly-imprinted SPE (MISPE) sorbents and also to determine riboflavin in foodstuffs [25] and herbicides in water samples [23].

According to the comparative data compiled in Table 1, FT-HPLC approaches feature better precision than off-line methods, as a consequence of the avoidance of the common intermediate steps of manual SPE applications. The RSD obtained with the FT-LC methods are better than those of on-line approaches previously reported. Also, as summarized in Table 2, the capability of the system to perform preconcentration and clean-up of one sample in parallel to the chromatographic analysis of that previously extracted ensures high-throughput sampling, the analysis cycles being reduced to a few min. Most FT-HPLC coupled methods reported, as shown in Table 1, provide shorter analysis time, higher analyst safety, lower reagent consumption and low waste generation by automating sample pretreatment prior to chromatographic separation.

3.2. Low-pressure chromatography

Research in LC columns has tremendously accelerated in recent years. An important direction of this research is the development of monolithic columns with high-porosity sorbent permitting high flow rates of mobile phase at low back pressures without losing efficiency. SIC and MSC are results of the coupling of SIA and MSFIA to monolithic columns, respectively. Both have proved to be excellent tools that exploit the capability of monolithic columns with relatively large pore sizes to effect separations without requiring high-pressure pumps [14,39].

SIC arose in 2003 [31,40] (see Fig. 2). In a typical SIC system, the monolithic column is coupled on a peripheral port of a selection valve, which may support the pressure when the liquid passes through the column (Fig. 1). However, usually the backpressure is too high, and the valve placed on the head of the syringe has to be replaced by a one-way connector and an additional solenoid valve with higher pressure resistance. SIC systems have mainly been applied to pharmaceutical analysis [31,39], and pesticides determination [30] (see Table 1).

MSC was developed in our group in 2007 [29,41] (Fig. 2). In MSC, when coupling monolithic columns to a multisyringe burette. it has also to be taken into account that standard solenoid valves cannot withstand pressures higher than 2 bar, and the solenoid valve at the head of the syringe has to be replaced by a one-way connector and an external solenoid valve that withstands higher pressure (6 bar) (see Fig. 1).

As shown in Table 1, MSC has been widely used in recent years, e.g., MSC systems were proposed for the on-line SPE and determination of hydrochlorothiazide and losartan potassium in water samples [28], oxalate determination with chemiluminescence detection in beer and urine [27] and thiazide diuretics determination exploiting SPE [26]. Table 2 shows the advantages of SIC and MSC over HPLC, such as the possibility of two-way and stopped eluent-flow directions, reduced use of organic solvents and the possibility of the analyzer being portable. An additional advantage of MSC is the ability to perform multi-isocratic chromatographic separations by the use of different mobile phases avoiding the need for gradients. However, HPLC provides higher resolution of the peaks and higher robustness, being able to analyze samples of greater complexity, as can be seen in Table 1. Furthermore, SIC and MSC would require a second piston pump, burette or syringe to carry out chromatographic separations in the gradient mode.

4. Flow-injection techniques coupled to gas chromatography

GC is often the method of choice for the analysis of volatile target analytes in complex samples, due to the speed of analysis, the separation efficiency and the wide range of selective and sensitive detectors available [2]. A major drawback of GC is the tedious sample preparation that most samples require, usually done manually. In this sense, some automatic approaches based on classical instrumental methods have been developed, and led to progress in coupled techniques.

One of the main difficulties when coupling flow systems with gas chromatographs arises from the relatively low sample volume to be inserted (a few μL). In this sense, when coupling a flow device to a gas chromatograph, the following considerations have to be taken into account:

(1) the volatility and the thermal stability of target analytes;
(2) the compatibility of the medium to be used with liquid samples;
(3) the extracted volume, which should be as small as possible in order to minimize analyte dilution and avoid decreasing the sensitivity as a result; and,
(4) the transfer of the analytes from the pretreatment module to the gas chromatograph, which should be quantitative [2].

Several interfaces have been implemented for this purpose, making it possible to inject large volumes or even whole extracts

Table 1
Examples of applications exploiting coupled flow and separation techniques for the analysis of samples

Analyte	Sample	Technique	Extraction technique	LOD µg L^{-1}	RSD (%)	AT (min)	Remarks	Ref.
			FT-HPLC					
Gemfibrozil	Pharmaceutical	SIA		0.002–0.008	2.5	1	Monolithic column 25 x 4.6 mm C18	[8]
Chloramphenicol	Waters	FIA	MISPE	0.015	-	-	C-18 column 150 x 4.6, 5 µm	[22]
Chrorotriazine herbicides	Waters and crude soil extracts	MSFIA-LOV	MISPE	0.02–0.04	6	30	C-18 column 100 x 4.6, 3.5 µm Renewable sorbent for SPE	[23]
Chrorotriazine herbicides	Waters and crude soil extracts	MSFIA	CNT/F (carbon nanotubes/fibers)	0.004–0.03	2	25	Assisted stirrer magnetic preconcentration C-18 monolithic column 100 x 4.6 Renewable sorbent for SPE	[10]
UV filters	Waters	MSFIA-LOV	SPE (Oasis HLB)	0.45–3.2	13	9	C-18 monolithic column 100 x 4.6 Renewable sorbent for SPE	[24]
Phenolic pollutants	Waters	MSFIA	SPE (Oasis HLB)	0.2–3	8	15	C-18 Chromolith column 100 x 4.6	[9]
Pharmaceutical residues	Water and human urine	MSFIA-LOV	SPE (Oasis HLB)	0.02–0.36	11	20	C-18 column 150 x 3.9, 5 µm Renewable sorbent for SPE	[11]
Riboflavin	Foodstuffs	MSFIA-LOV	MISPE	0.050	6	9.6	C-18 Chromolith column 100 x 4.6 Renewable sorbent for SPE	[25]
			FT-MSC					
Thiazide diuretics	Urine, well water and leachates	MSC	SPE (disks) C18 and ion exchange	3.0–60.0	3.5–4.9	5.0–12.0	Monolithic Precolumn 5 mm and column 25 x4.6 mm C18 Automated separation and pre-post-column pretreatment	[26]
Oxalate	Beer and urine	MSIC	-	25.000	5	1.25	Surfactant coated C18 monolithic column	[27]
Hydrochlorothiazide and losartan potassium	Water and wastewater	MSC	SPE (disks) C18 and cation exchange.	70.0–90.0	3	6.7	Monolithic column 25 x 4.6 mm	[28]
β-lactamic antibiotics	Pharmaceutical preparations	MSC	-	40000	0.6–2.3	-	Monolithic column 25x4.6 mm Similar chromatographic parameters to HPLC	[29]
Pesticides Fenoxycarb and permethrin	Veterinary pharmaceuticals	SIC		1000–2000	2.29–2.98	6.5	Monolithic column 10x4.6 mm	[30]
Salicylic acid and ester methylsalicylate	Pharmaceutical preparations	SIC		40–150	2.85–4.10	7	Chromolith C-18, 50x4.6 mm	[31]
			FT-GC					
Sterols	Oils	FIA	SPE (silica gel)	0.368	3.6	55	High pressure injection valve for HPLC Inj. V: 5 µL On-line derivatization	[32]
Drugs	Biological fluids	FIA	SPE (C18)	0.0005–0.01	4.6	20	High pressure injection valve for HPLC Inj. V: 4.6 µL	[33]
Phthalates	Water	In syringe	DLLME	0.035	5	10	Injection via air stream Inj. V: 3 µL Low pressure injection	[34]
PAHs	Water and leachate	MSFIA-LOV	DLLME	0.01–0.07	5	8	Injection via air stream Inj. V: 3 µL Low pressure injection	[13]
PCBs	Water and leachate	MSFIA-LOV	SPE (Bond Elut Plexa)	LOQ 0.006	6	45	PTV injection via air stream Inj. V: 150 µL	[12]
Organochlorine pesticides	Water	SIA	Hollow-fiber sorptive ME	0.001	4	40	PTV injection via air stream Inj. V: 150 µL	[18]
			FT-CE					
Nitrophenols	Leachate and water	MSFIA-CE	SPE (C18)	2364.7–5981.3	3.0–6.0	19	Hydrodynamic injection	[17]
Inorganic cations and anions	Water	SIA-CE	-	9.6–475	1.8–5.8	35	Hydrodynamic injection In field application	[35]
Amino acids and peptides		SIA-CE-LIF -	-	30.000	5	32	Electrokinetic injection via an in-line injection valve	[36]
Arsenic speciation	Shrimps and urine	SIA-CE-ICP-SFMS	Manual lyophilization and extraction	-	2.6	5	Hydrodynamic injection Reduction of As residues (87%)	[37]

Table 2
Main advantages and disadvantages of the coupling of flow and separation techniques

Technique	Advantages	Disadvantages
HPLC	- On-line sample pretreatment - Minimization of solvent consumption than switching column approaches - Cost-effectiveness in comparison to switching column approaches	- Need for specific software to control the sample pretreatment - The majority of reference methods are off-line - Limited use of materials with different particle diameter for SPE
GC	- On-line sample pretreatment - Simpler hardware and software devices than other on-line approaches - Minimization of reagent consumption and waste generation - Minimization of cross contamination - Improvement in analyst safety - No need of any modification in the GC injector port	- Limited use of materials with different particle diameter for SPE - Need for specific software to control the sample pretreatment - The majority of reference methods are off-line - Limited flow rate
SIC and MSC	- On-line sample pretreatment - Reduction of sample and reagent handling and cost per analysis compared with HPLC - Portability - Low cost instrumentation	- Limited flow rate - Limited column length and particle diameter - Limited number of analytes to separate - Limited use of materials with different particle diameter for separation and preconcentration
CE	- On-line sample pretreatment - Portability - Low cost instrumentation - Reduction of sample and reagent handling and cost per analysis	- Need for specific software to control the sample pretreatment - Lower sensitivity compared with other separation techniques

of pretreated samples into the GC, the most popular being on-column interfaces, the loop type and the programmable temperature vaporizer (PTV) [2,42]. The PTV injector offers a straightforward means for coupling FTs on-line to GC [12,18]. However, basic understanding of solvent effects and large-volume on-column injection is a prerequisite for developing such systems.

Another factor to take into account when coupling FTs and GC is the different aggregation state of the sample that defines the extraction method that will be used. In this sense, SPE was the first technique coupled on-line to GC in 1987 via an on-column interface [43] for the analysis of chlorinated pesticides in water samples, and is now one of the most used techniques in combination with GC (see Fig. 2). The materials used for SPE in these devices must have a specific diameter of particle, which allows the use of different flow rates without backpressure.

Diverse designs and configurations have been reported, where FTs are exploited as a front end to GC (see Table 1). Most applications date back to the 1990s, for the determination of different organic compounds [32,33,44]. The reduction in the number of publications with these coupled configurations can be attributed to the fact that the final eluate volume needs to be reduced prior to introduction into the GC capillary column, giving as a result a technically complex FT-GC coupling, difficult to optimize. For this reason, automatic approaches were developed {e.g., Prospekt-2 (Spark Holland, The Netherlands), Twin–PAL (Carrboro, NC, USA) [45]} to solve the inconvenience of FT systems. However, these robotic systems require modification of the PTV injector, whereby the GC can merely be used in combination with the analyzer.

Recently, a number of automated GC methods were developed using new configurations of FTs, such as MSFIA, LOV and in-syringe.

Automation with the MSFIA technique made it feasible to integrate multiple operations using a single multisyringe module, e.g., in off-line approaches in the determination of cocaine and benzoylecgonine in human urine [15] and beta blockers in human plasma [16], significantly reducing the time and the cost per analysis.

The versatility of coupling MSFIA-LOV on-line with GC was demonstrated in our group with several approaches applied to environmental samples, as shown in Table 1. The first was developed in 2009 by Quintana et al. [12], who designed an MSFIA-LOV-GC-MS system for determination of polychlorinated biphenyls in solid-waste leachates, using SPE with a renewable Bond Elut Plexa column (Fig. 2). This MSFIA-LOV-GC-MS system avoided an increase in backpressure in the flow system by minimizing the sorbent quantity and allowed the appropriate delivery of well-defined volumes of eluate. Boonjob et al. [18] also determined organochlorine pesticides in raw waters, using sorptive microextraction with a short single-strand polydimethylsiloxane hollow fiber integrated in an SIA network for automatic fluidic handling by programmable flow.

MSFIA has also been coupled to the in-syringe DLLME technique, resulting in a valuable tool for preconcentration and removal of the matrix effect. The proof-of-concept was developed in 2014 by coupling in-syringe DLLME and GC-MS for phthalates [34] (see Fig. 2) and PAH determination in environmental water samples and leachates [13], respectively (see Table 1). In all these approaches, a syringe containing air was connected to an injection valve used to inject an aliquot of the extracted organic drop that was transported into a loop and injected via an air stream. The main advantage of this coupling, as shown in Tables 1 and 2, is the increase in analysis throughput, due to the simultaneous performance of sample pretreatment and GC separation. Coupling FTs and GC achieves the same benefits as when automating sample pretreatment prior to HPLC.

5. Flow-injection techniques coupled to capillary electrophoresis

CE achieves low sample and electrolyte consumption, and experimental simplicity. Its sensitivity and precision can be improved by coupling with FTs [46] and temperature control. Most relevant advantages and disadvantages are summarized in Table 2. Coupling of pressure-driven flow and electrophoretic separation methods presents some technical challenges. The design of the interface that connects both analytical techniques is critical (see Fig. 1).

The first on-line FIA-CE systems were developed almost simultaneously [47,48] (Fig. 2) using two original interfaces. Both designs consist of a flow-through channel, into which one end of the CE separation capillary and an electrode are inserted. Kuban et al. fabricated an interface by precise drilling in a piece of poly(methyl methacrylate). In the other design, the conical-flow-through channel was an Eppendorf pipette tip placed vertically in a supporting vial. In the latter, it was important to keep the separation capillary as close as possible to the end of the conical flow inlet. These interfaces are currently widely used when coupling FIA or SIA to CE, sometimes with modifications. For example, the original FIA-CE system was modified by replacing the peristaltic pump with gravity flow [49], obtaining better RSD values from repeated injections, probably due to the avoidance of pulses by using gravity feed flow. Other authors

simplified the design of the interface to a three-channel T-shape piece using a tubular Pt electrode [50].

CE has also been successfully coupled to electrospray ionization mass spectrometry [51], atomic fluorescence spectrometry [52] and contactless conductivity detection for on-line analysis of metal cations [53]. In this system, the ends of the separation capillary and the electrodes were placed opposite each other in tubing that acted as flow-through channels.

In principle, the interfaces used for coupling SIA and CE are the same as in FIA-CE systems. The main advantage of SIA over FIA is the higher precision of the pressure generated by a syringe pump to pressurize the capillary, the lower flow rates available ($\mu L\ min^{-1}$), the possibility of using head column field amplified stacking by applying pressure and electrokinetic injection at the same time and that SIA can better handle limited amounts of sample. The first SIA-CE system was developed by Fang et al. in 1999 [54]. Since then, SIA-CE has been successfully coupled to different detection techniques, such as C4D [35], laser-induced fluorescence via a valve interface for on-line derivatization and analysis of amino acids and peptides [36], and an ICP sector field mass spectrometer reducing the As residues by 87% [37] (see Table 1).

CE was coupled to MSFIA for the first time in 2007 by Horstkotte et al. [55], as shown in Fig. 2. Another MSFIA-CE system was developed for preconcentration, separation, and determination of nitrophenols [17]. In this MSFIA-CE system, sample pretreatment and electrophoretic separation were successfully automated. A home-made photometric detection cell was used for on-capillary detection, using an optical fiber and an LED. Thus, by coupling CE and FTs, greener, more reproducible methods are achieved due to process downscaling and reduced analyst intervention.

6. Concluding remarks and future prospects

The coupling of FTs and chromatographic separation techniques provides the necessary selectivity and detection levels required to determine organic pollutants, leading to development of novel, fast, robust, reliable, sensitive and more environment-friendly methods. The advent of monolithic columns has opened up new possibilities in flow analysis, allowing the development of low-pressure chromatographic techniques.

The methodologies obtained through the coupling of FTs and separation techniques have advantages, such as:

(1) increased injection throughputs;
(2) high versatility;
(3) high robustness;
(4) new analytical improvements based on operating modes under non-stationary conditions;
(5) decrease in human exposure under hazardous chemical/physical sample pretreatments;
(6) more environment-friendly procedures obtained due to process downscaling; and,
(7) use of alternative detection systems with the concomitant simplification of the operating conditions.

However, of these coupled systems have not yet seen widespread use, given the lack of commercial instruments for on-line coupling and also requiring some adaptation and optimization as well as knowledge of the underlying principles. In LC-FT coupling, there have been more advances due to the similarities in aggregation state and physicochemical principles of LC and FTs. Nonetheless, sample pretreatment is still required prior to LC, SPE being the technique most used for this purpose. As a result, switching-column approaches have had more acceptance, leading to the advent of commercial devices.

Unfortunately, this is not the case for other separation techniques with more coupling restrictions, such as GC and CE, where further coupling strategies need to be developed to facilitate the general use of integrated systems.

Notwithstanding, the benefits of on-line coupling of separation and FTs are clear and the time invested in optimization is rapidly repaid in shorter analysis time, better reproducibility and improved detection limits.

Future prospects in this field seem to be focused on miniaturization and continuing the coupling of automated flow-based sample-treatment systems and separation techniques, and investigating new interfaces and coupling strategies.

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CHAPTER 2
OBJECTIVES

In this chapter, main and specific objectives of this thesis are presented.

2. OBJECTIVES

The main purpose of the present thesis is the development of new automated, accurate, precise, reliable and rapid analytical systems for monitoring the presence of parameters of environmental interest in water samples exploiting microextraction flow-techniques and separative techniques.

In addition, all the systems were designed aiming to achieve maximal robustness, simplicity, stability, minimal consumption of reagents, waste generation, and time of analysis, as well as the automation of the required sample treatment and the applicability to real processes.

Specific aims of this dissertation are given below, itemized per chapters.

Chapter 4

- Assess the potential use of 3-hidroxy-4-pyridinone chelator as nontoxic chromogenic reagent for iron determination using sequential injection analysis and spectrophotometry.
- Develop a microsequential injection lab-on-valve methodology using 3-hidroxy-4-pyridinone for iron speciation exploiting solid phase spectrophotometry.
- Compare the figures of merit of both methods, with and without the use of the SPE material for iron determination and speciation.

Chapter 5

- Develop a fully automated fluorimetric method for aluminium determination exploiting in-syringe dispersive liquid-liquid microextraction.
- Develop a fluorescence detector, a flow cell and, a heating device integrated into the holding coil to accelerate the reaction kinetics.
- Study the effect of implementing in-syringe magnetic stirring assistance for fully automated aluminium determination.
- Compare the figures of merit of both methods for aluminium determination.

Chapter 6

- Develop a simplified variant of the methylene blue active substances (MBSA) method exploiting in-syringe magnetic-stirring-assisted dispersive liquid-liquid microextraction with the novelty of setting the syringe up-side down in order to use chloroform as extraction solvent to achieve comparability toward the standard procedure for MBSA determination.
- Develop an in-syringe magnetic stirring assisted dispersive liquid-liquid micro-extraction method for fully automated determination of cationic surfactants in environmental water samples.

Chapter 7

- Develop a fast, simple, fully automated, cost-effective and environmental friendly method based on in-syringe magnetic-stirring-assisted dispersive liquid-liquid microextraction coupled to high performance liquid chromatography for the on-line preconcentration, separation and detection of UV filters.

CHAPTER 3

MATERIALS AND METHODS

In this chapter, the instrumentation, detection systems, the software, and the multivariate optimization methodology used are presented.

3. MATERIAL AND METHODS

3.1. Flow instrumentation

The flow instrumentation used has been separated in two groups. On the one side the instrumentation used for sequential injection LOV (chapter 4) [1, 2] from FIAlab Instruments (Medina, WA, USA) and, on the other side the instrumentation used in the MSFIA systems developed (chapter 5-7) [3-6] from Crison Instruments S.A. (Alella, Spain). These were used for the development of automated analytical methods for laboratory and monitoring analysis.

Both FIAlab and Crison instruments were connected to a personal computer for remote operational control via the serial RS232C interface accomplished by the software FIAlab or AutoAnalysis, respectively. Thus, the instrumentation used during the development of this thesis is described in detail below.

3.1.1. Sequential injection lab-on-valve instrumentation

A FIAlab-3500 analyzer from FIAlab Instruments (Medina, WA, USA), shown in Figure 3.1, was used to develop the SI-LOV analyzer used in this thesis (chapter 4) [1, 2].

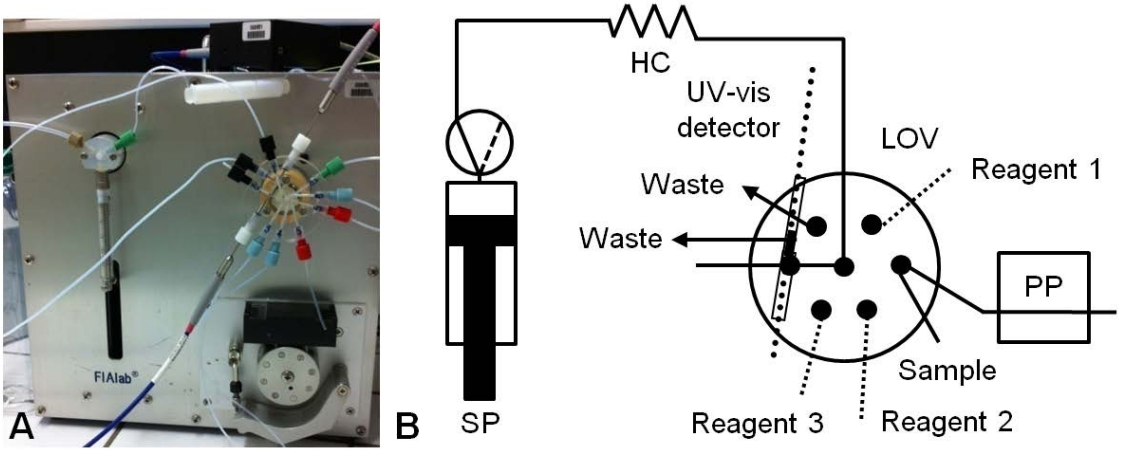

Figure 3.1 A) Image of a FIAlab-3500 system. B) Schematic illustration of the developed SI-LOV system. HC: holding coil, PP: peristaltic pump, SP: syringe pump.

The SI-LOV analyzer consisted of a box containing a bi-directional SP (2.5 mL of volume), a bi-directional variable speed PP, and a LOV manifold mounted on top of

a six-port MPV. The high resolution bi-directional SP is the main component used to aspirate and propel the solutions from the MPV to the system. The syringe is equipped with a two way commutation valve on the top of it. Moreover, syringes of 50, 100, 250, 500 µL, 1.0, 2.5, 5.0, 10, and 25 mL can be implemented. In our flow system the 4 channel PP was employed as auxiliary propulsion device to circulate the sample solution or the bead injection suspension permitting a flow rate of 0.3-2.8 mL/min per channel.

The LOV body was made of Ultem, a very resistant material to a variety of chemicals. This type of valve acts as a stream selector, connecting just one side port to the central one at a time. The central port is commonly connected to a HC. Furthermore the LOV piece had an integrated flow cell with four outlets capable of accommodating optical fibers. These were mounted axially to perform spectrophotometric detection. The optical path length can be varied by changing the position of the optical fiber as can be seen in Figure 3.2. Thus, it is possible to obtain a light path between 2 and 10 mm. A path length of 10 mm was used.

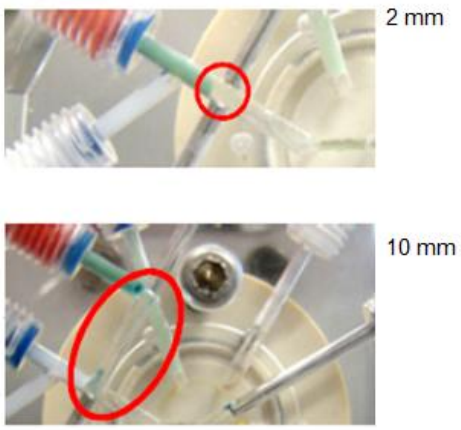

Figure 3.2 Representation of the different light path dimensions that can be obtained in the LOV used.

The FIAlab-3500 system includes multiple RS-232 ports, A/D data collection channels, and 24 VDC relays. Peripheral compatible analytical instruments, such as autosamplers, additional SPs, additional PPs, external multiposition and injection valves, can be implemented directly with the FIAlab-3500 simply by connecting them to its rear panel.

3.1.1.1. Manifold components

The flow network of the manifold was constructed of flexible tubes of chemical resistant PTFE of 0.8 and 1.5 mm inner diameter (id), including a HC. Moreover, Tygon tube of 0.8 mm id was used in the PP. All connections were made by means of polyvinylidene fluoride (PVDF) connectors. Particular tube lengths are detailed in following chapters according to particular manifolds used in each flow system.

3.1.1.2. FIAlab software

The FIAlab software for Windows 5.0 (Figure 3.3) was used. Its core functions are designed to communicate with a wide range of peripheral devices and instruments. Thus, FIAlab software can be used to control FIAlab instruments, for data collection from the FIAlab-3500 system and to control compatible peripheral devices. The family of external devices controllable by FIAlab for Windows include: a SP, a MPV, a PP, an USB 2000 Ocean Optics spectrometer, and data acquisition devices.

Figure 3.3 Layout of the FIAlab for Windows 5.0 program user interface (www.flowinjection.com).

3.1.2. Multisyringe flow injection analysis instrumentation

The MSFIA systems developed in this thesis (chapter 5-7) exploited the versatility of the multisyringe pump and the software AutoAnalysis connecting in series several instruments, e.g. MSP, MPV, IV and autosamplers from Crison Instruments. All these developed systems had in common the use of a MSP as liquid driver.

MSP consist of a conventional automatic titration burette which can be equipped with up to four syringes (Figure 3.4 A). Pistons of the syringes are mounted on a common steel bar driven by a single step per motor. Thus, all pistons are moved simultaneously and unidirectional for either liquid delivering (dispense) or aspirating (pick up). Each syringe has a three-way solenoid valve (N-Research, Caldwell, NJ, USA) at the head, which facilitates the application of multicommutation schemes. For each syringe (S1-S4) both operations are feasible either with the head valves activated (ON) or deactivated (OFF) allowing four kinds of liquid displacement: ON-dispense, OFF-dispense, ON-pick-up and OFF-pick-up (Figure 3.4 B and C).

Generally, the position ON is connected to the system manifold and the position OFF to a solution reservoir. The short time required for valve switching, i.e. only 35 ms, allows change of the valve position even during flow operation. The valves can withstand a backpressure of 2 bars. High chemical robustness is provided by the use of resistant polymers poly(ethylene-co tetrafluoroethylene) (head valves) and PTFE (piston heads, poppet flaps).

The step motor reaches total displacement corresponding to 5000, 16000 or 40000 steps. In this thesis 5000 and 16000 steps MSPs were used achieving total displacement between 8-329 s and 20-999 s, respectively. Thus, the MSP allows precise volume handling and a wide flow rate range (0.9-37.5 mL/min). Syringes of 0.5, 1, 2.5, 5, 10 and 25 mL can be implemented, enabling a wide range of combinations. In the works presented, syringes of 5 mL were used.

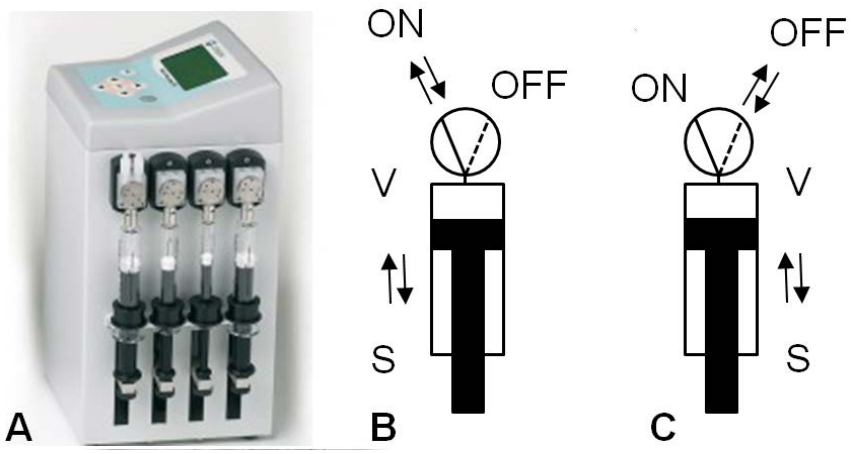

Figure 3.4 A) Side view of a multisyringe burette (Crison Instruments) B) Activated solenoid valve: "ON" position and C) Deactivated solenoid valve: "OFF" position.

The multisyringe burette has four backside ports (V5-V8, Figure 3.5) which enable the power of additional external multicommutation valves, micropumps, motors or other instruments either directly or via a relay allowing remote software control. This amplifies the possibilities to construct sophisticated flow networks. Each port provides 12 V with a maximal current of 0.5 A.

Figure 3.5 Backside connections of a multisyringe pump.

Moreover, Crison instruments, e.g. MPVs, IVs and autosamplers, can be connected in serial through the back port "peripheral" of the multisyringe pump.

MPV modules (Crison) (Figure 3.6) can be equipped with 6, 8, or 10-port rotary MPVs made of chemical resistant polymers polyetheretherketone (PEEK) (stator) and PTFE (rotor). In the systems developed 8 and 10 port rotatory MPVs were used.

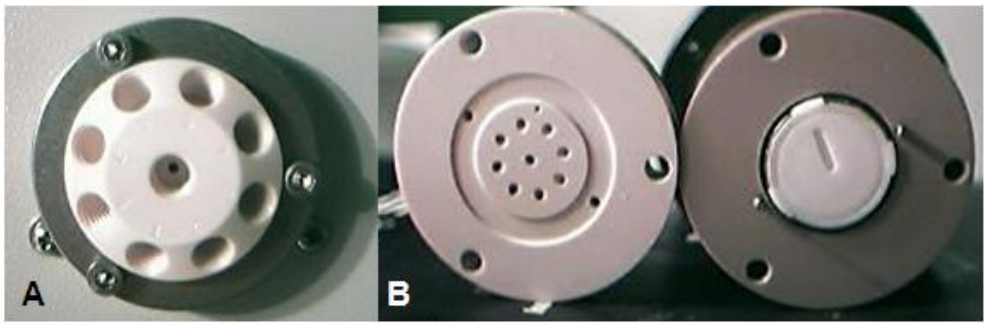

Figure 3.6 A) Multiposition valve used in SIA systems and B) inner parts of the valve.

The IV module used (Figure 3.7) is a 6-port rotary IV. IVs have two different positions, load (Figure 3.7 B) and inject (Figure 3.7 C), allowing the injection of a selected plug into a carrier stream, e.g. it can be used to interface flow systems and HPLC so that when the valve is in load position, sample flows through the external loop while the carrier flows directly through to the chromatographic column, and when the valve is switched to inject position, the sample contained in the sample loop is injected onto the carrier stream going to the chromatographic column.

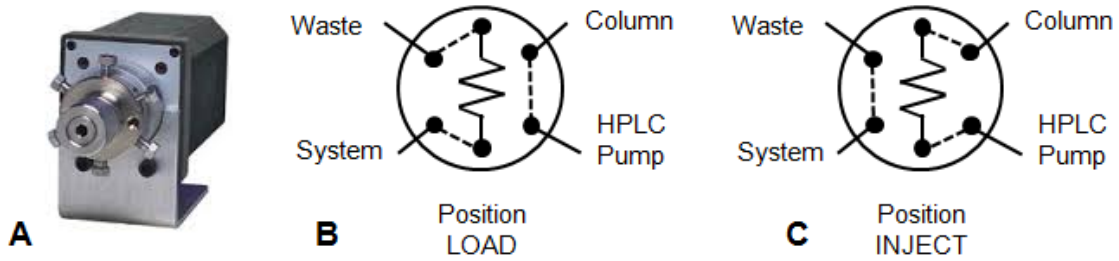

Figure 3.7 A) injection valve, B) schematic representation of load position and C) inject position.

The autosampler (Figure 3.8) can be equipped with a 45-position turntable for tubes of 15 mL or a 15-position turntable for 120 mL beakers. Both of them were used for optimization, interferences studies or calibrations during the development of this thesis.

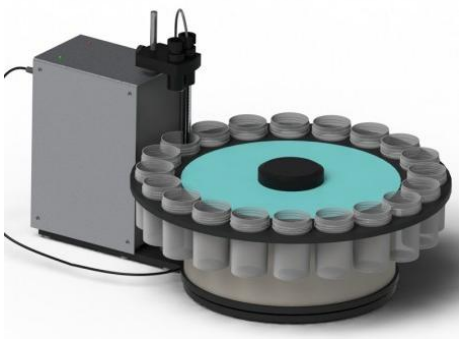

Figure 3.8 Autosampler.

This instrumentation is compatible with a wide variety of detection techniques. The software AutoAnalysis permits data acquisition and processing from UV-VIS spectrophotometry, fluorescence, chemiluminescence and electrochemical detectors. In our case, UV-VIS spectrophotometry and fluorescence were used. These detection techniques are described in more detail in section 3.3.

3.1.2.1. Control of temperature

In one of the systems developed (chapter 5) [3, 6] it was necessary to control the temperature to carry out the reaction between the analyte and the chelant. Thus, a device to control the temperature was implemented (Figure 3.9). It consisted in a brass support for the tight insertion of a chemical inert 12 cm long glass capillary (1.5 mm id, 2 mm outer diameter (od)) used as flow channel, and a commercial halogen light bulb (12 V, 20 W) used as heating source. Temperature control and bulb powering was done via a negative temperature coefficient (NTC) thermistor probe and a thermostat control circuit from CEBEK – Fadisel SL (Barcelona, Spain Ref. I-81), respectively. A temperature hysteresis of less than 1°K was achieved by increasing the value of the original feed-back resistor on the operational amplifier of the thermostat circuit to 2 MΩ.

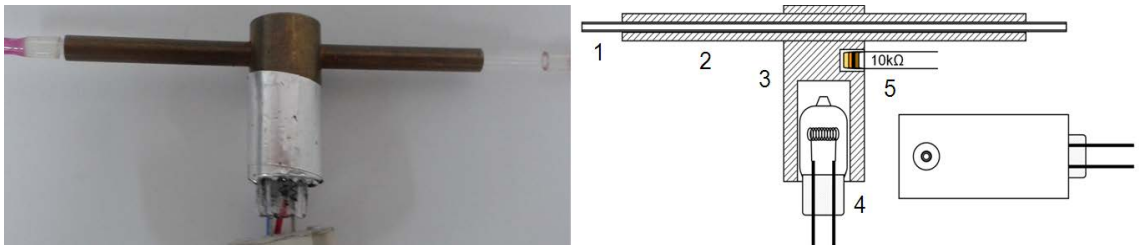

Figure 3.9 A) Photograph of the heating device. B) Detailed representation of heating device as both cross-section cut top view and side view. Elements: (1) glass capillary,(2) brass tube, (3) brass cylinder, (4) halogen light bulb, and (5) NTC resistance used as sensor.

3.1.2.2. In-syringe magnetic-stirring-assisted dispersive liquid-liquid microextraction device

Two different sample treatments to isolate and preconcentrate the analytes of interest in a suitable medium prior detection have been used in this thesis, i.e. solid phase microextraction and in-syringe MSA-DLLME [3, 4, 6]. In-syringe DLLME was improved by implementing MSA devices developed in our laboratory to increase the dispersion

between phases.

As described previously, in- syringe MSA-DLLME is a way of automating DLLME inside the syringe of a system composed of a MSP, a magnetic stirring system and a MPV in order to aspirate and dispense sequentially the reagents and sample. The movement of the stirrer is controlled by computer via the MSA system which is depicted in Figure 3.10.The MSA system consists of four main parts, a small magnetic stirring bar placed inside the syringe (A), an external magnetic stirring device (Figure 3.11 A, B) (B) which is placed around the syringe, a motor (M) (Figure 3.11 C) which forces the rotation of the external device, and a circuit (C), which controls the ON / OFF and the engine revolution through one of the auxiliaries exits of the back of the multisyringe.

The magnetic micro stirring bar had the following dimensions: 10 mm length and 3 mm diameter placed inside the syringe. The top position of the syringe piston was adjusted in such a way, that a gap of about 5 mm was left when emptying the syringe to avoid any damage or breakage when completely emptying the syringe. Furthermore, this space allows free rotation of the stirring bar even when the piston is in the upper position.

Two magnet drivers were designed, depending on the position of the MSP (up or upside-down) to create the rotating magnetic field within the syringe. These are shown in Figure 3.11. If the MSP is up, the external device consists of two rings made of Delrin® used as bearings. These could be easily placed onto the syringe, with the bottom ring sliding on the flange of the syringe glass barrel. Two M4 steel screws of 80 mm in length were used as spacers and connection between both rings. This assembly can rotate freely around the syringe longitudinal axis. Thus, by placing two neodymium magnets (5 mm x 4 mm od) on top of the screws, a magnetic field in the syringe and along its whole length was obtained.

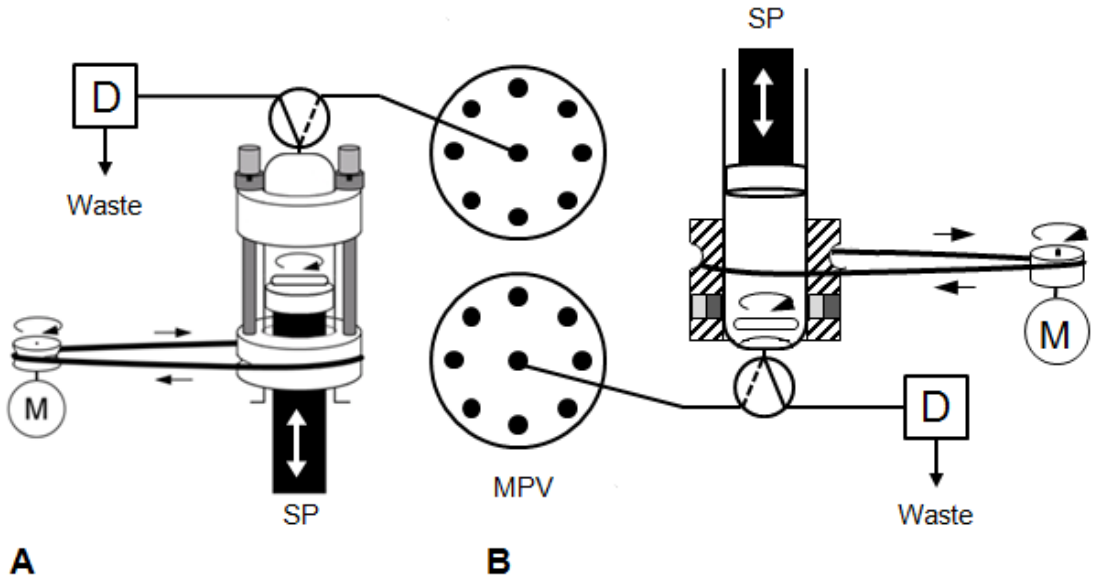

Figure 3.10 A) Magnet driver placed onto the syringe barrel with the multisyringe pump up. B). Magnet driver placed around the syringe body with the multisyringe pump down. D: detector, M: motor, MPV: multiposition valve, SP: syringe pump.

This magnetic force was sufficient to attract and force the rotation of the stirring bar inside the syringe independently from the position of the syringe piston when turning. The bottom ring had a groove for a rubber band, which allowed to propel the external device with a low-cost DC motor. The DC motor was activated using a homemade relay and a regulation circuit board through an auxiliary supply port of the multisyringe module. The circuit to control the motor is given in Figure 3.12.

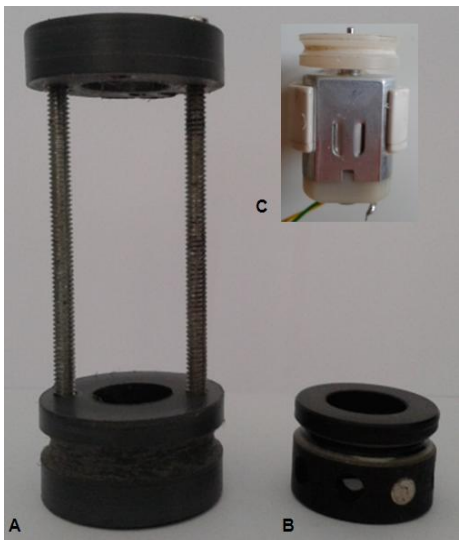

Figure 3.11 A) External device for up multisyringe, B) external device for upside-down multisyringe and C) motor.

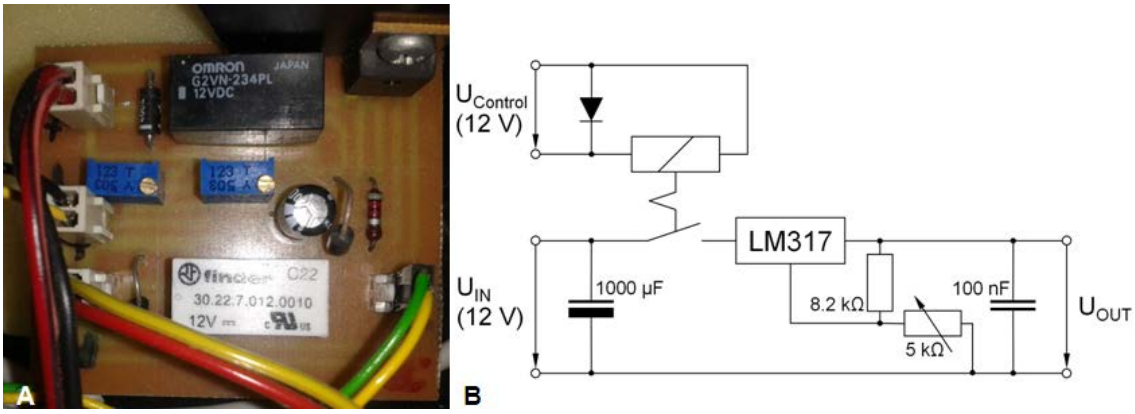

Figure 3.12 A) Image and B) scheme of voltage regulator for the stirring DC motor.

If the MSP is upside-down, the same device was employed but just with one ring as external driver holding two small neodymium magnets facing each other creating a rotating magnetic field around the syringe's body (Figure 3.11 B).

3.1.2.3. Manifold components

The flow network manifold was constructed of flexible tubes of chemical resistant PTFE of 0.8 and 1.5 mm id, including HCs. Particular tube lengths are given at each developed manifold. All connections were made by means of PVDF connectors, except cross-junction, which are made of PMMA or PEEK.

3.1.3. AutoAnalysis software

The software package AutoAnalysis 5.0 was fundamentally described by Becerra et al. (1999) [7], developed in our group "Analytical Chemistry, Automation and Environment" and is commercialized by Sciware Systems S.L. (Bunyola, Spain). Thus, instrumental control, data acquisition and processing were carried out with this software which is written in Delphi and Visual C++ and offers a window-based graphical surface.

The distinctive feature of this software based on dynamic link libraries (DLLs) at 32 bits is the possibility of using a single and versatile application without further modification for whatever instrumentation and detection system needed. It involves a basic protocol which allows the implementation of specific and individual DLLs, addressing the

configuration of the assembled flow analyzer. Thus, being a very flexible tool, easy to handle by non-specialised users.

3.1.3.1. Hardware configuration

The establishment of a communication protocol or "channel" (e.g. RS232, RS485, USB), incorporation and configuration of the connected hardware (e.g. MSP, spectrometer) are carried out via individual DLLs. These are installed and loaded as required for each system forming an individual configuration. Thus, modification of the instrumentation assembly requires a minimum of adaptation effort (Figure 3.13). Program versatility is only limited by the availability of the required DLLs. Up to now, DLL for seven communication channels and 30 devices are available including atomic fluorescence, fluorescence, spectrometric and electrochemical detectors, autosampler, syringe-, peristaltic-, and solenoid-micropumps, valve modules, and I/O, A/D, or D/A PC digital cards for the connection and communication with other devices. In this work, DLLs for the communication channels Ocean Optics (USB) and Serial Crison (RS232) were used, also DLLs for the following instruments control were used, i.e. automatic multisyringe Crison, automatic valve module Crison, IV Crison, autosampler Crison, and spectrometer Ocean Optics. After loading the required configuration for the connected instruments, they can be addressed in each method and corresponding command forms become available in the editor window in addition to the basic functions. Data processing is the same independently of the instruments or configuration loaded. At the same time, data processing and method development are centralized, providing ease of use for the users by minimizing the time and efforts required to incorporate a new instrument to the system.

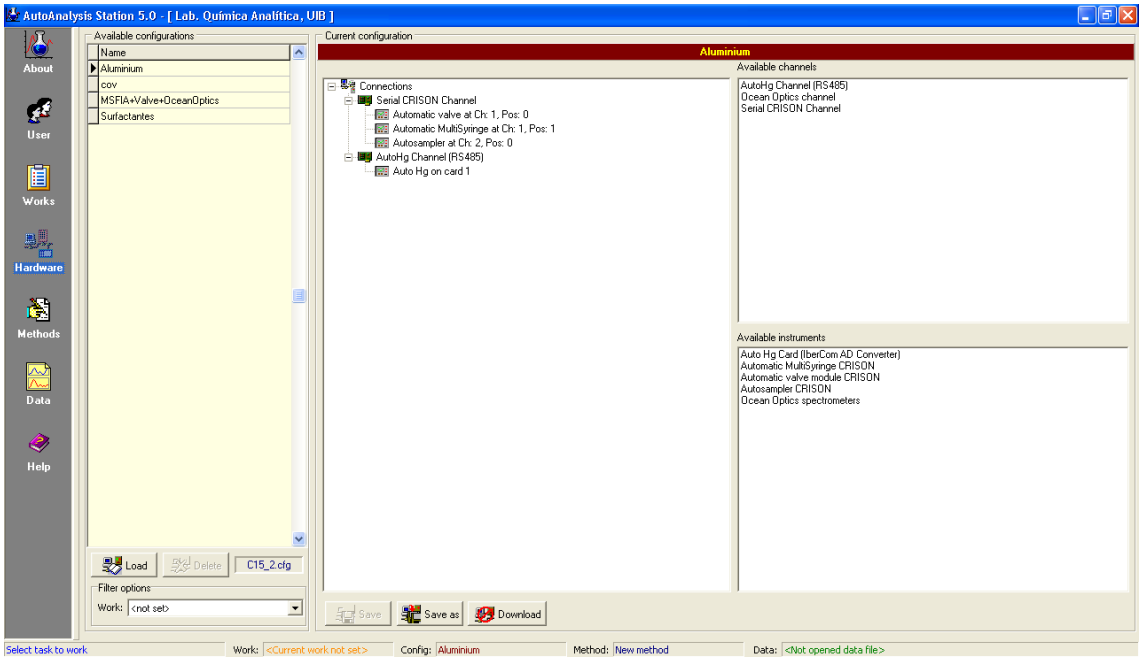

Figure 3.13 Hardware window, where channels and instruments are loaded to establish a configuration.

3.1.3.2. Method edition

The analytical method is created by sequencing functions (e.g. wait, variable) or instructions for the instruments by their selection from a pull-down menu and assigning specified commands to them (Figure 3.14). This provides a variability of programming functions for method creation including procedures, loops, indexing, basic calculations, variables, user input, waiting steps, event marking, comments, conditional enquiries, and on-line data processing. Further functions enable method test execution, definition of detection thresholds, manual data processing, such as calibration, data smoothing, spectral information processing, peak adjustment, and data export. Furthermore, this software allows the use of conditionals which enables the development of smart systems.

Procedures are prior-created instruction protocols, which can be loaded by all methods within the same hardware configuration. Main advantages are higher method clearness by apparent shorting, module-like programming, and repeated application in the same method.

The window “Execute” enables method initiation, pause, stop, as well as the control of the execution and continuous data acquisition. Zoom, scale, and shift functions are also available. Taking advantage of these features allows the creation and optimization

of highly versatile applications. AutoAnalysis allows multitasking operations such as simultaneous method execution and data processing, multiple loading and working with other programs running at the same time. It is important to note that operations can be carried out either exclusively, i.e. the next instruction is not processed until the previous one finishes, or no-exclusive, i.e. the next instruction is processed already after initialization of the previous operation, which is then executed in background.

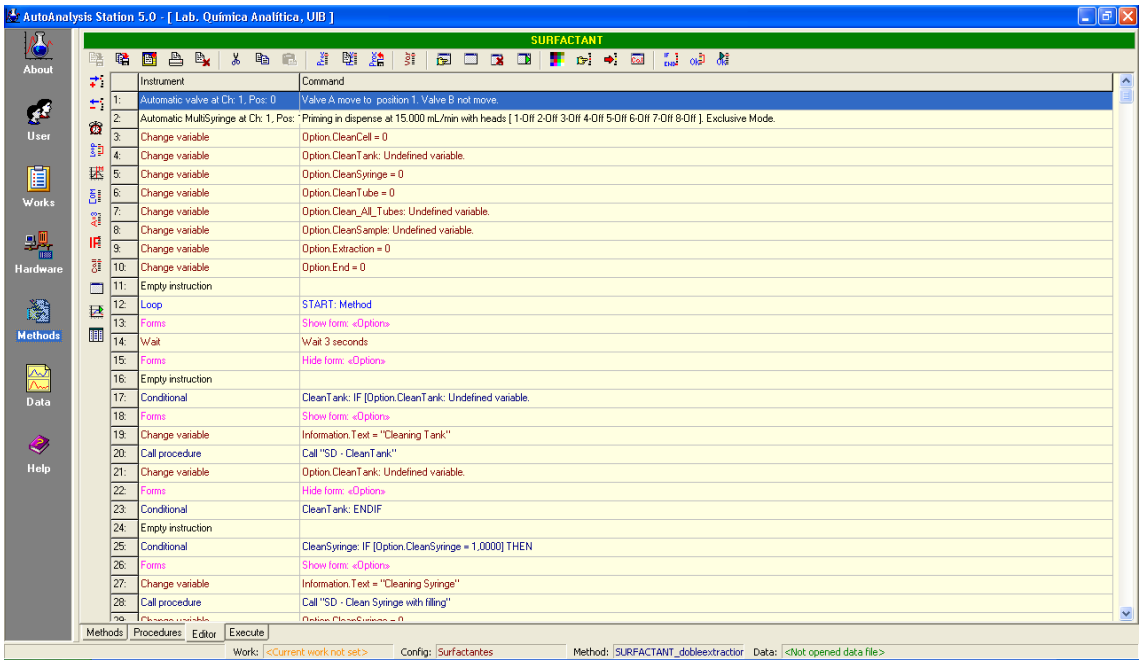

Figure 3.14 Method edition window.

3.1.3.3. Data processing

AutoAnalysis offers tools for on-line data processing and after method termination such as zoom functions, visualization of the original acquired data as well as of smoothed functions. It allows peak height and peak area calibration, data export and saving, basic calculations, and overlay with prior acquired data (Figure 3.15). For peak maximum, start-, and end-point identification, thresholds for the numerical first and second derivative and peak height minimum can be defined. Manual, mouse-drag peak correction, adding of non-identified peaks, or elimination of error-peaks is further possible.

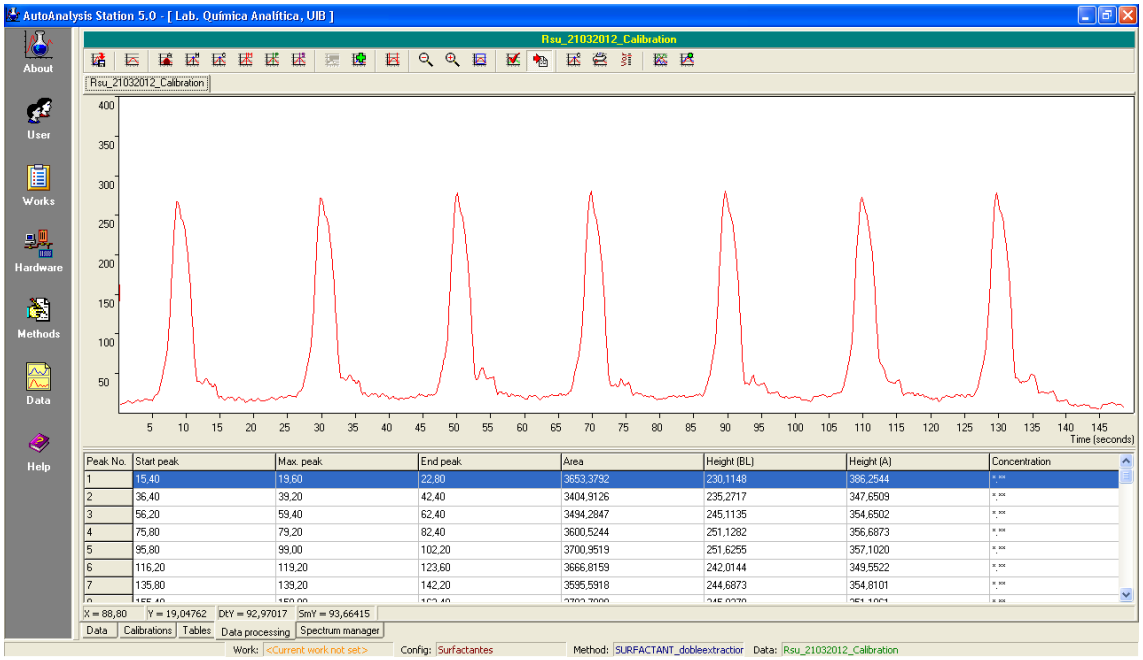

Figure 3.15 Fiagram and register table of the analytical signals.

3.2. Liquid chromatography instrumentation

A HPLC instrument, equipped with a quaternary Pump (600), UV/vis Detector (2996) and a column oven, from Waters (Torrance, CA, USA) was used. Separations were carried out using a phase reverse Symmetry® C18 analytical column (250 mm x 3 mm id 5 µm) preceded by a C18 guard column (5 mm x 4.6 mm id), both from Waters. Reversed phase column is based on silica limiting the pH range to 2-8. HPLC system and data management were controlled by Empower software (Waters).

3.3. Detectors

3.3.1. Spectrophotometric detector

UV-VIS spectrophotometric detection was carried out using a miniature optical fiber spectrophotometer *Coupled Charge Device* array sensor type and an USB 2000 spectrophotometer from Ocean Optics Inc. (Dunedin, USA). A DH-2000-BAL UV/VIS/NIR light source from Ocean Optics Inc. and an optical fiber of 400 µm core diameter were also used.

In some of the works developed (chapter 4) [1, 2] the flow cell was integrated in the LOV between two optical fibers that were mounted axially having a 10 mm optical path, as it is shown in Figure 3.16.

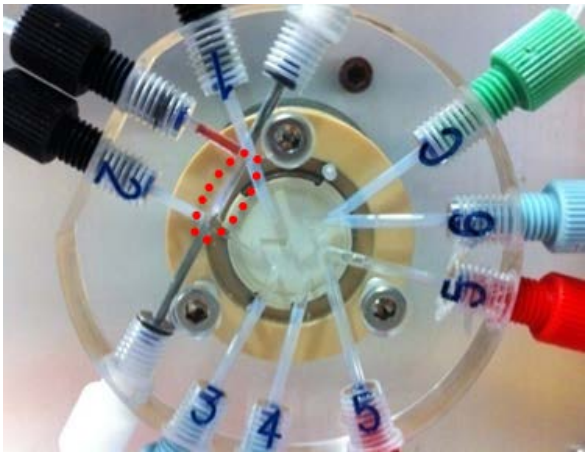

Figure 3.16 Lab-on-valve with integrated flow cell with a 10 mm optical path.

In other works (chapter 6) [4, 5] a flow cuvette of 1 cm optical path length and 1.5 mm flow channel diameter from Hellma Analytics (Müllheim, Germany) and a fiber-optic cuvette support from Ocean Optics was implemented.

3.3.2. Fluorimetric detector

For fluorescence measurements (chapter 5) [3, 6], a special detection cell was designed. This is schematically shown in Figure 3.17 [6]. Shortly, it comprised a glass tube of 3 mm id used as detection flow cell channel. A bright green Light-Emitting Diode (LED) with an emission wavelength of 500 nm, powered by a mobile phone charger, was used as excitation light source and placed on-top of the detection flow cell (3, 3.5 cm, 5 mm od, 3 mm id) with a metal-film band-pass interference filter between them. The emission light is filtered by a long-path filter and detected from a photomultiplier tube. The photomultiplier tube (PMT) from Hamamatsu Phototonics K.K. (Hamamatsu, Japan, Ref.: HS5784-04) was used for detection of fluorescence emission and was mounted in perpendicular position onto the detection flow cell.

The interference band-pass filter of 500±10 nm (reference NT62-091) and the long-pass glass filter of 580 nm cut-off wavelength (reference NT66-042) were purchased from Edmund Optics (Barrington, NJ, USA) and placed between the LED and the glass

tube, and the glass tube and the PMT, respectively. A control unit from Sciware Systems SL was used for PMT supply and data readout.

A signal amplifier module was used to supply the PMT with a feedback voltage of 0.3–0.8 V and to obtain a signal gain (*G*) linear over the range 1–1000. Best results were obtained with a voltage of 0.8 V.

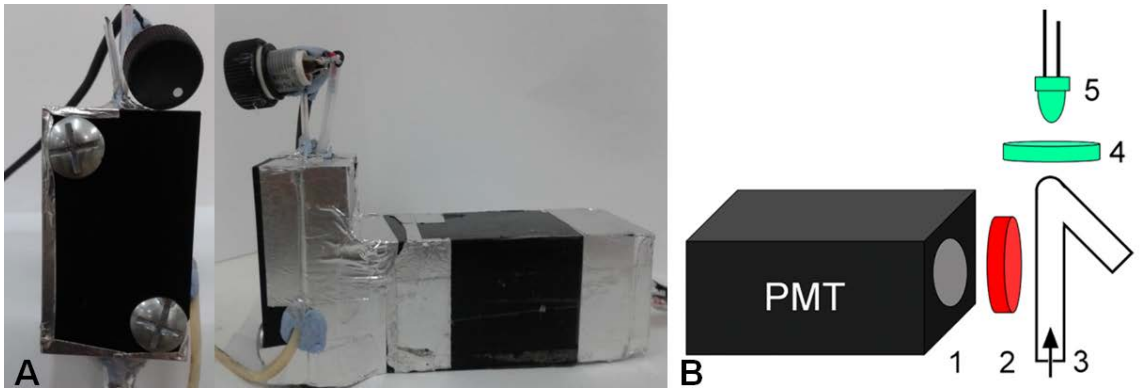

Figure 3.17 A) Detection cell for fluorescence measurements. B) Scheme of the detection fluorescence cell used. (1) Photomultiplier tube, (2) long-path filter, (3) glass tube, (4) band-pass interference filter and (5) green LED.

3.4. Experimental design-Multivariate optimization

The term optimization in analytical chemistry usually refers to a process based on instructions that allow obtaining the best conditions for an analytical method. These are often expressed through mathematical methods to maximize or minimize any specific property of the system under study [8, 9]. Optimization in analytical chemistry has been carried out by monitoring the influence of one factor at a time on an experimental response. While only one parameter is changed, others are kept at a constant level. This optimization technique is called one-variable-at-a-time. Thus, part of the optimization in this thesis was done in univariate approach. However, by univariate optimization possible significant interactions between factors can be missed, which is risky of misinterpreting the results and, in the worst case to be far from the optimal values.

In order to overcome this limitation, multivariate statistic techniques, in which different values of factors are varied simultaneously, can be employed. Multivariate optimization allows extracting more accurate information with the performance of a minimum number of experiments taking into account the interactions between factors.

In multivariate experimental designs, the knowledge of the system plays a major role because the number of experiments to be performed corresponds to an exponential function, where the factors studied are in the exponent. Therefore, including unnecessarily factors in the multivariate study leads to exponentially increase the number of experiments, without any utility. Usually optimization strategies are a combination of univariate methods for those factors which do not interact with other; and multivariate for those expected to have significant interactions. By comparing univariate design with multivariate design, it is possible to say that:

- Experimental design takes into account the interactions among the variables, while the univariate design does not.

- Experimental design provides a global knowledge (in the whole experimental domain), while the univariate design gives a local knowledge (only where the experiments have been performed).

- In each point of the experimental domain, the quality of the information obtained by experimental design is higher than the obtained by the univariate design.

- The number of experiments required by an experimental design is smaller than in a univariate approach.

As general rule, when the experimental variables and the responses have been defined, the experiments can be planned and performed in such a way that a maximum of information is gained from a minimum of experiments. The objective of modelling the response is to establish a function in which the dependant variable is the response and the independent variables are the factors that influence it. The screening is the first step. It is used to see the tendency of the system and which factors have a significant effect upon the response. After determining the significant factors, the optimum operation conditions are attained by using more complex experimental designs including quadratic terms.

The mathematical model found after fitting the data to a function can sometimes not satisfactorily describe the experimental domain studied. The more reliable way to evaluate the quality of the model fitting is by the application of analysis of variance (ANOVA).The central idea of ANOVA is to compare the variation due to the treatment

(change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses. From this comparison, it is possible to evaluate the significance of the regression used to foresee responses considering the sources of experimental variance. Moreover, as replicates of the central point are made, it is possible to estimate the pure error associated with repetitions.

Another way to evaluate the model is the *lack of fit test (LOF)*. If the mathematical model is well fitted to the experimental data, LOF should reflect only the random errors inherent to the system. Several models including linear and quadratic models are checked in order to choose the one which fits better to the experimental data. To apply a lack of fit test, the experimental design must be performed with authentic repetitions at least in its central point. Summarizing, a model will be well fitted to the experimental data if it presents a significant regression and a non-significant lack of fit. The lack of fit is directly related to the model quality.

3.4.1. Screening

The objective of this stage is to observe how factors influence the responses being really useful to reduce the number of experiments required by studying the effect and interactions of a preliminary selection of factors. This information enables the analyst to fix the factors with a negligible effect upon the response and to readjust the range of study of the significant factors [10].

In screening studies linear or second order interaction models are common, such as full factorial, fractional factorial, factorial at various levels or Taguchi orthogonal array designs. In this thesis, full factorial designs were used, since these permit to investigate influences of all experimental variables, factors, and interaction effects on the response or responses. Three centre points were included in order to minimize the risk of missing non-linear relationships in the middle of the intervals and to determine confidence intervals.

In a full factorial design, it is necessary to determine how many factors are of interest, and how many levels of each factor have to be studied. k^n experiments will be involved, being “n” the number of factors and “k” the number of levels. So, the first step is to establish the experimental domain; there is no guarantee that the results will be valid

outside this region. The second step is to choose a design. Basically, choose the "k" levels depending on the number of factors to be studied "n". Two level (k = 2) designs were used to optimize the methods developed. Two-level full factorial designs are used in the estimation of first-order effects, but they fail when additional effects, such as second-order effects, are significant. Therefore, central points in two-level factorial designs are required for evaluating the curvature.

In conclusion, the evaluation of the screening, such as p-value for variables, curvature, lack of fit, correlation coefficients and residuals, allows the selection of the representative variables and its ranges. Nonetheless, in order to determine a critical point (maximum, minimum, or saddle) it is necessary to use a polynomial function containing quadratic terms (response surface models).

3.4.2. Response surface

Taking into account the screening results, the non-significant factors are not included in the response surface multivariate optimization [11]. If necessary, these factors can be studied in a univariate way, modifying the experimental domain.

Response surface models make possible to predict how variation in the values of the variables affects the response and therefore, to choose the values that provide the best operational response.

As stated before, to determine the optimum conditions it is necessary that the polynomial function contains quadratic terms. For quadratic models, the critical point can be characterized as maximum, minimum, or saddle. It is possible to calculate the coordinates of the critical point through the first derivate of the mathematical function, which describes the response surface and equates it to zero.

Among the more known second order symmetrical designs are the three-level factorial design, Box–Behnken design, central composite design (CCD), and Doehlert design. In this thesis central composite design was used. Central composite designs are the result of superimposing a factorial design at 2 levels (2^n) on a star design ($2n + C_0$, being C_0 the replicates of the central point). As a result, the total number of runs is $2^n + 2n + C_0$. In Fig 3.18, a special distribution of CCD for three factors is represented. These combinations define the linear, quadratic and interaction terms of the model together with an estimation of the error in the terms.

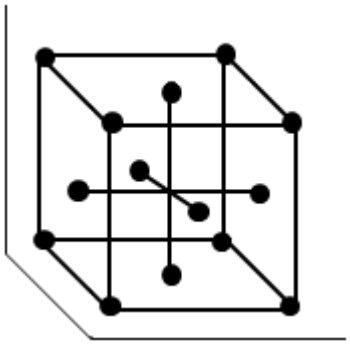

Figure 3.18 Face centered CCD for three variables and central points.

Another interesting tool of multivariate optimization is the desirability function (D) which permits multiple response evaluation. It was proposed by Derringer and Suich in 1980 [12], and nowadays it has become increasingly popular. Desirability is defined as the geometric mean, weighted or otherwise, of the individual desirability functions of each response (d_i), which is obtained from the transformation of the individual response of each experiment. The scale of the desirability function ranges between d=0 (for an undesirable response) and d=1 (for completely desirable one). D is calculated combining the individual desirability values by applying the geometric mean: $D = (d_1 \times d_2 \times \ldots d_m)^{1/m}$. An algorithm is then applied to the D function in order to determine the set of variable values that maximize it. Thus, the simultaneous optimization process is reduced to find the levels of factors that demonstrate the maximum overall desirability. The application of desirability functions in analytical chemistry brings advantages such as efficiency, economy, and objectivity in the optimization of multiple response procedures. This function has been used during the optimization of analytical systems, which involve several responses.

In conclusion, experimental design provides useful information making easier to understand the problem.

Design of experiments and results evaluation were done with the software package STATISTICA 8.0. Particular information regarding each method optimization is given in more detail in their respective chapters.

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CHAPTER 4

DEVELOPMENT OF GREEN ANALYTICAL METHODS FOR IRON DETERMINATION AND SPECIATION

4. Development of green analytical methods for iron determination and speciation

4.1. General remarks about iron

Iron is the fourth most abundant element in the Earth's crust [1], being an essential element for most forms of life on earth. In recent years, there have been several studies showing that iron is a limiting factor for phytoplankton growth in some areas of the ocean and also they suggest that fertilization of the oceans with iron could be the more feasible option to remove atmospheric CO_2 and reduce the greenhouse effect [2]. In fact, this element plays a crucial role in many biological and microbiological processes. However, high levels of iron are associated with an increased risk of cancer, heart disease, and other illness such as endocrine problems, arthritis, diabetes and liver disease [3].

Although iron is a non-toxic metal its speciation in certain samples is gaining interest. Generally, iron is present in the Fe (III) state, and its salts are readily hydrolysed to insoluble forms. Fe (II) is highly soluble but thermodynamically unstable being rapidly oxidized to Fe (III) [1]. As it is mentioned above, Fe (III) is known as one of the essential trace metals for many living organisms and it is important to determine trace amounts of iron in water for deeper knowledge of environmental protection, hydrogeology and some chemical processes. The determination of iron in water samples is usually carried out in routine quality controls as limits on its total content are imposed by legislation. In drinking water, the European Union directive set a limit of 200 µg/L [4]. Thus, it is very important to develop precise, rigorous and robust analytical methods with low limits of detections (LODs) for iron determination and speciation in order to assure the public health and to understand its biogeochemical cycle and the interaction between iron species and phytoplankton growth [5].

The concentration of iron in natural waters is highly variable and influenced by many factors. The main source of iron in natural waters is from weathering and leaching of rocks and soils. Figure 4.1 shows a schematic diagram of the biogeochemical cycle of iron in the ocean. The major sources of iron in the ocean come from atmospheric deposition, river transport, submarine hydrothermal processes of regeneration in the continental shelf and the emergence of subsurface water enriched with iron [6]. While flocculation and precipitation processes scavenge most of the iron transported from rivers, marine organisms also consume significant amounts of iron from seawater.

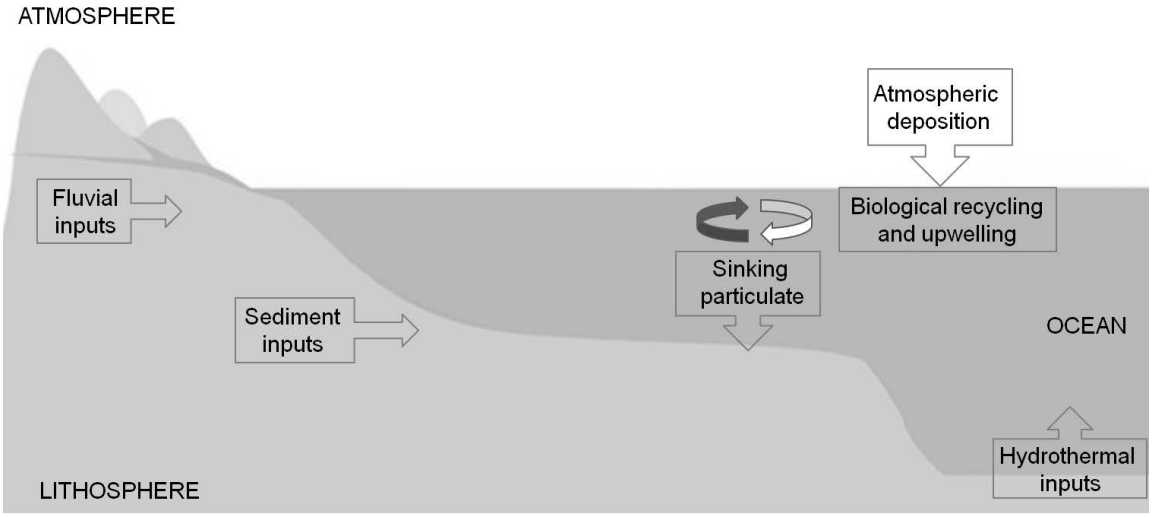

Figure 4.1 Schematic diagram of the biogeochemical iron cycle in the ocean.

The comprehensive effect of these factors results in a large iron concentration gradient in estuarine and coastal regimes, from nmol/L to µmol/L. Iron determination is particularly difficult because of the complexity of the matrix and also because of different physical, chemical and biological processes involved [5]. In seawater, iron exists in various physicochemical forms, and physical fractionation is usually carried out using membrane filtration techniques (0.2 to 0.45 µm, cellulose acetate or polycarbonate) to differentiate the dissolved and particulate fraction. Main applications for iron determination and speciation have been focused in seawater [7], in food science [8] and in pharmaceutical formulations [9].

4.2. Detection techniques for iron

In natural waters, when aiming to measure iron at low levels, many well-established analytical techniques such as atomic absorption spectrometry (AAS) [10] and inductively coupled plasma - mass spectrometry (ICP-MS) [11], are the most popular choices. However, these techniques require expensive instrumentation and have low tolerance to physical/matrix interferences, making its application difficult, especially with samples with high salt contents such as sea or estuarine waters. Furthermore, iron speciation is not possible with these techniques. In this context, spectrophotometric tecniques have been used as alternative methods for iron monitoring in environmental samples.

The main drawback of spectrophotometric methods for iron determination and speciation is that these usually employ highly toxic reagents, such as: thiocyanate, 1,10-phenantroline, bathophenantroline, 2,2-bipyridyl, eriochrome cyanine R and cetyltrimethylammonium [12]. In this context, alternatives involving greener reagents are demanded. Therefore, we decided to develop a more environmental friendly method for iron determination by using newly synthesized iron ligands, i.e. bidentate 3-hydroxy-4-pyridinone (3,4-HPO) [13] chelators, as chromogenic reagents.

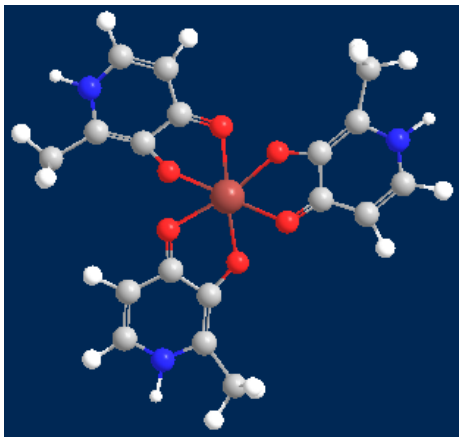

Figure 4.2 3-hydroxy-4-pyridinone-iron complex

These ligands are well known, mainly by their biomedical applications [14]. They are particularly attractive for pharmaceutical purposes since their structure allows tailoring of their hydrophilic/lipophilic balance (HLB) without significantly changing its chelating properties [15]. Variations in HLB can be achieved by simply introducing appropriate substituents on the endocyclic nitrogen atom of the pyridinone ring leading to the optimal lipophilicity for delivery or removal of metal ions in the human body. The 3,4-HPO are hard ligands that bear two oxygen coordinating atoms showing a very high capacity to trap Fe (III) and comparatively low affinity for Fe (II). The use of 3,4-HPO ligands as iron colorimetric reagents proved to be an environmental friendly effective alternative for the quantification of iron content in natural waters [16]. However, the main drawback reported in previous studies was the low solubility of the ligand together with the need of using the ligand in a 3:1 ligand:iron stoichiometry [16], which implies a higher ligand solution volume, as the limited solubility prevents an increase in ligand concentration. So, in order to tackle this limitation, a more soluble and hexadentate 3,4-HPO ligand [17] was explored [7] providing 1:1 stoichiometry and a lower kinetic lability, being a potential alternative chromogenic reagent for iron determination. Moreover, increased sensitivity and lower LOD were obtained.

4.3. Iron extraction and preconcentration techniques

Moreover, waters with high salt content, such as seawater, matrix effect and low concentration levels (3-112 ng/L), can pose a real challenge and preclude direct iron determination. To overcome these difficulties separation and/or preconcentration of the analyte is often necessary and especially useful in spectrophotometric methods providing higher selectivity and sensitivity.

Nowadays, SPE is the most widely utilized technique for preconcentration and separation of metals due to its high enrichment factors, low organic solvents consumption, safety with respect to hazardous samples, flexibility and ease of automation.

There are several commercial SPE materials. Most commonly used solid phases for iron extraction and preconcentration are C18 [18], Sephadex [19], Amberlite XAD- 4 [20], immobilized on fluorinated metal alkoxide glass - 8-hydroxyquinoline (MAF- 8HQ) [21], (8-HQ) [22], Chelex100 [23] and nitrilotriacetic acid (NTA) Superflow resins [24]. The latter, NTA Superflow resin was originally designed for high throughput sample clean-up procedures based on the affinity chromatography concept. It represents several advantages such as, analyte recovery at low pH and the supported high flow rates which are very interesting for using in applications where high volumes of sample have to be processed and a high sampling rate is needed. Recently, NTA Superflow resin was applied for retaining iron in a preconcentration procedure for water analysis [24, 25]. In both works the NTA Superflow resin column not only acted as preconcentration unit but also allowed matrix removal. In addition, Páscoa et al. [25] demonstrated that NTA Superflow resin has superior stability than Chelex 100. In our particular case, the implementation of a SPE step helped to improve the LOD by preconcentration and by the sample clean-up, since without SPE the LOD was not low enough to determine iron in seawater due to the high interference of chlorine in these samples. Furthermore, NTA Superflow resin has a high affinity for Fe (III) and very low to Fe (II) allowing iron speciation in the second developed work presented below.

4.4. Automation of the proposed methodology by flow analysis techniques

As mentioned in the introduction section (chapter 1) sample pretreatment steps are tedious and time consuming. These difficulties can be solved by automating the analytical protocol exploiting flow analysis techniques. Thus, several solid phases have been implemented in flow systems in order to carry out the analyte preconcentration and therefore, achieve lower LODs and at the same time, improve tolerance to potential interferences [26].

For instance, a FIA system using Chelex-100 and 1,10-phenantroline as chromogenic reagent was developed for iron speciation in water samples [27]. Other authors developed a MPFS system for iron speciation using ammonium thiocyanate as chromogenic reagent and chelating disks for SPE [28]. Same authors proposed a MCFIA system for iron speciation in water samples using the resin Chelex-100 [23]. Chelex-100 and NTA Superflow resins were compared in a MSFIA system for iron speciation in water samples based on a colorimetric detection, testing two colorimetric reagents, i.e. ferrozine and ammonium thiocyanate [25]. μSI-LOV system exploiting BI (NTA Superflow resin) was developed for determination total iron in wine using SCN^- as chromogenic reagent [29]. More recently, a SIA system for iron speciation in water samples was developed using a synthesised hexadentate 3,4-HPO chelator and the NTA Superflow resin not only for preconcentration but also for matrix elimination [7].

Also it has to be taken into account that the fact that iron is present everywhere causes risk of contamination during sampling, filtration, storage, and analysis. In this context, flow analysis techniques, as closed systems, play a crucial role because provide simplified sample handling, and reduced contamination risks, together with increased accuracy, precision, sample throughput, reproducible sample injection, controlled dispersion of the sample zone, low LODs, low reagent and sample consumption.

Thus, with the aim of developing more environmental friendly analytical methods for iron determination and speciation, we studied the applicability of 3,4-HPO ligands as green colorimetric reagents. First we developed two systems, a SIA and μSI-LOV system which not only exploited a non-toxic chromogenic reagent but also permitted reducing the reagents consumption and so the waste generation (350 μL per analysis in μSI-LOV). The use of this new chelant with μSI-LOV made possible to determine iron in the range of 0.10-1.00 mg Fe/L with a LOD of 7 μg Fe/L. However, no speciation was achieved. Thus, aiming to expand the applicability of the developed method to

water samples with high salt content, we developed a μSI-LOV-SPS method for iron speciation in different bathing waters, coastal and inland. The NTA Superflow resin was placed into the flow cell for assembling the preconcentration column where Fe (III) was retained prior the reaction with 3,4-HPO. In Table 4.1 are summarized the most relevant figures of merit of these developed works. With the μSI-LOV-SPS approach, minimization of the high salinity interference in coastal water samples and a linear working range suitable for lower concentrations of iron were attained. The developed method enabled a LOD of 8.5 μg /L Fe with a linear range between 20-100 μg /L Fe, with low sample consumption and effluent production, 400 μL and 2.15 mL respectively. The use of SPS provided selectivity, allowing speciation and expanding the method applicability to samples with high salt content, and with lower levels of iron concentration due to preconcentration and on-column detection.

Table 4.1 Comparison between the μSI-LOV system and μSI-LOV-SPS system methodologies for iron determination.

	μSI-LOV system	**μSI-LOV-SPS system**	
Parameter	Iron Fe (III)	Iron Fe (III)	Total Iron
Reagent consumption per assay			
3, 4 – HPO (mg)	0.48	0.48	0.48
$NaHCO_3$ (mg)	0.11	0.34	0.34
H_2O_2 (μg)	-	-	4.4
Sample (μL)	50	400	400
Waste production per assay (mL)	0.350	2.15	2.15
Determination rate (h^{-1})	90	14	13
Dynamic range (μg/L)	100-1000	20-100	20-100
LOD (μg/L)	7	8.5	8.5
Repeatability (RSD %)	1.4	2.1	3.7
Samples	River water	River water Seawater	River water Seawater

More detailed information is given below in two original research papers result of these investigations which were published in scientific international journals with high impact factor.

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4.6. Original paper 1

Title: Exploiting the use of 3,4-HPO ligands as nontoxic reagents for the determination of iron in natural waters with a sequential injection approach

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Exploiting the use of 3,4-HPO ligands as nontoxic reagents for the determination of iron in natural waters with a sequential injection approach

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ABSTRACT

In this paper, the use of 3-hydroxy-4-pyridinone (3,4-HPO) chelators as nontoxic chromogenic reagents for iron determination is proposed. The potential application of these compounds was studied in a sequential injection system. The 3,4-HPO ligands used in this work were specially designed to complex iron(III) at physiologic pH for clinical applications. The developed sequential injection method enabled to study the reaction conditions, such as buffering and interferences. Then, to further improve the low consumption levels, a microsequential injection method was developed and effectively applied to iron determination in bathing waters using 3,4-HPO ligands. The formed iron complex has a maximum absorbance at 460 nm. The advantage of using minimal consumption values associated with sequential injection, together with the lack of toxicity of 3,4-HPO ligands, enabled to present a greener chemistry approach for iron determination in environmental samples within the range 0.10–2.00 mg Fe/L with a LOD of 7 μg/L. The overall effluent production was 350 μL corresponding to the consumption of 0.48 mg of 3,4-HPO ligand, 0.11 mg of $NaHCO_3$, 0.16 mg of HNO_3 and 50 μL of sample. Three reference samples were assessed for accuracy studies and a relative deviation $< 5\%$ was obtained. The results obtained for the assessment of iron in inland bathing waters were statistically comparable to those obtained by the reference procedure.

1. Introduction

Iron is an essential element for most forms of life on earth and for that reason it is a micronutrient and not considered a water pollutant. Nevertheless, it is very important to monitor the iron content in waters since high levels of iron may result in aesthetic (odour and taste), cosmetic (colour) and technical (damage to water equipment) effects [1]. The visual impact of the reddish colour in recreational waters, namely bathing waters may result in negative economical impact due to public opinion. Inland bathing waters present a challenging matrix due to the expected diversity of parameters and variability in concentration levels. In fact, these waters are often highly stressed due to recreational activities, so the efficient monitoring of parameters such as iron represents a valuable contribution to the overall environmental assessment. Furthermore, the established relationship between the iron content and algae blooms emphasises on the importance of public acceptance concerning the mentioned recreational waters.

The assessment of iron is often carried out by molecular absorption spectrophotometry using highly toxic species: thiocyanate, 1,10-phenantroline, bathophenantroline, 2,2-bipyridyl, eriochrome cyanine R, and cetyltrimethylammonium [2]. In this context, and aiming the use of nontoxic reagents for spectrophotometric determinations of iron, we are presently exploring the use of 3-hydroxy-4-pyridinone (3,4-HPO) chelators as chromogenic species for iron. These ligands are well known, mainly by reason of their biomedical applications; they are particularly attractive for pharmaceutical purposes since their structure (Table 1) allows tailoring of their hydrophilic/lipophilic balance (HLB) without significantly changing its chelating properties.

Variations in HLB can be achieved by simply introducing appropriate substituents on the endocyclic nitrogen atom of the pyridinone ring thus leading to the optimal lipophilicity for delivery or removal of metal ions in the human body. The 3-hydroxy-4-pyridinones are hard ligands that bear two oxygen

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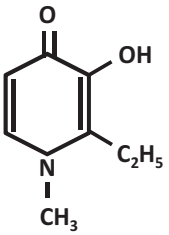
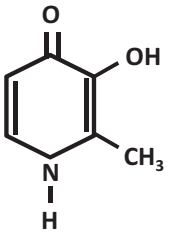
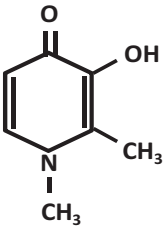
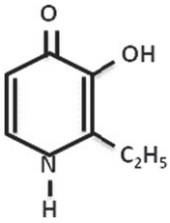
Table 1
The 3,4-HPO bidentate ligands tested as colorimetric reagents for iron determination with the respective chemical formula and molecular weight.

3,4-HPO Ligand	2-methyl-3-hydroxy-4-pyridinone	1,2-dimethyl-3-hydroxy-4-pyridinone	2-ethyl-3-hydroxy-4-pyridinone	1-methyl-2-ethyl-3-hydroxy-4-pyridinone
Abbreviation	Hmpp	Hdmpp	Hetpp	Hempp
Structure				
Chemical formula	$C_6H_7O_2N$	$C_7H_9O_2N$	$C_7H_9O_2N$	$C_8H_{11}O_2N$
Molecular weight	126.14	139.15	139.15	153.16
pKa	$pK_{a_1} = 3.62 \pm 0.05$ $pK_{a_2} = 9.48 \pm 0.05$	$pK_{a_1} = 3.69 \pm 0.01$ $pK_{a_2} = 9.61 \pm 0.03$	$pK_{a_1} = 3.63 \pm 0.04$ $pK_{a_2} = 9.62 \pm 0.05$	$pK_{a_1} = 3.53 \pm 0.02$ $pK_{a_2} = 9.46 \pm 0.05$

coordinating atoms and consequently show a very high capacity to trap iron(III) and comparatively low affinity for iron(II).

The values of iron(III) stability constants are in the range $35.0 < \log \beta_3 < 37.0$ and $12.0 < \log \beta_2 < 15.0$ for iron(II). Affinity for other metal (III) and metal (II) ions can be found in the literature and show that these ligands may be of use in the development of new methods to monitor iron [3,4]. The use of 3,4-HPO ligands as iron colorimetric reagents requires a detailed study of the reaction conditions and a comprehensive interference study. In order to be considered an effective alternative, similar (or better) sensitivity should be obtained when compared to commonly used reagents together with non-significant interferences.

Within this context, in this paper, the potential use of these chelators as chromogenic reagents for iron was studied. Due to the advantages of using flow-based techniques for carrying out spectrophotometric measurements, this study was performed in a sequential injection (SI) system [5]. A sequential injection analysis method for the determination of iron was developed and a thorough study of the 3,4-HPO ligands as iron colorimetric reagents was carried out. The conditions for the colorimetric reaction, the sensitivity and selectivity of the 3,4-HPO/Fe chelates were assessed and critically compared to others involving commonly used chromogenic reagents. Aiming for a greener chemistry approach on the application to natural waters, namely inland bathing waters, downscaling of the SI method led to the development of a microsequential injection lab on a valve (μSI-LOV) method [6]. A detailed assessment of the possible interferences was carried out and the limits of the chelation reaction were tested. The downscaling enabled to decrease both the sample/reagents consumption and effluent. The μSI-LOV method was developed using a lab on valve manifold known for the robustness in handling significantly low volumes, between 5 and 50 μL [7].

2. Materials and methods

2.1. Reagents and solutions

The 3,4-HPO ligands were synthesised according to the methods published in the literature [3]. All solutions were prepared with analytical grade chemicals and boiled deionised water (specific conductance less than 0.1 μS/cm).

Saturated ligand solutions were obtained by dissolution of approximately 2 mg of the synthesised ligand in 100 mL of water corresponding to a concentration of 20 mg/L which is higher than the solubility value thus ensuring the saturation of the solution. The ligand solution used in the μSI-LOV method was a 1.25 dilution of the 20 mg/L solution, resulting in a final concentration of ≈15 mg/L, also a saturated solution.

The buffer solutions of hydrogen carbonate 0.10 and 0.25 M were prepared by dissolving 420 mg and 1.05 g of sodium hydrogen carbonate in 50 mL of water, respectively. The pH was set to 10.5 with 0.5 M sodium hydroxide.

An iron(III) stock solution, 10 mg/L, was prepared by diluting the atomic absorption standard of 1000 mg/L. Working standards in the dynamic range 0.1–2.0 mg/L were weekly prepared from dilution of the stock solution in 0.03 M of nitric acid.

A stock solution of iron(II) was prepared from the solid iron(II) ammonium sulphate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$) to a final concentration of 18.2 mg/L. This stock solution was diluted to obtain an intermediate solution of 4.6 mg/L, which was used to prepare the working standards in the dynamic range 0.1–2.0 mg/L (in 0.03 M of nitric acid).

2.2. Sequential injection manifold and procedure

The sequential injection manifold developed for iron(III) determination with 3,4-HPO ligand and study of the reaction is depicted in Fig. 1.

Solutions were propelled by a Gilson Minipuls 3 peristaltic pump, equipped with PVC pumping tube connected to the central channel of a ten port selection valve (Valco VICI Cheminert C25-3180EUHB). All tubing connecting the different components was made of PTFE (Omnifit), with 0.8 mm i.d. A personal computer (HP Pavilion zt3000) equipped with a National Instruments DAQcard—DI0 interface card, running a homemade software, was used to control the selection valve position and the peristaltic pump direction and speed.

An Ocean Optics USB 4000 charged coupled device detector (CCD), equipped with a pair of 400 μm fibre optic cable and a Mikropack DH-2000 deuterium halogen light source and a Hellma 178.710-OS flow-cell with 10 mm light path and 80 μL inner volume, was used as the detection system. Data acquisition was performed through the Ocean Optics—Spectrasuite software at 459 nm.

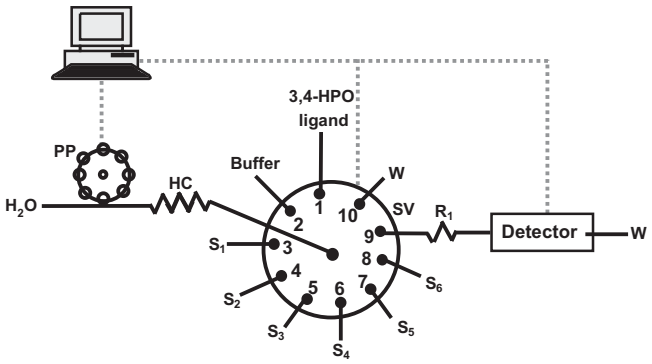


Fig. 1. Sequential injection manifold to study the 3,4-HPO ligand as a colorimetric reagent for iron determination; SV, 10 port selection valve; HC, holding coil with 300 cm of length; PP, peristaltic pump; S_i, iron(III) standards; R_1, reaction coil with 6 cm; and W, waste.

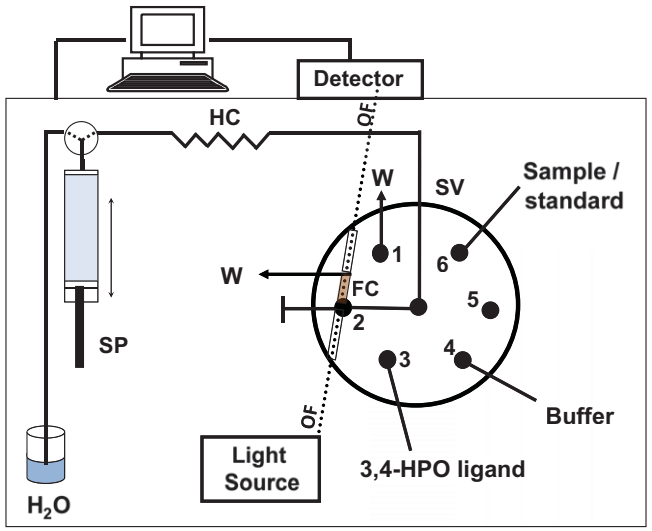


Fig. 2. Microsequential injection-Lab on valve, μSI-LOV, manifold for the determination of iron(III) in bathing waters using the 3,4-HPO ligand as a colorimetric reagent; SV, 6 port selection valve; HC, holding coil with 1.5 m of length; SP, syringe pump; FC, 1 cm flow cell; OF, optical fibbers; W, waste.

2.3. Microsequential injection manifold (lab on valve) and procedure

Aiming for further miniaturisation and decrease of consumption volumes, a microsequential injection lab on valve methodology, μSI-LOV, was also developed and is depicted in Fig. 2.

The μSI-LOV system was a FIAlab-3500 (FIAlab Instruments) consisting of a bi-directional syringe pump (2500 μL of volume), a holding coil and a lab-on-valve manifold mounted on the top of a six-port multi-position valve. As a detection system, a USB 2000 Ocean Optics, a CCD spectrophotometer equipped with fibre optics (FIA-P200-SR, 200 μm), and a Mikropack DH-2000-BAL deuterium halogen light source was used. FIAlab for Windows 5.0 software on a personal computer (HP Compact) was used for flow programming and data acquisition.

The sequence of steps with the respective time and volumes used for both methodologies is shown in Table 2.

The first step was the aspiration to the holding coil of 3,4-HPO ligand (step A), followed by the aspiration of the buffer and the standard (steps B and C). Mixing was promoted by the flow reversal while propelling the aspirated plugs towards the detector (step D). For the μSI-LOV, due to the reduced size of aspiration plugs and absence of reaction coil, mixing mainly occurs in the holding coil.

Table 2
Protocol sequence for both the developed methodologies, SI and μSI-LOV, for iron determination with 3,4-HPO ligand as a colorimetric reagent.

Step	Port		Time (s)	Flow rate (μL/s)		Volume (μL)		Description
	SI	μSI-LOV	SI	SI	μSI-LOV	SI	μSI-LOV	
A	1	3	4	60	25	240	40	Aspiration of ligand
B	2	4	2	15	10	30	5	Aspiration of buffer
C	3–8	6	5	60	25	300	50	Aspiration of standard
D	9	2	20	60	20	1200	350	Propelling to detector

2.4. Water samples—inland bathing waters

Water samples from inland bathing areas were collected in polyethylene plastic bottles of 0.5 L capacity at about 20 cm depth. The samples, acidified at collection according to the collection procedure [8], were introduced directly in the developed system without filtration.

2.5. Accuracy assessment

The collected inland bathing waters were spiked and analysed using the atomic absorption method (APHA 3111B) [8] and the results were compared to those obtained with the developed μSI-LOV method. For further accuracy assessment, results obtained with the proposed μSI-LOV system were compared to the certified values of three certified water samples, ERM-CA010a (hard drinking water), ERM-CA021a (soft drinking water) from LGC standards and NRC-CNR SLRS-4 (River water) from the National Research Council Canada.

3. Results and discussion

3.1. Preliminary studies

The 3,4-HPO bidentate ligands form complexes with iron(III) of the type FeL_3. The stepwise formation of the complex results in a sequence of colour products according to the number of ligands, 1, 2 and 3, bound to the metal ion. The final complex (FeL_3) shows a maximum of absorbance at 460 nm, and was the one used for iron(III) quantification. There were four 3,4-HPO bidentate ligands available with the same affinity for iron(III) and different solubility values. The reaction sensitivity and kinetics were expected to be quite similar but some studies were carried out to ensure the choice of the most appropriate ligand. The tested compounds, used in the initial studies, are shown in Table 1 together with the formulae, molecular weight and acidity constants [3].

Based on the reported solubility for Hdmpp [4] of 14 mg/L, a first approach to the ligand concentration was made. A 6 mg/L ligand solution, about half the maximum concentration, was prepared to ensure that both higher and lower concentrations could be tested. Then, 1 mL of this solution was added to 1 mL of iron(III) standards, 0.5 and 1 mg/L, and no absorbance signal was observed. Because significantly lower concentrations of iron(III) were aimed, another ligand solution was prepared with a concentration of about the reported solubility value, 15 mg/L, a saturated solution. In these conditions, the same procedure was carried out (1 mL of ligand solution added to 1 mL of iron(III) standard) and colour was observed for the 1 mg/L standard ($A=0.035$). Aiming for the highest sensitivity possible, saturated solutions were used for the remaining preliminary

studies and the ligand concentration study was revisited in the flow analysis studies.

Saturated solutions (≈ 20 g/L) were prepared for the four ligands and the first experiments were carried on by mixing 1 mL of ligand solution with 1 mL of the 10 mg/L iron(III) standard solution. Scans were made directly on all the mixtures without any pH adjustment and are shown in Fig. 3. Analysis of the spectra showed that the maximum absorption was observed at 515 nm thus indicating the need for pH adjustment in order to ensure stoichiometric formation of the complex FeL_3, whose maximum of absorption is generally observed at 460 nm. Considering the values of the acidity constants of the ligands (Table 1) we chose to adjust the pH of the ligand solution to 10 prior to the addition of the iron(III) solution. Using the latter procedure, the maximum of absorbance was shifted from 515 nm to 459 nm thus confirming the stoichiometric formation of FeL_3 and the need of including a buffering solution in the system.

Aiming for the application of these ligands for iron quantification in flow analysis, it was important to evaluate the kinetics of the complex formation. The absorbance of a buffered mixture of 1 mL saturated ligand solution and 0.5 mL of 10 mg/L iron(III) standard was measured for 1 min. This procedure was repeated for the four ligand solutions and colour was observed almost immediately after mixing, indicating the complex formation. After the observed initial reaction, there was no significant absorbance increase during the measured time (1 min). In fact, for the ligands Hmpp and Hempp, the final absorbance was the same as the initial value and for Hdmpp and Hetpp ligands there was a slight increase (< 8%).

These preliminary studies enabled to set some basic conditions: the preparation of saturated ligand solutions and the need of buffering the complex formation at pH ≈ 9.5. Although similar results were obtained for all the four tested ligands, given the need of the highest concentration possible the two most hydrophilic ligands (Hmpp and Hdmpp) were used in flow studies.

3.2. Sequential injection method

The advantageous characteristics of sequential injection analysis concept namely versatility, automation and low reagent consumption, made it an appropriate choice for automation of the new analytical application of 3,4-HPO ligands for iron determination. So, a SI manifold was designed for the study of the complex formation.

3.2.1. Physical parameters

Having established the need for three solutions (ligand solution, buffer and sample) the aspiration sequence was assessed. The choice of keeping the buffer in between the ligand and sample ensured buffering the sample prior to the reaction. So, the tested sequences were as follows: ligand–buffer–sample/standard (LBS) and sample/standard–buffer–ligand (SBL). The results obtained with the sequence LBS presented a fourfold increase in sensitivity so that was the chosen sequence. With the established aspiration sequence the volumes of each plug was studied. The buffer volume was the first to be studied with three volumes tested, 30, 60 and 90 μL (Fig. 4). The sensitivity decreased with the increase of the buffer volume, so 30 μL was the volume chosen. Lower volumes

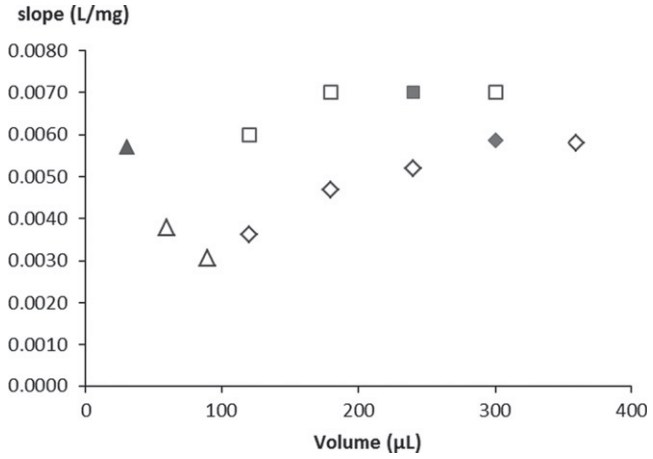


Fig. 4. Study of the influence of the volume of ligand (□), buffer (△) and sample (◇) on the sensitivity of the iron determination; the chosen volumes are represented in full black.

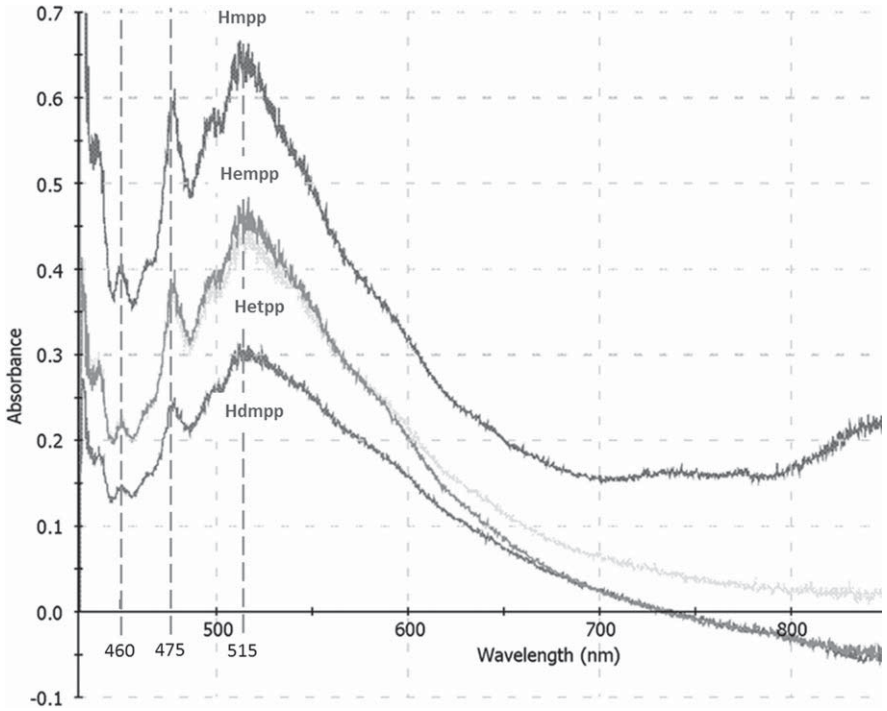


Fig. 3. Visible spectra of the 3,4-HPO ligands.

were not tested as 30 μL normally represents the minimal reproducible value to be used in SI methods [9]. Then, the sample volume was studied within the range 120–360 μL, and the sensitivity increased up to the volume of 300 μL, so that was the chosen value (Fig. 4). Finally, the volume of ligand solution was also studied. From the tested volumes of 120, 180, 240 and 300 μL the volume of 240 μL was chosen to ensure an excess of ligand as the sensitivity increased up to 180 μL (Fig. 4).

In order to reinforce the previous choices, limits of detection and quantification were calculated using two volumes of sample/standard, 300 and 360 μL, and two volumes of ligand, 240 and 300 μL. The previously set values, 300 μL for sample and 240 μL for ligand, proved to be an appropriate choice as they provided the lowest limits.

3.2.2. Chemical parameters

3.2.2.1. Ligand solution. The results observed in the section "Preliminary studies" showed a significant increase in sensitivity when a saturated ligand solution was used. Nevertheless, due to the potential complications from using a saturated solution in a flow system, the study of the ligand concentration was revisited using the SI method.

First, calibration curves with saturated solutions, 20 mg/L, of Hmpp and Hdmpp (two most hydrophilic ligands) were compared to calibration curves with solutions of the same ligands diluted to half and a 42% decrease in sensitivity was observed. Then, for a combined evaluation of the effect in both the sensitivity and the intercept, several dilutions were made from the saturated ligand solutions (Fig. 5).

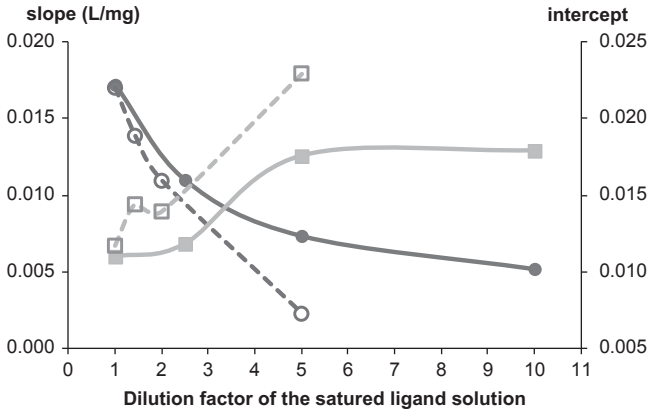


Fig. 5. Study of the influence of the 3,4-HPO concentration; Hmpp, full lines with ●, for the slopes (sensitivity) and ■ for the intercepts; Hdmpp, dashed lines with ○, for the slopes (sensitivity) and □, for the intercepts.

The results, similar for both Hmpp and Hdmpp showed that the increase of the dilution factor resulted not only in a sensitivity (slope) decrease but also an increase of the intercept and consequently of the limit of detection.

The results confirmed the choice of using a saturated solution of ligand in order to obtain the maximum sensitivity. Despite the use of saturated solutions, no clogging problems were observed and the repeatability of the calibration curve slope (RSD = 5.2% for four calibrations in consecutive days) proved that no major problems occurred.

Afterwards, in order to choose one of the two, a comparison was made between the two most soluble 3,4-HPO ligands, Hmpp and Hdmpp, (Table 1). Two calibration curves were made, one with the Hmpp ligand and another with the Hdmpp ligand and the results showed a similar sensitivity. In fact, the obtained slopes were quite comparable (RD = 1.4%) and the intercepts were also quite alike (RD = 1.5%). Without major differences between the two tested ligands, Hmpp was chosen as the most hydrophilic one.

3.2.2.2. Buffer solution. The requirement of buffering the complex formation was established in the section "Preliminary studies". Aiming to buffer the reaction at pH ≈ 9.5, buffer solutions were prepared with higher pH due to the acidity of iron(III) standard solutions (pH ≈ 2). Two buffer compositions, at pH = 10.5, were compared: 0.5 M hydrogen carbonate and 0.5 M hydrogen phosphate. Although the sensitivity doubled with the hydrogen phosphate buffer, the same occurred to the intercept, resulting in a significant increase of the detection and quantification limits. So, a hydrogen carbonate solution was chosen as buffer and a study of its concentration was carried out. The tested concentration range was 0.05–0.5 M and, although the sensitivity increased up to 0.25 M, the intercept increased continuously with the increase in concentrations. In this context, 0.1 M was chosen, as it represented a 72% increase to 0.05 M and was only 12% lower than 0.25 M, maintaining the detection limit below 0.1 mg/L.

3.3. Microsequential injection lab on valve method (μSI-LOV)

Aiming for the application to environmental samples, the concern for a greener analytical procedure led to the down scaling to μSI-LOV. In fact the advantage of μSI-LOV is the substantial reduction in reagent and sample consumption coupled to extremely low effluent production. Consequently, a 6 fold volume reduction was made from the previous set volumes (*physical parameters* section) as shown in Table 2. A further reduction could not be employed, as 5 μL (buffer volume) was previously reported as the minimal volume to produce an acceptable reproducibility [10].

Table 3
Features of the developed methodologies for iron(III) determination in water samples using 3,4-HPO ligand as a colorimetric reagent.

Method	Dynamic range (mg/L)	Typical calibration curve $A = m\,[Fe^{3+}] + b$	LOD (μg/L)	LOQ (μg/L)	Quantification rate (det./h)	Reagent/sample consumption	Effluent production (μL)
SI	0.30–2.00	$y = 0.0251\ (\pm 0.0020)\ [Fe^{3+}] + 0.0155\ (\pm 0.0057)$ $R^2 = 0.999$	83	277	102	3.4 mg Hmpp 0.25 mg $NaHCO_3$ 0.57 mg HNO_3 300 μL	1200
μSI-LOV	0.10–1.00	$y = 0.0488\ (\pm 0.0008)\ [Fe^{3+}] + 0.0030\ (\pm 0.0004)$ $R^2 = 0.999$	7	24	90	0.56 mg Hmpp 0.11 mg $NaHCO_3$ 0.10 mg HNO_3 50 μL	350

The μSI-LOV main characteristic, the detector (flow cell) located in the valve, justifies the study of the flow rate of propelling to the detection as there is no reaction coil. So different flow rates were tested (20, 25 and 30 μL/s), corresponding to different reaction times, and as expected there was a slight increase with the decreasing flow rate so the flow rate of 20 μL/s was chosen. However, as the sensitivity increase was not significant ($<10\%$), lower flow rates were not tested to avoid the decrease of the quantification rate.

The study of carbonate concentration (buffer solution) was revisited, as it had been a compromise solution (*chemical parameters* section). Calibration curves were established for carbonate concentrations of 0.1, 0.25 and 0.5 M and, once again, the sensitivity increased up to 0.25 M (over 30% higher than with 0.1 M). Nevertheless opposite to what was previously observed, the intercept was lower (49% lower than with 0.1 M) so 0.25 M carbonate was chosen as the buffer solution.

The ligand concentration was also revisited because using μSI-LOV implies practically no dispersion raising possibility of lower concentrations. Dilutions from the saturated Hmpp solution were made, dilution factors from 1 to 5, and for a dilution factor of 1.25 the sensitivity was statistically the same (relative deviation 1.4%) decreasing significantly (relative deviation $>5\%$) for the other dilution factors. So the ligand concentration used was a 1.25 dilution of the saturated solution corresponding to ≈ 15 mg/L, still a saturated solution.

3.4. Features of the developed SI and μSI-LOV methodologies

After the detailed studies for the determination of iron based coloured complex formed with 3,4-HPO bidentate ligand, the characteristics of the developed methods were summarised (Table 3).

The limits of detection and quantification, LOD and LOQ, were calculated according to IUPAC recommendations [11,12]. For the SI method, three (LOD) and ten (LOQ) times the standard deviation of ten consecutive injections of deionised water were used for the calculation. As for the μSI-LOV method, four calibration curves were established to calculate the limits based on three (LOD) and ten (LOQ) times the standard deviation of the intercept.

The dynamic range of the SI method was established based on the calculated LOQ and up to the limit of the linear response. For the μSI-LOV method a calibration curve with eight standards, ranging from 0 to 4 mg/L, was made to assess the dynamic linear range defined as 0.1–1.0 mg/L (Table 3).

The determination rate was calculated based on the time spent per cycle. A complete analytical cycle took about 0.59 min for the SI method and 0.67 min for the μSI-LOV method. An analytical cycle is the sum of the time needed for each step plus the time necessary for the port selection in the selection valve. The presented consumption values, for reagents and sample, and the effluent production were calculated per determination.

Considering the lower limits obtained and the significant reduction of reagents and sample consumption and effluent production, the μSI-LOV was used for the interferences study and sample application.

3.5. Application of the μSI-LOV methodology to iron determination in natural waters

Although in natural waters iron is mostly found as iron(III), evaluating the response of the developed μSI-LOV method to iron(II) was important to assess.

A variety of iron standards, with the same final iron concentration, were prepared: iron(III) standards; iron(II) standards; iron(III) standards with iron(II) concentration constant and iron(II) standards with iron(III) concentration constant. The obtained results proved that both iron forms were effectively complexed with the 3,4-HPO ligands as all the slopes were not significantly different, relative deviations $<5\%$ (ESI, Fig. 1S). This feature clearly indicated that the total dissolved iron was being determined and was probably the result of working with a carbonate buffer at a pH = 9.5.

3.5.1. Study of possible interferences

Due to the nature of the colorimetric reaction, the possible interference of several bivalent and trivalent cations was assessed. Besides, the application to water samples justified testing other major ions commonly present in waters, namely nitrate, nitrite and sulphate. The tested concentrations were based on maximum values mentioned in both Portuguese [13] and international legislation [8]. Exception was made for chloride, as the tested values correspond to the expected values in estuarine and marine waters, 6 g/L and 19.2 g/L, respectively.

The solutions of the tested cations were obtained from proper dilution of atomic absorption standards except for cobalt(II), which were obtained by dilution of a stock solution prepared from solid cobalt sulphate. As for the anions, the tested concentrations were obtained from proper dilution of stock solutions prepared from the respective solids: sodium chloride, sodium nitrate, sodium nitrite and sodium sulphate.

Several standards, with 400 μg/L of iron(III) and the tested concentration of interfering ions, were prepared and analysed with the developed μSI-LOV method. The obtained absorbance values of the standard with and without interfering ion were registered and the interference percentage calculated (Table 4).

Overall, for expected values in natural waters, no significant interferences were observed as most of the interference percentages were below 5%. Exceptions were observed for the highest concentrations tested of calcium, magnesium and cobalt with

Table 4
Assessment of possible interfering ions in the determination of iron according to legislated values; UNFAO, United Nations Food and Agriculture Organisation.

Possible interfering ion	Legislation maximum values		Tested concentration of interfering ion in a standard 0.4 mg Fe^{3+}/L (mg/L)	Interference (%)
	UNFAO (mg/L)	Portugal (mg/L)		
Al^{3+}	5[a]	20[a]	5.10	−0.5
			25.6	−7.6
Ca^{2+}	15[b]	50[b]	10.0	−1.6
			25.0	−12.2
Co^{2+}	0.1[a]	10[a]	0.10	−0.7
			5.0	−14.4
Cu^{2+}	1.3[a]	5[a]	1.00	1.1
			5.00	−9.6
Mg^{2+}	5[b]	50[b]	30.0	−6.9
			50.0	−11.8
Mn^{2+}	0.2[a]	10[a]	0.20	−0.3
			10.0	−1.4
Ni^{2+}	0.2[a]	2[a]	0.20	0.0
			2.0	7.6
Zn^{2+}	2[a]	10[a]	1.00	−0.8
			10.0	1.3
Cl^-	–	–	6000	−4.9
			19,200	5.7
NO_3^-	–	50[a]	25.0	0.9
			50.0	2.5
NO_2^-	–	0.1[b]	0.050	1.6
			0.10	4.8
SO_4^{2-}	–	–	2.00	−0.7
			2000	2.8

[a] Irrigation waters.
[b] Streams waters.

interference percentages slightly over 10%. However, it is important to emphasise that the concentration ratios of iron/interferent represent over 1:60, 1:120 and 1:10 respectively, values that are not expected to occur in natural waters. In the end, the specificity of the 3,4-HPO ligand to iron was shown even for highly disadvantageous conditions, namely much higher concentrations of possible interfering cations (ESI, Fig. 2S).

It is important to stress the acceptable interference percentage obtained with the expected chloride concentration in sea waters, 19.2 g/L, indicating the possibility for application to those waters.

3.5.2. Method validation and application to bathing water samples

For accuracy assessment of the developed method, three certified water samples were analysed and the results compared to the certified value. Two of the certified samples were drinking waters: a hard drinking water, ERM-CA010a and a soft drinking water, ERM-CA021a with certified values in iron content of 236 ± 6 μg/L and 196 ± 2 μg/L, respectively. The obtained concentrations with the developed μSI-LOV method were 249 ± 9 μg/L for the ERM-CA010a, corresponding to a 5% relative deviation, and 192 ± 9 μg/L for the ERM-CA021a, corresponding to a relative deviation of −2%. Another certified water sample was analysed, a river water sample NRC-CNR SLRS-4, with an iron content of 103 ± 5 μg/L and the relative deviation obtained was 3% as the concentration calculated with the developed method was 107 ± 9 μg/L.

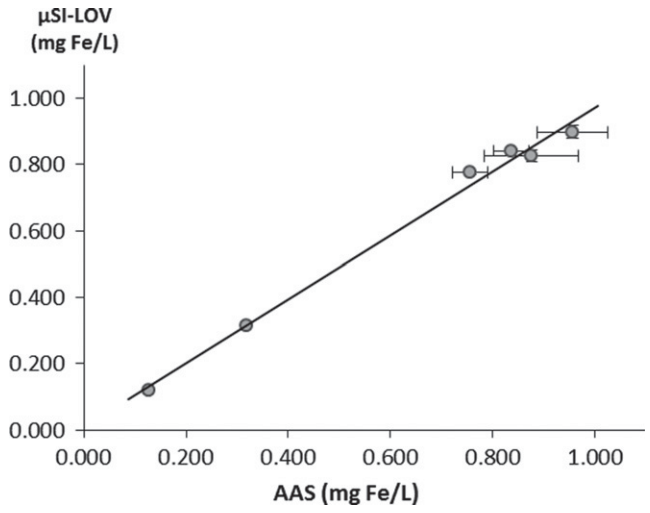


Fig. 6. Scatterplot for the comparison of the results obtained with the developed μSI-LOV method and the atomic absorption method.

For further accuracy assessment, six spiked water samples were analysed by the reference procedure, atomic absorption spectrometry (AAS) [8], and by the developed μSI-LOV methodology and a linear relationship between $C\mu_{SI\text{-}LOV}$ (mg/L) and C_{AAS} (mg/L) was established. The results were plotted (Fig. 6) and the equation found was: $C_{\mu SI\text{-}LOV} = 0.962\ (\pm 0.041) \times C_{AAS} - 0.009\ (\pm 0.025)$, where the values in parenthesis have 95% confidence limits.

These figures show that the estimated slope and intercept do not differ statistically from values 1 and 0, respectively. Therefore, there is no evidence for systematic differences between the two sets of results [14].

Additionally, several samples of inland bathing waters were collected at pH ≈ 2 [8] and spiked with iron(III) to final concentrations of 250 and 750 μg/L; volumes of 0.25 and 0.75 mL of a 10 mg/L iron(III) standard were used, respectively. Recovery percentages, presented in Table 5, were calculated according to IUPAC [15] and the average was 100% with a standard deviation of 3%. A statistical test (*t*-test) was used to evaluate if the mean recovery value did significantly differ from 100% and for a 95% significance level the calculated *t*-value was 0.094 with a correspondent critical value of 2.593. The statistical results indicate the absence of multiplicative matrix interferences.

The repeatability was assessed by calculation of the relative standard deviation (RSD) obtained by the mean of ten consecutive injections of sample. For a spiked water sample, the calculated RSD was 1.4% (0.814 ± 0.012 TDI mg/L), and for a certified sample it was 4.1% (0.103 ± 0.002 TDI mg/L).

4. Conclusions

The use of 3,4-HPO ligands as iron colorimetric reagents proved to be an environmental friendly effective alternative for the quantification of iron content in natural waters. As far as we know, 3,4-HPO ligands were used for the first time as chromogenic reagents for iron. The sensitivity obtained enabled fairly low detection and quantification limits, namely 7 and 24 μg/L respectively. In fact, although some previously described methods report much lower detection limits [16–18], they include preconcentration steps and rely on pollutant and toxic reagents, namely DPD. Iron being a nontoxic analyte, it is particularly important to have an environmentally friendly reagent as an alternative to those commonly used for its analysis: thiocyanate ($\varepsilon \approx 2.4 \times 10^4$ L/mol cm), 1,10-phenantroline ($\varepsilon \approx 1.1 \times 10^4$ L/mol cm), bathophenantroline ($\varepsilon \approx 2.2 \times 10^4$ L/mol cm),

Table 5
Recovery percentages calculated from the application of the developed μSI-LOV method to the iron determination in inland bathing waters; G, conductance; TDS, total dissolved solids; TDI, total dissolved iron; and SD, standard deviation.

Sample ID	pH	G (μS/cm)	TDS (mg/L)	Initial TDI (mg/L) SD	Added mg Fe^{+3}/L	Found TDI (mg/L) SD	Recovery % SD
Pi1	8.00	45	29	0.043 ± 0.005	0.25	0.283 ± 0.003	96.0 ± 1.2
					0.75	0.815 ± 0.015	103 ± 2
Pi2	7.00	51	33	0.056 ± 0.005	0.25	0.315 ± 0.008	104 ± 3
					0.75	0.830 ± 0.037	103 ± 5
Pi3	7.17	44	28	0.011 ± 0.003	0.25	0.260 ± 0.006	99.6 ± 2.3
					0.75	0.809 ± 0.028	106 ± 4
Pi4	6.82	39	26	0.060 ± 0.022	0.25	0.304 ± 0.014	97.6 ± 5.6
					0.75	0.794 ± 0.047	97.9 ± 6.2
Pi5	6.89	35	23	0.040 ± 0.004	0.25	0.287 ± 0.018	98.8 ± 7.2
					0.75	0.783 ± 0.022	99.1 ± 2.9
Pi6	6.87	33	21	0.032 ± 0.015	0.25	0.272 ± 0.007	96.0 ± 2.8
					0.75	0.782 ± 0.017	100 ± 2

2,2-bipyridyl ($\varepsilon \approx 8.7 \times 10^3$ L/mol cm), eriochrome cyanine R ($\varepsilon \approx 3.3 \times 10^4$ L/mol cm) and eriochrome cyanine R combined with cetyltrimethylammonium ($\varepsilon \approx 1.27 \times 10^5$ L/mol cm) [2]. With the use of the 3,4-HPO ligands ($\varepsilon \approx 4.7 \times 10^3$ L/mol cm), there is a decrease in sensitivity. Even so, some previously described flow methods using those reagents [19–21] present higher detection limits.

The choice of sequential injection as a flow technique enabled to perform the detailed study of the complexation reaction with a fast and automatic method. The complex formation proved to be almost immediate, no absorbance increase was observed after the initial colour formation, which is an excellent characteristic for a flow analysis application. Furthermore, the downsizing to micro-sequential injection Lab on valve method enabled reducing the consumption values to a minimum with only a minor decrease of the determination rate, about 10 determinations per hour. The developed method was successfully applied to natural waters, namely inland bathing waters, after accuracy validation. Although with the microsequential injection Lab on valve method a quantification limit < 30 μg/L was attained (24 μg/L), adequate for inland waters, it was not enough to cover the reported range expected for sea waters (10–100 μg/L [22]). However, the application to these waters was a realistic possibility due to the low interference observed for chloride values expected in those samples.

The developed work enabled to prove the effectiveness of 3,4-HPO ligands as a selective, nontoxic reagents for iron determination as a "more sustainable" alternative. The latter should be further explored, namely with incorporation of preconcentration steps like the mentioned works [16,18,19,21] aiming for lower detection limits.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.02.058.

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4.6.1. Supplementary information – paper 1

Figure 1S. Comparison of the slopes of calibration curves obtained when the standards were composed of: S Fe(III), iron (III) standards; S Fe(II), iron(II) standards; Mix S1, increasing concentrations of iron(III) and iron(II), ratio Fe(III)/Fe(II) 1:1; Mix S2, increasing concentrations of iron(III) with constant concentration of iron(II)= 0.46 mg/L; Mix S3, constant concentration of iron(III)= 0.40 mg/L with increasing concentrations of iron(II); the dashed lines represent the standard deviation of the calibration curve slope obtained with iron(III) standards.

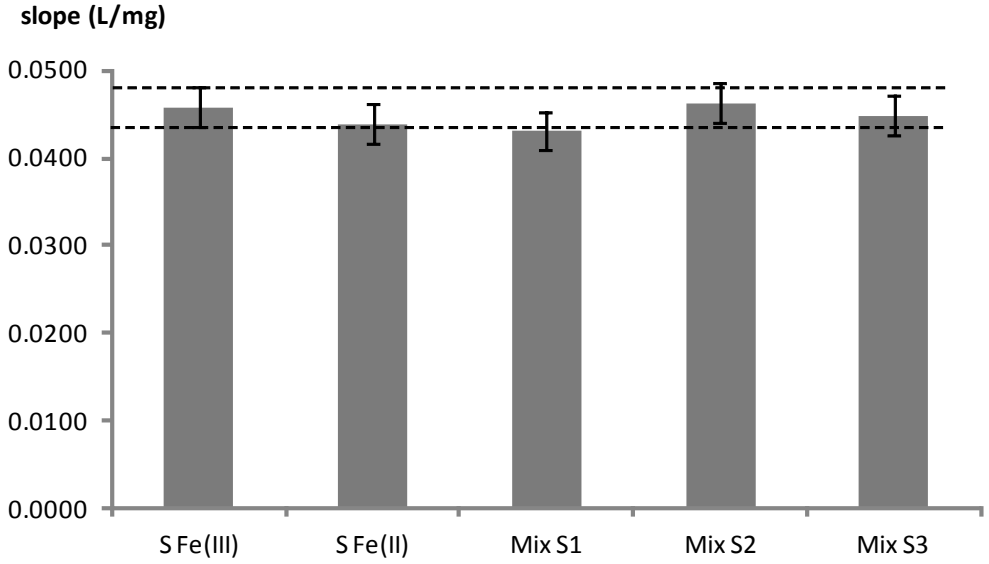
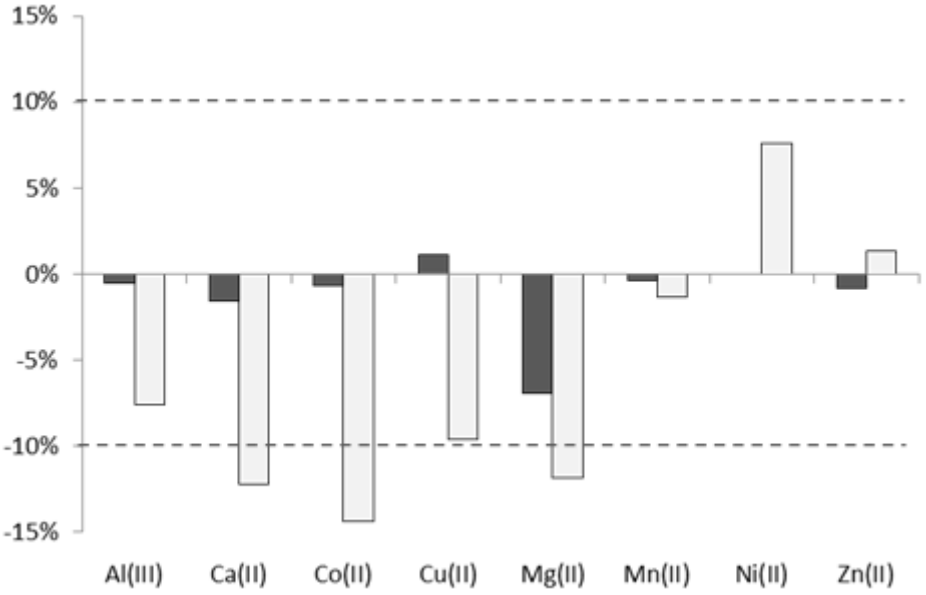


Figure 2S. Percentage of interference on the analytical signal of the possible interfering cations; the dark bars correspond to the minimum concentrations tested: 5.1 mg/LAl^{3+}, 10 mg/LCa^{2+}, 0.10 mg/LCo^{2+}, 1.0 mg/LCu^{2+}, 30 mg/LMg^{2+}, 0.20 mg/LMn^{2+}, 0.20 mg/LNi^{2+}, 1.0 mg/LZn^{2+}; the light bars correspond to the maximum concentrations tested: 25 mg/LAl^{3+}, 25 mg/LCa^{2+}, 5.0 mg/LCo^{2+}, 5.0 mg/LCu^{2+}, 50 mg/LMg^{2+}, 10 mg/LMn^{2+}, 2.0 mg/LNi^{2+}, 10 mg/LZn^{2+}.



4.7. Original paper 2

Title: Iron speciation by microsequential injection solid phase spectrometry using 3-hydroxy-1 (H)-2-methyl-4-pyridinone as chromogenic reagent

Authors: Ruth Suárez, Raquel B. R. Mesquita, Maria Rangel, Víctor Cerdà and António O. S. S. Rangel

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Iron speciation by microsequential injection solid phase spectrometry using 3-hydroxy-1(H)-2-methyl-4-pyridinone as chromogenic reagent

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ABSTRACT

The speciation of iron using the newly synthesized 3-hydroxy-1(H)-2-methyl-4-pyridinone by solid phase spectrophotometry in a microsequential injection lab-on-valve (μSI-LOV-SPS) methodology is described. Iron was retained in a reusable column, Nitrilotriacetic Acid Superflow (NTA) resin, and the ligand was used as both chromogenic and eluting reagent. This approach, analyte retention and matrix removal, enabled the assessment of iron (III) and total iron content in fresh waters and high salinity coastal waters with direct sample introduction, in the range of 20.0–100 μg/L. with a LOD of 9 μg/L. The overall effluent production was 2 mL, corresponding to the consumption of 0.48 μg of 2-metil-3-hydroxy-4-pyridinone, 0.34 mg of $NaHCO_3$, 16 mg of HNO_3, 4.4 μg H_2O_2 and 400 μL of sample. Four reference samples were analyzed and a relative deviation < 10% was obtained; furthermore, several bathing waters (#13) were analyzed using the developed method and the results were comparable to those obtained by atomic absorption spectrophotometry (relative deviations < 6%).

1. Introduction

To monitor the concentration of iron in natural waters is crucial to the knowledge of its distribution of the element in the environment. Moreover, it is important to determine not only the total iron content but also the fraction of both oxidation states: ferrous ion, Fe(II), and ferric ion, Fe(III) due to their dissimilar biological activity and toxicity. Therefore, for iron speciation in natural waters, direct measurements using atomic absorption or ICP techniques are not useful. Instead, spectrophotometric detection can be used for the speciation of iron in a more straightforward and economic way, if suitable chromogenic agents are employed; in fact, similar detection limits can be achieved, in the order of micrograms per liter [1]. However, most of the commonly used reagents for the spectrophotometric determination of iron, namely 1,10-phenanthroline, bathophenanthroline and eriochrome cyanine R [2], are highly toxic, and so alternatives using benign reagents are needed. In our previous work [3], the analytical application of 3-hydroxy-4-pyridinone chelators as chromogenic reagents for iron quantification was studied. A sequential injection method was developed and the 3-hydroxy-1(H)-2-methyl-4-pyridinone ligand proved to be the best choice for a flow analysis application. Although the detection limit was adequate for the iron assessment of natural waters, the application was limited to freshwaters. Additionally, no iron speciation was achieved.

In this work, the use of a 3-hydroxy-4-pyridinone (3,4-HPO) ligand in solid phase spectrophotometry (SPS) approach, combined with a microsequential injection analysis configuration is proposed to achieve iron speciation in both fresh and coastal waters. The chosen approach of SPS aimed to efficiently tackle the complexity of the target matrices, namely coastal waters. The analyte is retained by solid phase extraction and the matrix discarded to waste. After perfusion of the solid material with the ligand, the complex was measured and subsequently directed to waste. In fact, the matrix elimination resulting from the SPS approach [4] enhanced the sensitivity of the spectrophotometric method [5].

To accomplish SPS, a NTA resin was used as solid phase due to its favourable characteristics for this purpose: being relatively transparent to radiation and its affinity for iron (III). Actually,

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NTA resin has been effectively used for retaining iron in a pre-concentration procedure for water analysis [6–8] and in solid phase spectrophotometry detection [9,10]. The proposed method for iron speciation comprises two cycles: (i) direct perfusion of the resin with the ligand for the determination of iron (III); (ii) performing a preliminary in-line mixing of the sample with peroxide and subsequent retention of total iron. To the best of our knowledge, it is the first time that a 3-hydroxy-4-pyridinone ligand, the synthesized 3-hydroxy-1(H)-2-methyl-4-pyridinone, is used to perform SPS detection.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and boiled Milli-Q water (resistivity $>18\ M\Omega$ cm, Millipore, Bedford, MA, USA).

A stock solution of 10 mg/L iron(III) standard was prepared by dilution of the 1000 mg/L atomic absorption standard (Spectrosol, England). Working standards, 0.02–0.1 mg/L in 0.03 mol/L HNO_3, were prepared by dilution of the stock solution.

The 3-hydroxy-1(H)-2-methyl-4-pyridinone ligand (Hmpp) was synthesized as previously described [11]. The ligand solution was prepared by dissolution of Hmpp to a final concentration of 15 mg/L, corresponding to a saturated solution [3]. A carbonate buffer solution, 0.5 mol/L, was prepared by dissolving 4.2 g of $NaHCO_3$ (Panreac, Spain) in 100 mL of water and adjusting the pH adjusted to 10.5 with NaOH. The Hmpp reagent was prepared every other day by mixing the Hmpp solution with the carbonate buffer, in a ratio 4:1.

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 in the flow cell for iron(III) retention.

Nitric acid 1 mol/L was prepared from the concentrated solution ($d=1.39$; 65%, Merck) and used as washing/conditioning solution to ensure an acidic pH for the pre-concentration.

A hydrogen peroxide solution, 12.8 mmol/L, was prepared from the concentrated solution (perhydrol, 30% H_2O_2, Merk) and used for iron(II) oxidation.

For interference assessment studies, the solutions of the tested ions were obtained from: Al^{3+}, dissolution of the solid $AlK(SO_4)_2 \cdot 12H_2O$ (Steinheim, Germany); Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, dilution from the respective atomic absorption standards 1000 mg/L (Spectrosol, England).

2.2. Sample collection and preparation

Water samples from inland and coastal bathing areas (ESI Table. S1) were collected in polyethylene plastic bottles of 0.5 L capacity at about 30 cm depth. The samples, acidified at collection to pH ≈ 2 (with HCl) according to the collection procedure [1], were introduced directly in the developed system without filtration.

2.3. Microsequential injection manifold and procedure

The microsequential injection lab-on-valve solid phase spectrometry (μSI-LOV-SPS) manifold developed is depicted in Fig. 1.

The μSI-LOV system was a FIAlab-3500 (FIAlab Instruments) consisting of a bi-directional syringe pump (2500 μL of volume), a holding coil and a lab-on-valve manifold mounted on the top of a six-port selection valve.

The detection system comprised a USB 2000 Ocean Optics CCD spectrophotometer, fiber optics cables (FIA-P200-SR, 400 μm), and a Mikropack DH-2000-BAL deuterium halogen light source. FIAlab for Windows 5.0 software on a personal computer (HP Compact) was used for flow programming and data acquisition. The bead column was obtained by packing the NTA superflow resin between the two optical fibers, 10 mm optical path (Fig. 1B). To prevent any resin loss, a PTFE stopper (aligned with the central channel) and a PEEK tube, with inner diameter of 127 μm (#1535 Upchurch scientific), were used (Fig. 1B). All tubing connecting the different components of the flow system was of polytetrafluoroethylene (PTFE) with 0.8 mm inner diameter, including a 1.5 m of holding coil.

The protocol sequence with the respective volumes used for both iron(III) and total iron determinations is shown in Table 1.

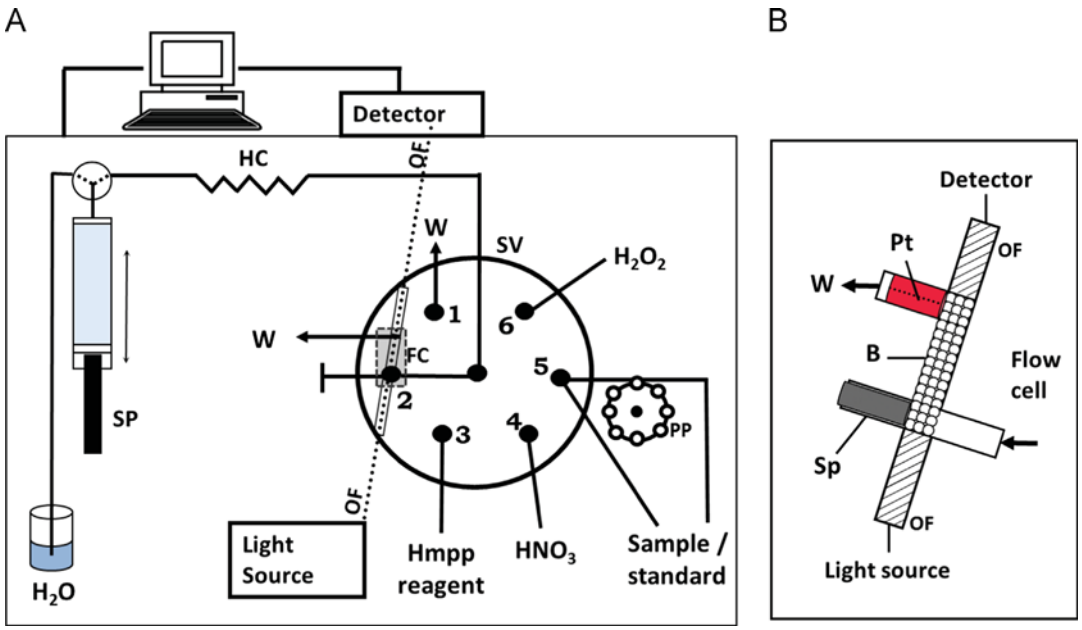

Fig. 1. Microsequential injection manifold developed for the solid phase spectrometry determination of iron with Hmpp: (A) Schematic representation: SP, syringe pump; SV, six port selection valve; HC, holding coil; PP, peristaltic pump; FC, flow cell; W, waste; OF, optical fiber; (B) Detailed scheme of the flow cell where the black arrows represent the flow direction: Sp, PTFE stopper, Pt, PEEK tube with 127 μm inner diameter; B, packed beads column of NTA resin; OF, optical fiber.

Table 1
Protocol sequence for the developed μSI-LOV-SPS for the determination of iron with Hmpp ligand.

Step	Operation	SV position	Volume (μL)	Flow rate (μL/s)
A	Fill the syringe with carrier	–	1000	200
B[a]	Aspirate H_2O_2	6	5	10
C	Aspirate sample/standard	5	400	25
D[a]	Aspirate H_2O_2	6	5	10
E	Propel sample or sample mixture through the NTA column in the flow cell	2	600	10
F	Aspirate of the Hmpp reagent	3	40	25
G	Propel the Hmpp reagent through the NTA column in flow cell with the retained iron and absorbance measurement	2	350	10
H	Aspirate HNO_3	4	250	60
I	Propel HNO_3 through the NTA column in the flow cell for washing/conditioning the column	2	1000	10

[a] Steps present only for the determination of total iron.

After filling the syringe pump with carrier (step A), the sample/standard was aspirated and propelled through the flow cell packed with NTA resin (steps C and E) for retaining iron(III). Then, the Hmpp reagent was aspirated to the holding coil and sent through the column, removing iron from the resin beads by forming the colored, 3,4-HPO iron(III) complex to be measured (steps F and G).

For the determination of total iron, two extra steps were included in the protocol sequence. After filling the syringe pump (Step A), the sample/standard was aspirated between two plugs of peroxide (Steps B–D), promoting the oxidation of iron(II) to iron (III). Then, the oxidized sample/standard was sent to the flow cell packed with NTA resin and iron(III) was retained (Step E).

Afterwards, the determination was carried out as previously described (Steps F and G).

At the end of each cycle, the NTA column was washed and conditioned with nitric acid in order to achieve similar initial experimental conditions for each cycle (steps H and I).

2.4. Reference procedure

The collected bathing waters, both inland and coastal, were analyzed using the atomic absorption method (APHA 3113B) [1] and the results were compared to those obtained with the developed μSI-LOV-SPS method.

For further accuracy assessment, results obtained with the proposed μSI-LOV-SPS system were compared to the certified values of four certified water samples. A river water certified reference material (NRC-SLRS-4), a surface water reference material (NIST-SPS-SW2) and two drinking waters (CA-021a and CA-010a) were analyzed for the evaluation of the accuracy of the developed method.

3. Results and discussion

The reaction between Hmpp and iron(III) has been previously studied and effectively applied in a sequential injection and a microsequential injection procedure [3]. However, in this work, the aim was to achieve a lower dynamic range, to enable iron speciation and also to extend the application to saline samples. In this context, a SPE step was explored using the NTA resin (beads) packed in the flow cell of a microsequential injection analysis lab-on-valve unit. The pre-concentration was attained by propelling the sample/standard solution through the packed column of beads in the flow cell followed by perfusion of the beads with the Hmpp reagent (Hmpp in carbonate buffer).

3.1. Preliminary studies

In our previous work [3], the Hmpp solution and the carbonate buffer solution were mixed in-line to improve reagent stability. In this work, due to the number of available ports, the Hmpp solution and carbonate buffer were previously mixed to produce the Hmpp reagent (described in Section 2.1).

The Hmpp concentration, corresponding to a saturated solution (15 g/L), and the aspiration volume (40 μL) were set from the previous work [3]. Different carbonate concentrations were tested, ranging from 0.03 to 0.1 mol/L; the sensitivity increased up to 0.1 mol/L. Higher concentrations were not tested as they produced a degradation of the Hmpp reagent, easily observed by a color (yellowish) increase.

The stability of this solution was studied by comparing calibration curves of four consecutive days. The results showed no significant impact on the sensitivity (slope variation $< 1\%$ for the first three days and 5% by the fourth day) but a major increase in the detection limit (intercept tripled by the third day). This feature could be explained by the degradation of the ligand with a consequent blank increase. So, an option was made to prepare Hmpp reagent every other day.

3.2. Iron retention in the NTA resin

The NTA resin (beads) was used in a reusable approach: at the beginning of the working day, the flow cell was packed by propelling the beads suspension to the optical path. Whenever a visual decrease in the column size was observed, resulting from the loss of some smaller beads to the waste due to continuous propelling, the column was refilled and/or repacked. The packed column could be used for 2 days, about 180 determinations, with no need for refilling or repacking.

There are two possible approaches when working with solid phase spectrometry: resin beads can be discharged after each measurement in a bead injection approach (BI) or reused in a pre-packed column approach. The latter was chosen as a less expensive (resin saving) option. Having set the column packing procedure, the preparation of the Hmpp reagent and the respective volume to be used, parameters for the SPE step were assessed. The flow rate of propelling through de beads was set as previously reported [9]: 10 μL/s.

3.2.1. Regeneration of the column

Due to the option of a reusable column approach, it was necessary to ensure complete elution of the metal after the measurement and guarantee identical conditions for each cycle. Nitric acid 1 mol/L was used as eluent/conditioner and different volumes were tested: 75, 100, 125, 250 μL, followed by a 4 fold volume of water for rinsing. The efficiency of the washing/regeneration process was assessed by estimating the repeatability (RSD) of a 0.800 mg Fe^{3+}/L standard. The volume of 250 μL resulted in a RSD $< 2\%$ so that was the chosen volume.

3.2.2. Column breakthrough

The column breakthrough corresponds to the maximum amount of iron(III) retained in the beads. Using an iron(III) standard of 0.4 mg/L, increasing amounts of iron(III) were loaded in the packed beads column by executing consecutive cycles with increasing volumes. The corresponding absorbance values were plotted against the mass of iron(III) loaded in the beads (ESI Fig. S1). The signal increased up to 56 ng of iron(III) as the absorbance for both 56 ng and 64 ng of iron(III) was statistically the same ($A=0.042\pm0.003$ and $A=0.043\pm0.002$, respectively). Therefore, 56 ng was considered as the maximum amount of iron(III) retained in the beads column.

3.2.3. Sample/standard volume

Having established the maximum amount of iron(III) to be retained in the NTA resin, the influence of sample volume on the sensitivity was studied. First, a wide range of volumes: 40, 60, 100, 200, 400 and 600 μL were assessed by comparing the signal of an 80 Fe^{3+}μg /L standard. The volumes of 400 and 600 μL produced the higher signals obtained (increase of > 35% compared to the other tested volumes), so, calibration curves with those volumes were compared. The sample volume of 400 μL was chosen as it resulted in a calibration curve with a higher slope value (7% increase) and a lower intercept value (5% decrease) than the calibration curve obtained with a sample volume of 600 μL, thus meaning more sensitivity and lower detection limit.

3.3. Iron speciation

The ligand Hmpp can complex both iron(II) and iron(III), due to its mild oxidizing capacity [3]. So, it was necessary to ensure that only iron(III) was retained in the NTA resin in order to achieve speciation.

3.3.1. Determination of iron(III)

The dispensed volume, of sample/standard plus carrier, propelled through the NTA resin was studied to guarantee that non-retained ions were washed out. Although iron(II) was not expected to be retained, if the dispensed volume was not sufficient, it could remain in the column dead volume and complex with the Hmpp ligand. Two iron(III) standards of 60 μg/L were prepared, one containing iron(II) 110 μg/L. The absorbance values for both standards, with different dispensed volumes, 440, 600, 800 μL (corresponding to 10%, 50%, 100% over the sample/standard volume) were compared. The results obtained showed that 440 μL of dispensed volume was not enough to wash out the non-retained iron(II) from the NTA resin. In fact, the signal for the standard containing both iron(II) and iron(III) was higher (relative deviation = 30%) than the signal for the iron(III) standard (ESI Fig. S2). However, for the dispensed volumes of 600 and 800 μL, the registered signals were the same for both standards (relative deviations < 3%), showing that iron(II) was not retained in the NTA resin (ESI Fig. S2). In order to minimize the waste production, a dispensed volume of 600 μL was chosen.

3.3.2. Determination of total iron - concentration of H_2O_2

In order to determine the total iron content, it was necessary to oxidize the Fe(II) to Fe(III), as Fe(II) was not retained in the beads. Hydrogen peroxide was chosen as the oxidizing agent for the determination of total iron and two steps were added to the analytical cycle, in order to sandwich the sample between two oxidant plugs [9]. The volume of oxidant was 5 μL per plug, the reported minimum volume to attain an effective overlapping [12].

The concentration of hydrogen peroxide was studied within the range 2–64 mmol/L in order to attain a complete oxidation of iron (II) to iron(III). The signal of an iron(II) standard of 110 μg/L was registered for the different peroxide concentrations and compared to the signal obtained for an iron(III) standard with the same concentration (Fig. 2). The results showed that 12.8 mmol/L was the minimal hydrogen peroxide concentration to obtain the same signal with equimolar standards of iron(II) and iron(III).

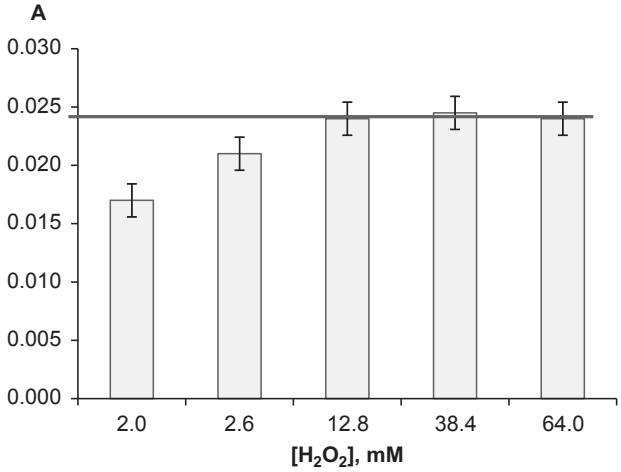

Fig. 2. Study of the effect of the hydrogen peroxide concentration on the signal obtained for 110 μg Fe^{2+} /L standard solution, grey bars; the black line represents the signal obtained for an iron(III) standard with the same concentration.

3.4. Interferences study

3.4.1. Salinity interference assessment

The effect of salinity was studied for the salinity values, 0, 5, 15 and 35, comparing calibration curves using standard solutions with those salinity values. These standard solutions were prepared by adding sodium chloride to the previously used standards to achieve a final concentrations of 0, 22, 45 and 112 g/L of NaCl. The calibration curve resulting from pure iron standards was compared to the calibration curves obtained from the iron standards with added NaCl. The estimated slopes of the curves were assessed at the confidence intervals at 95%. The quality of the regression was tested by residual analysis (i.e. randomness and normality) and by the coefficient of correlation, R^2, which was above 0.987 in all cases. No statistical difference, at 95% confidence level, was observed between the calibration curves with complete overlapping of the slope values (ESI Fig. S3), thus indicating no salinity interference. Therefore, calibration with pure iron standard solutions can be used to analyze samples with higher salinity. So, the developed methodology of iron speciation is applicable to sea water samples as well as to river and estuarine waters.

3.4.2. Possible interference of other bivalent cations

A detailed study of the possible interfering species for the colorimetric determination of iron(III) with the Hmpp ligand was accomplished in the previous work [3]. So, only potential interferences on the SPE step were evaluated, namely for other metal ions that could also be retained in the NTA resin. The metal ions that are likely to be present in natural waters such as aluminium (III), calcium(II), magnesium(II), copper(II) and zinc(II) were those whose interference was tested. Several standard solutions were prepared with the same concentration of iron(III), 60 μg/L, and different concentrations of the foreign metal ions. The tested concentrations were based on the values from the Environmental Protection Agency (EPA) for natural waters and from United Nations Food and Agriculture Organization (UNFAO) for irrigation waters [1]. The signal obtained from the standards with an inter-

fering ion were compared to those obtained with a pure iron(III) standard (Table 2).

No significant interference (< 5%) of the tested metal ions for the tested concentrations (Table 2) was found. Exception made for 1.00 mg/L of copper(II) with an interference percentage of over 10%. However, that concentration is not expected in natural waters.

3.5. Figures of merit

The features of the developed method, namely dynamic range, limit of detection, determination rate and reagent consumption, are summarized in Table 3.

The typical calibration curve corresponds to a mean of four calibration curves with the standard errors between brackets. The LOD was calculated as three times the standard deviation of the intercept ($n=5$), according to IUAPAC recommendation [13]. The quantification rate was calculated in the time spend per cycle plus the time needed for equipment operation. The repeatability was assessed by calculating the relative standard deviation (RSD) of two bathing water samples, one inland and one coastal. The reagent consumption values presented were calculated per determination. A sample consumption of 400 µL and an effluent production of about 2 mL per cycle was obtained.

3.6. Application to natural waters

3.6.1. Accuracy assessment for total iron determination

For the accuracy assessment studies, the protocol sequence was the one for the determination of total iron described in Table 1. In order to evaluate the developed methodology accurateness, four certified water sample were analyzed: two drinking waters, CA-021a and CA-010a, a surface water, NIST-SPS-SW2, and a river water certified material, NRC-SLRS-4 (Table 4). Since the certified value was above the dynamic concentration range, the certified samples were diluted prior to analysis. The repeatability was also evaluated by the calculation of the relative standard deviation (RSD).

The results obtained, RD < 10%, validate the determination of total iron attained with the developed microsequential injection lab-on-valve methodology.

For further accuracy assessment, several river and sea water samples (♯13) were assessed with the developed µSI-LOV methodology (µSI-LOV) and the results compared with those obtained by the reference procedure, atomic absorption spectrometry (AAS) (Fig. 3).

A linear relationship between the results obtained with the developed µSI-LOV ($[Fe^{3+}]_{\mu SI\text{-}LOV}$) and the reference procedure ($[Fe^{3+}]_{AAS}$) was established and the equation found was: $[Fe^{3+}]_{\mu SI\text{-}LOV}=1.004\ (\pm 0.100)\ [Fe^{3+}]_{AAS}+0.000\ (\pm 0.007)$, where the values in parenthesis are the 95% confidence limits. These figures show that the estimated slope and intercept do not differ from the values 1 and 0, respectively. Thus, there is no evidence for systematic differences between the two set of results [14]. Furthermore, the relative deviations (RD) between the results obtained with the developed methodology and the reference procedure were calculated and the values obtained, RD ≤ 10%, proved that there were no significant differences between the two sets of results (ESI Table. S2).

3.6.2. Iron speciation in bathing waters

The proposed method was applied to several bathing waters, both inland and coastal, for iron speciation (Table 5). The determination of iron(III) was carried out without the oxidation of the analyte. For the determination of total iron, the inclusion of hydrogen peroxide, steps B and D of the protocol sequence detailed in Table 1, ensured the oxidation of iron(II) to iron(III) so the total iron content of the sample was retained in the NTA beads. The calculation of the iron(II) content was calculated by subtracting the iron(III) content from the total iron content (Table 5). The total iron content of the samples previously determined with the reference procedure (AAS) was also included in the table.

The iron content of some samples, Pi2 and P2, were slightly above the dynamic range of the developed method but the

Table 2
Study of possible interference from metal cations in the registered signal of a 60 µg /L iron(III) standard.

Tested cation	Average values in streams [1]	Legislation limits EPA[a]/UNFAO[b] [1]	Tested concentration (mg/L)	Signal interference (%)
Ca^{2+}	15 mg/L	–	25	−2.3
Mg^{2+}	4 mg/L	–	10	4.0
Al^{3+}	400 µg/L	50 µg/L (EPA) 200 µg/L[c] (UNFAO)	2.50	−3.3
Cu^{2+}	4–12 µg/L	1.3 mg/L (EPA) 200 µg/L (UNFAO)	0.50 1.00	−3.8 −14.0
Zn^{2+}	20 µg/L	2 mg/L (EPA) 5 mg/L (UNFAO)	10	2.3

[a] Environmental protection agency.
[b] United Nations Food and Agriculture Organization, irrigation waters.
[c] Value for minimal risk.

Table 4
Certified water samples assessed by the developed µSI-LOV-SPS method; RSD, relative standard deviation; RD, relative deviation.

Certified sample ID	µSI-LOV (mg Fe/L ± SD)	RSD (%)	Certified value (mg Fe/L ± SD)	RD (%)
CA-021a	0.199 ± 0.006	3.0	0.196 ± 0.002	−1.6
CA-010a	0.254 ± 0.011	4.2	0.236 ± 0.003	−7.8
SLRS-4	0.096 ± 0.001	0.8	0.103 ± 0.005	6.4
SPS-SW2	0.103 ± 0.002	2.1	0.100 ± 0.001	−3.2

Table 3
Features of developed microsequential injection methodology.

Dynamic range (µg/L)	Typical calibration curve A = slope × mg Fe^{3+}/L + b	LOD (µg/L)	Quantification rate (h^{-1})	RSD (%), (µg/L ± SD)	Reagent consumption
20.0–100	$A=0.229\ (\pm 0.007)\ [Fe^{3+}]+0.007\ (\pm 0.001)$ $R^2=0.997\ (\pm 0.003)$	8.5	14 13[a]	2.1% (75.6 ± 1.4)[b] 3.7% (88.6 ± 3.3)[b]	0.48 µg Hmpp 0.34 mg $NaHCO_3$ 15.8 mg HNO_3 4.4 µg H_2O_2[a]

[a] For the determination of total iron.
[b] Sample concentration values in brackets.

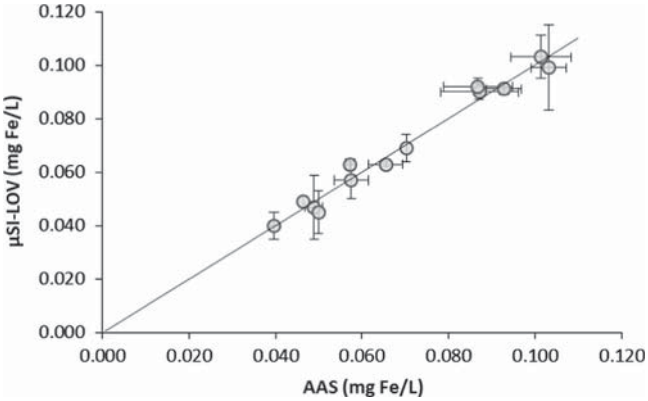

Fig. 3. Accuracy assessment comparing the total iron concentration in river and sea water samples calculated using the developed methodology (μSI-LOV) and using atomic absorption spectrometry (AAS); the line represents the optimal correlation (slope=1 and intercept=0).

Table 5
The developed μSI-LOV-SPS methodology was applied to iron speciation in bathing waters; SD, standard deviation; AAS, atomic absorption spectrometry; RD, relative deviation.

Sample		μSI-LOV			AAS	RD (%)
Type	ID	μg Fe^{3+}/L ± SD	μg Fe^{2+}/L	μg Fe/L ± SD	μg Fe/L ± SD	
Inland beach	Pi1	46.0 ± 1.4	16.9	62.9 ± 1.2	65.5 ± 3.6	−4
	Pi2	78.6 ± 2.4	24.7	103 ± 8	101 ± 7	2
	Pi3	46.9 ± 1.0s	18.6	48.7 ± 1.5	46.5 ± 0.0	5
	Pi4	84.0 ± 8.5	6.30	90.3 ± 2.7	87.1 ± 8.6	4
	Pi5	39.0 ± 0.7	29.8	68.8 ± 4.0	70.2 ± 0.0	−2
	Pi6	30.7 ± 7.3	9.8	40.4 ± 4.5	39.7 ± 1.0	2
Coastal beach	P1	45.9 ± 5.0	48.3	94.2 ± 2.3	–	–
	P2	86.3 ± 4.2	23.7	110 ± 5	–	–
	P3	80.4 ± 6.4	14.2	91.7 ± 3.3	86.8 ± 8.1	6
	P4	75.8 ± 3.1	15.3	91.1 ± 1.5	92.8 ± 4.5	−2

results were still in agreement with the reference procedure (when performed).

4. Conclusions

The developed microsequential injection lab-on-valve methodology for iron speciation in bathing waters proved to be an effective, real time, reliable tool for the environmental monitoring of iron. To the best of our knowledge, this is the first application of the 3-hydroxy-4-pyridinone, the 3-hydroxy-1(H)-2-methyl-4-pyridinone (Hmpp) ligand, in a solid phase spectrophotometric (SPS) determination. Although this ligand has been previously successfully used as a selective chromogenic reagent in a greener approach to the spectrophotometric determination of iron [3], its use with the solid phase extraction of the metal ion was a highly advantageous improvement. A more than 4.5 fold sensitivity increase was achieved. The choice of NTA resin for retaining iron (III) attested to be appropriate as it enabled to establish a suitable dynamic range (20.0–100 μg/L) and a direct introduction of the natural water samples. Furthermore, the choice of SPS approach widens the application range to high salinity samples. The combination of the SPE step with the SPS determination, attained by the packing of the beads (NTA resin) in the flow cell, was possible due to the high affinity of the Hmpp reagent for iron which ensured the complete removal of the metal ion following the detection. This feature allowed employing a reusable approach, minimizing reagent consumption and overall analysis cost.

The most remarkable output is to have a single methodology for iron speciation in both low salinity and high salinity water samples, with direct introduction of the sample. Aiming for the environmental monitoring of iron distribution it becomes feasible to compare results from different water sources by assessing all target samples with the same experimental procedure.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.03.059.

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4.7.1. Supplementary information – paper 2

Table S1. Bathing waters **s**amples characterization and sampling locations; Temp., temperature; G, conductivity; DO, dissolved oxygen; TDS, total dissolved solids; Long., longitude; Lat., latitude.

Sample type	Sample ID	Multiparameter probe						Geographical coordinates	
		Temp. (°C)	pH	G (µS cm^{-1})	Salinity	DO, O_2 (mg L^{-1})	TDS (mg L^{-1})	Long.	Lat.
Inland bathing waters	Pi1	11.38	8.00	45	≤ 0.02	11.70	29	41.81	-8.42
	Pi2	13.48	7.00	51	≤ 0.02	10.98	33	41.81	-8.42
	Pi3	14.38	7.17	44	≤ 0.02	10.74	28	41.84	-8.37
	Pi4	11.48	6.82	39	≤ 0.02	11.78	26	41.60	-8.46
	Pi5	10.56	6.89	35	≤ 0.02	11.92	23	41.61	-8.41
	Pi6	9.34	6.87	33	≤ 0.02	12.22	21	41.61	-8.38
Coastal bathing waters	P1	12.48	7.20	48	31.06	10.29	31	41.15	-8.68
	P2	12.67	7.90	49	32.28	11.05	32	41.16	-8.68
	P3	12.62	7.91	50	32.74	11.74	32	41.16	-8.69
	P4	12.81	7.87	47	32.34	11.83	32	41.17	-8.69

Figure S1. Calculation of the maximum amount of iron(III) retained in the NTA resin, breakthrough of the beads column. The signal stabilized at 56 ng of iron(III).

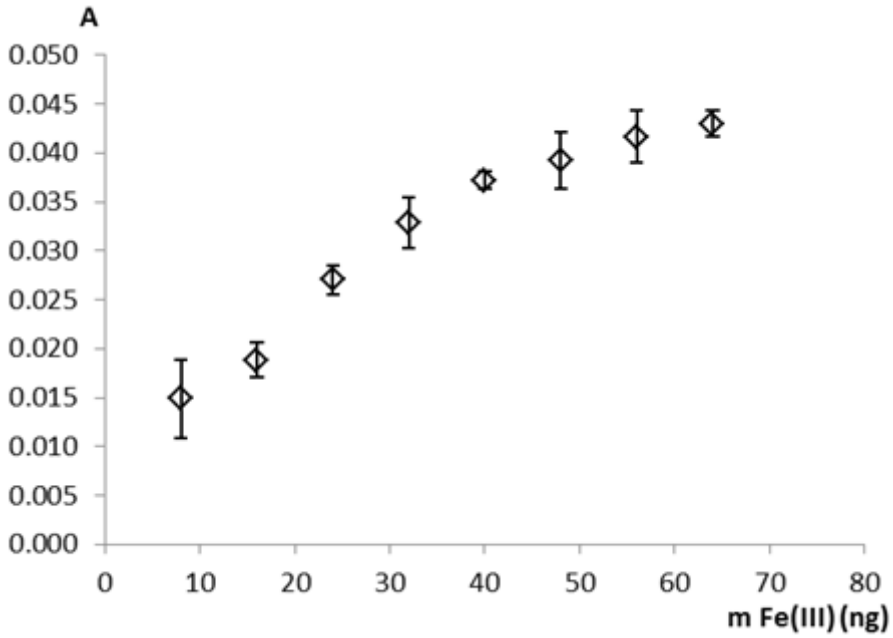

Figure S2. Study of the dispensed volume in effective washing of the beads column; comparison of the signal obtained for a 60 Fe^{3+}µg /L standard, white bars, and for standard with 60 Fe^{3+}µg /L and 110 Fe^{2+}µg/L, grey bars.

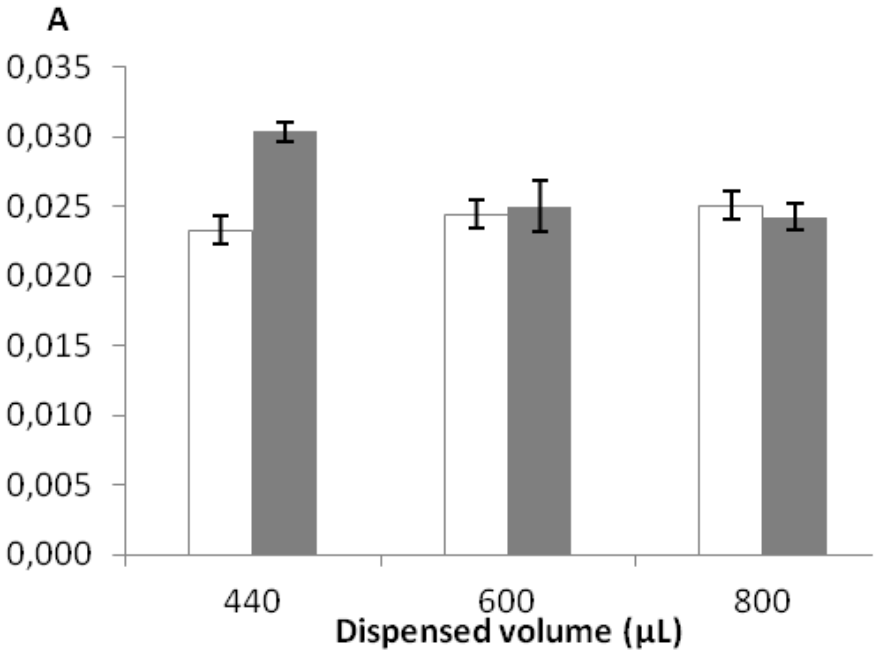

Figure S3. Study of the possible interference from salinity; plotting of the slopes of calibration curves using standards with different concentrations of sodium chloride.

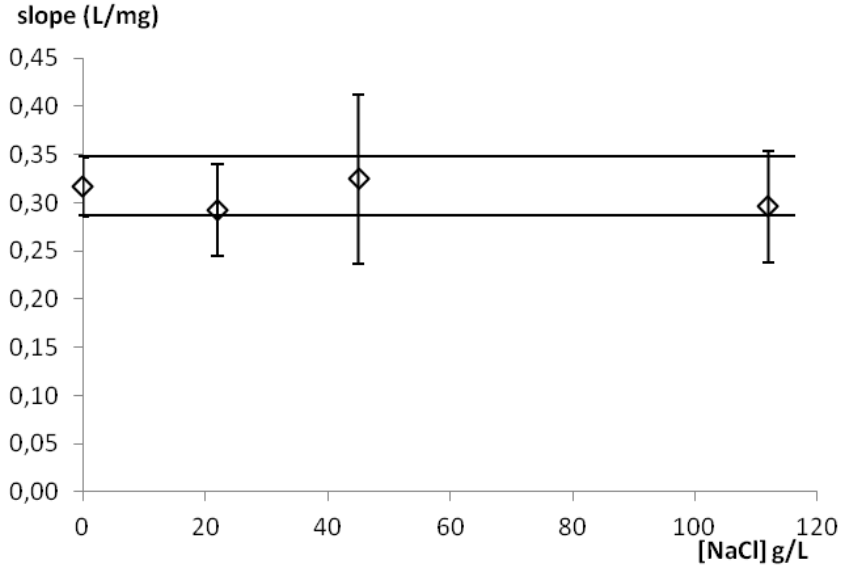

Table S2. Accuracy assessment by comparison of the results obtained with the developed methodology (μSI-LOV) with those obtained with the reference procedure, atomic absorption spectrometry (AAS); RD, relative deviation.

Sample ID	AAS (mg Fe/L)	μSI-LOV (mg Fe/L)	RD (%)
1	0.057 ± 0.001	0.063 ± 0.002	10.1
2	0.101 ± 0.007	0.103 ± 0.008	1.6
3	0.087 ± 0.009	0.090 ± 0.003	3.6
4	0.049 ± 0.002	0.047 ± 0.012	-4.0
5	0.040 ± 0.001	0.040 ± 0.005	1.9
6	0.087 ± 0.008	0.092 ± 0.003	5.7
7	0.093 ± 0.004	0.091 ± 0.002	-1.9
8	0.065 ± 0.004	0.063 ± 0.001	-4.0
9	0.103 ± 0.004	0.099 ± 0.016	-4.0
10	0.046 ± 0.000	0.049 ± 0.002	4.8
11	0.058 ± 0.004	0.057 ± 0.007	-0.1
12	0.070 ± 0.000	0.069 ± 0.005	-2.1
13	0.050 ± 0.000	0.045 ± 0.008	-10.7

CHAPTER 5

IN-SYRINGE DLLME METHODS FOR ALUMINIUM DETERMINATION

5. In-syringe dispersive liquid-liquid microextraction methods for aluminium determination

5.1. General remarks about aluminium

Aluminium (Al) is the third most abundant element in the Earth's crust and is a non-essential element [1]. Aluminium is widespread throughout the environment, e.g. air, plants, and food. Consequently it is present in all natural waters in a wide variety of chemical forms. Thus, due to the extensive uses and wide occurrence of aluminium in nature, monitoring of aluminium in water is of great relevance.

During last years, there has been an increasing interest in the study of the toxicity of aluminium and its effects in aquatic ecosystems, plants and humans [2]. In addition, aluminium concentration has been identified as a major factor that limits plant growth of many species [3] in acidic soils by inhibiting root growth. High aluminium concentration in the human body can also produce many clinical disorders, e.g. it is believed to cause renal failure in patients undergoing hemodialysis treatment [4]. Moreover, it has a potential neurotoxic effect [5], its intake is connected with a variety of human pathologies including, Alzheimer's disease, autism and Parkinson's disease [6]. According to the World Health Organization (WHO), the permissible level of aluminium in drinking water is 200 µg/L [7].

An outline of aluminium biogeochemical cycle is represented in Figure 5.1. It is important to understand the importance of aluminium marine geochemistry for several reasons, e.g. in oceanography, Al (III) concentration data can be used to trace atmospheric dust deposition and thus to estimate the entry and deposition of other essential elements, such as iron, which do have biological functions and are readily up-taken [8]. Al (III) salts present a low solubility in water, so the concentration of aluminium in water depends on the characteristics of the sample. In seawater the content of Al depends on the salinity and is relatively low, varying commonly between 0.03-2 µg/L. River waters contain much higher Al amounts that range broadly from 2 to over 1000 µg/L, depending on surrounding rocks and soils. In the surface layer of seawater may be found Al at higher levels due to the atmospheric deposition and higher scavenging rate. Further Al sources are river effluents and anthropogenic emissions.

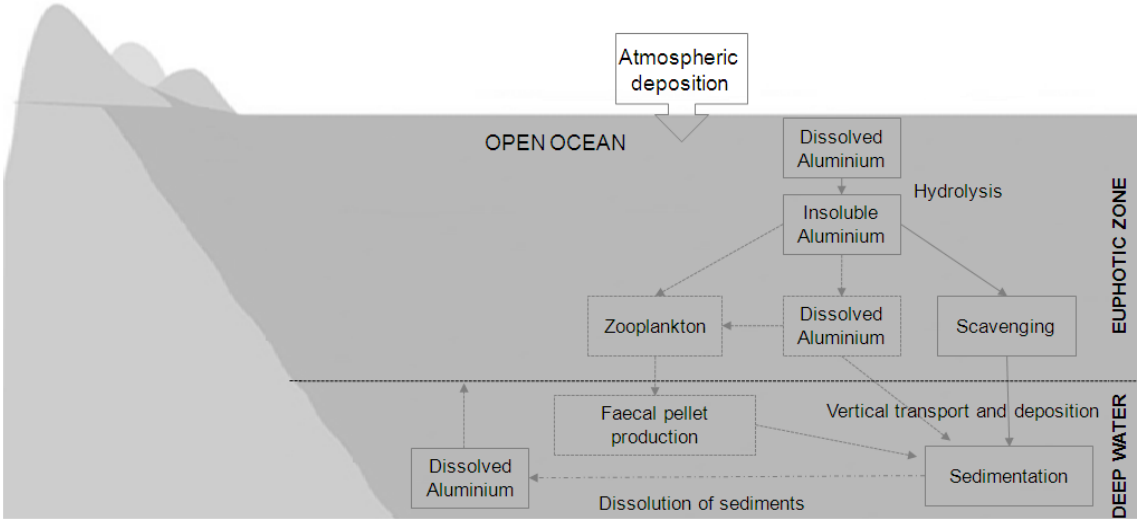

Figure 5.1 Aluminium biogeochemical cycle.

The speciation of Al in the aquatic systems depends on several parameters, such as pH, dissolved organic carbon and total concentration of different ligands. In these systems Al exists mainly as: free Al^{3+}, Al–hydroxide complexes ($AlOH^{2+}$, $Al(OH)_2$, $Al(OH)_3$ and $Al(OH)_4^-$, monomeric F complexes (e.g. AlF^{2+}, AlF_2^+, AlF_3), and monomeric SO_4^{2-} complexes, and in association with naturally occurring organic ligands. It has been indicated by laboratory bioassay that monomeric inorganic Al^{3+}, $AlOH^{2+}$, and $Al(OH)_2^+$ are the most toxic forms, whereas Al-F and Al-Org can reduce or diminish toxicity. Therefore, the development of methods for speciation of Al in surface waters is critical and has received considerable attention [9].

Thus, in order to protect human health and to ensure environmental safety, it is essential to establish simple, rapid, sensitive and environmental friendly methods for aluminium monitoring at trace levels in biological, environmental and food samples.

5.2. Detection techniques for aluminium

The determination of aluminium is generally carried out using classical techniques such as graphite furnace - atomic absorption spectrometry (GF-AAS) [10], and inductively coupled plasma - atomic emission spectrometry (ICP-AES) [11]. Although, these methods provide high selectivity, they are expensive in terms of both instrumentation and operating costs. Thus, low cost and rapid methods which imply less chemicals consumption have been developed, e.g. spectrophotometric or fluorimetric methods. These techniques have been widely used for chemical analyses in a number of fields.

This fact is because of their inherent sensitivity, simplicity, ease of automation, cost effectiveness, and to some extent selectivity. Also when coupled to flow analysis techniques, automation can be accomplished, leading to increased reproducibility and a decrease in cost.

Fluorimetric detection provides higher sensitivity than spectrophotometry, while spectrophotometric methods usually employ simpler instrumentation. Typical spectrophotometric determination of Al is characterized by the use of quercetin [12] as the colorimetric reagent, forming a stable complex free from interfering species. Other typical chelating reagents include pyrocatechol violet [10] and eriochrome cyanine [13]. On the other hand, most fluorescence determinations involve the use of fluorogenic ligands since aluminium is not fluorescent by itself. The formation of highly fluorescent chelates through the combination of a metal ion and an organic ligand proves to be a sensitive and specific method for the determination of many metals. Many fluorogenic reagents have been used for the determination of aluminium based on the formation of metal complexes. The most popular ligand is lumogallion (LMG) [14, 15] despite other compounds have also been studied varying the selectivity and sensitivity according to different applications, e.g. 8-HQ [16], morin [17], and, salicyladehyde picolinohydrazone [18]. LMG binds with Al (III) for increased fluorescence emission through the reaction shown in Figure 5.2. LMG acts as a planar tridentate ligand in the reaction, two phenolic oxygen ions and the azo group apparently being bound to the metal, forming complex rings in aromatic linkage. The maximum excitation/emission for this complex is 502/588 nm , respectively [19].

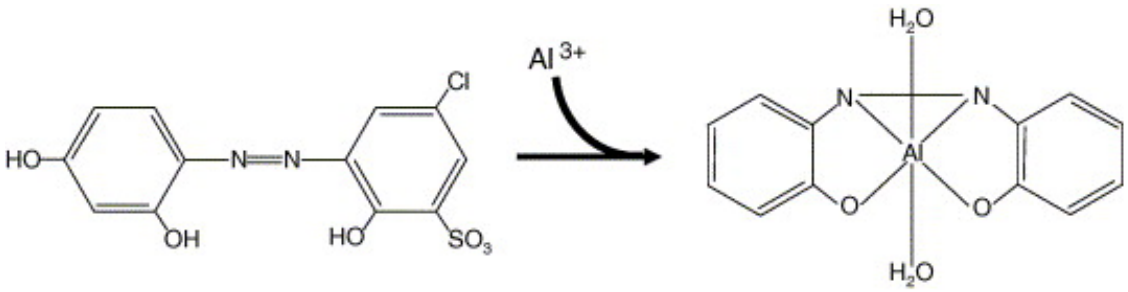

Figure 5.2 Reaction of lumogallion with Al^{3+} to form the lumogallion-aluminium complex.

5.3. Aluminium extraction and preconcentration techniques

It is generally impossible to determine directly aluminium in environmental samples because of interfering species, and/or the concentration of the analyte being below the detection limit of the instrument. Thus, despite advances made in detection instrumentation, trace metal analysis most often requires some form of separation and preconcentration [20]. Most widely used techniques for the separation and preconcentration of aluminium include LLE [16] and SPE [10, 21]. Although SPE is often praised for its avoidance of solvents and for being less environmental harmful than LLE, μ-LLE techniques overcome typical problems of SPE such as potential clogging, long extraction times, and higher costs while minimizing the environmental impact of classical LLE. Reported μ-LLE techniques used for the determination of Al^{3+} include single drop microextraction (SDME) exploiting 8-HQ [22]. Also, cloud point extraction (CPE) [23] has been employed for the preconcentration of trace aluminium forming the complex of Al with xylidyl blue prior to its determination by flame atomic absorption spectrometry (FAAS). And most recently, DLLME combined with stopped-flow spectrofluorometry [24] was applied to determine Al using oxine as a chelating agent.

5.4. Automation of the DLLME method for aluminium determination

Accurate determination of dissolved aluminium in seawater is difficult due to the complexity of the matrix and the trace concentrations at which the metal exists. Hence, with the aim of developing fully automated, sensitive and selective methods for aluminium determination in seawater using in-syringe dispersive liquid-liquid microextraction, several investigations were performed. Flow based methods based on spectrophotometric or fluorimetric detection are of interest being an acceptable alternative due to their relative low cost, simple operation, and widespread diffusion of equipment. Thus, the fluorimetric detection exploiting LMG for aluminium determination was selected due to its excellent sensitivity and minimal interferences.

In addition, two devices were specially made for these works, a fluorescence detector and flow cell and a heating device integrated into the holding coil to accelerate the reaction kinetic between Al^{3+} and LMG. These are explained in detail in chapter 3 in section 3.3.2 and 3.1.2.1, respectively and in the supporting information provided below (section 5.6.1).

After a wide study of extraction and dispersing solvents, the Al-LMG complex was extracted by DLLME using hexanol as extraction solvent and ethanol as dispersing solvent in a 1:8 % v/v mixture.

In the first developed assembly exploiting in-syringe DLLME, the complete analytical procedure including sampling, buffering, reaction of the analyte with fluorescence reagent LMG, extraction, phase separation, and quantification was completely automated and carried out within 4 min. Under optimum experimental conditions, the LOD was 8.0 ± 0.5 nmol/L Al and an RSD of 1.5% was achieved for eight replicate determinations of 200 nmol/L Al. The calibration graph using the preconcentration system was linear up to 1000 nmol/L. It was successfully applied to the determination of aluminium in coastal seawater samples. Analyte recoveries from 97 % to 113 % proved the applicability and adequateness of the analyzer system to seawater samples.

Despite the good performance of the in-syringe DLLME method developed we decided to investigate and develop an in-syringe MSA-DLLME system for aluminium determination based on the same reaction. This new approach was developed by our research group. It is based on the use of a magnetic stirring bar inside the syringe of an automated syringe pump, allowing rapid and homogeneous mixing of a sample with the required reagents within a short time avoiding the use of the dilution chamber and of the disperser solvent. Thus, the whole procedure including reagents and sample mixing were performed within the syringe body, allowing the reduction of organic solvent required (since no disperser is needed) and so a higher sample volume to be processed, achieving a higher preconcentration factor, higher sensitivity and shorter analysis time as can be seen in Table 5.1.

The LOD was 6.1 nmol/L, and a wide linear range was attained, e.g. up to 1.1 µmol/L. An average recovery of 106.0 % was achieved for coastal seawaters. The whole extraction and detection time, including cleaning of the syringe, was 210 s, and only 150 µL of *n*-hexanol and 4.1 mL of sample were required.

Thus, as can be seen in Table 5.1 a better analytical performance was achieved with the in-syringe MSA-DLLME method, since a lower reagents consumption was attained, together with a faster determination rate, due to the enhanced mixing efficiency provided by the agitation system avoiding the use of disperser solvent and speeding up the steps of the procedure. Moreover, syringe cleaning can be performed fast and

efficiently, due to the stirring. It should be pointed out, that no memory effect was observed and cleaning with water was sufficient to avoid cross-over contamination at the change of sample or standard solution.

Table 5.1 Comparison between the dispersive liquid-liquid microextraction and magnetic stirring- assisted dispersive liquid-liquid microextraction methodologies for aluminium determination.

	DLLME system	**MSA-DLLME system**
Reagent consumption per assay		
Lumogallion (ng)	930	310
n-hexanol (μL)	120	150
Ethanol (μL)	830	-
Sample (mL)	3.9	4.1
Determination rate (h^{-1})	13	17
Dynamic range (nmol/L)	1000	1100
LOD (nmol/L)	8.0	6.1
Repeatability (RSD %)	1.5	3.3
Samples	Seawater	Seawater Pond water

More detailed information is given below in two original research papers result of these investigations which were published in international journals with high impact factor.

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5.6. Original paper 1

Title: Fully-automated fluorimetric determination of aluminium in seawater by in-syringe dispersive liquid-liquid microextraction

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Fully-Automated Fluorimetric Determination of Aluminum in Seawater by In-Syringe Dispersive Liquid−Liquid Microextraction Using Lumogallion

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Supporting Information

ABSTRACT: A sensitive and selective automated in-syringe dispersive liquid−liquid microextraction (DLLME) method is presented. It was successfully applied to the determination of aluminum in coastal seawater samples. The complete analytical procedure including sampling, buffering, reaction of the analyte with fluorescence reagent lumogallion (LMG), extraction, phase separation, and quantification was completely automized and carried out within 4 min. DLLME was done using *n*-hexanol as an extracting solvent and ethanol as a dispersing solvent in a 1:8 v/v percent mixture. The Al−LMG complex was extracted by an organic solvent and separated from the aqueous phase within the syringe of an automated syringe pump. Two devices were specially developed for this work. These were (a) the fluorescence detector and accompanying flow cell for the organic phase enriched with the reaction product and (b) a heating device integrated into the holding coil to accelerate the slow reaction kinetics. The limits of detection (3σ) and quantification (10σ) were 8.0 ± 0.5 nmol L^{-1} and 26.7 ± 1.6 nmol L^{-1}, respectively. The relative standard deviation for eight replicate determinations of 200 nmol L^{-1} Al^{3+} was <1.5%. The calibration graph using the preconcentration system was linear up to 1000 nmol L^{-1} with a correlation coefficient of 0.999. Ambient concentrations of samples were quantifiable with found concentrations ranging from 43 to 142 nmol L^{-1}. Standard additions gave analyte recoveries from 97% to 113% proving the general applicability and adequateness of the analyzer system to real sample analysis.

Aluminum is the third element in order of abundance in both the pedosphere- and lithosphere-forming part of minerals, rocks, and clays.[1] Consequently, it is present in all natural waters. The Al^{3+} cation does not have any biological function, and Al^{3+} salts show a low solubility in water throughout, resulting in a low natural concentration level down to a few nanomoles per liter for open ocean seawater.

In oceanography, Al^{3+} concentration data can be used to trace atmospheric dust deposition and thus to estimate the entry and deposition of other essential elements such as silica or iron, which do have biological functions but are readily uptaken. Further Al^{3+} sources are river effluents and anthropogenic emissions.

Besides, there has been increased interest during the last several decades in the toxicity of Al^{3+} and its effect on plants,[2] aquatic ecosystems,[3,4] and humans.[5,6] Consequently, knowledge of Al^{3+} concentration levels in both biological and environmental media is of current interest. As a result, the development of novel, simple, robust, and transportable analytical instrumentation for fast, sensitive, and environmentally friendly methods is of high interest.

Lumogallion (LMG), 4-chloro-6-(2,4-dihydroxyphenylazo)-1-phenol-2-sulfonic acid, a tetradentate ligand that coordinates with Al^{3+} was first introduced as a selectivity fluorescence reagent for Al^{3+} complexation in the 1970s.[7] During the following years, the analysis of Al^{3+} with LMG was gaining widespread acceptance.[8−13] The Al−LMG complex offers excellent sensitivity with minimal interference. Consequently, it has been successfully used for the determination of Al^{3+}, even in complex and high-salt matrices such as body fluids[14] and seawater.[15] LMG further presents an important advantage over morin, another often-used fluorescence reagent for aluminum. It has lower hydrophilicity, which allows liquid−liquid extraction of the Al−LMG complex for analyte enrichment.[10]

Flow techniques (FT), divided in respect of their operation scheme, instrumentation, and flow pattern, have proven to be excellent tools for the automation of laboratory procedures. Outstanding advantages over batch-wise robotic automation are the performance of the complete procedure in a closed compartment (i.e., tubing manifold) and a self-cleaning process of this system by a continuous or semicontinuous flush with the

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carrier. This and a general gain in reproducibility and sample frequency are excellent conditions for analysis of trace concentrations of abundant elements such as aluminum. Unsurprisingly, the Al–LMG reaction has been automated using FT and successfully applied to field monitoring.[4,9]

Due to the low concentrations of Al^{3+} found in environmental water samples, analyte enrichment techniques have to generally be used such as liquid–liquid extraction (LLE)[16] or solid phase extraction (SPE).[17−19] While SPE is often praised for its avoidance of solvents and is less environmentally harmful than LLE, the quantity of crude required for cartridge fabrication is often forgotten.

Micro-LLE techniques overcome typical problems of SPE such as potential clogging, long extraction times, and higher costs while minimizing the environmental impact. Reported micro-LLE techniques used for the determination of Al^{3+} include single drop microextraction (SDME),[20] cloud point extraction (CPE),[21] and most recently, dispersive liquid–liquid microextraction (DLLME).[22,23]

The DLLME is a novel extraction technique, first reported in 2006[24], of rapidly increasing interest. Its simplicity and fastness are probably the most attractive benefits of this technique. It is based on the rapid injection of a solvent mixture into the aqueous sample by which one component of the solvent dissolves nearly instantaneously (i.e., the dispersion solvent) while the second component (i.e., the extraction solvent) remains and is disrupted into a cloud of fine droplets. The simultaneous enormous increase of the interaction surface with the sample enables efficient mass transfer of the analyte into the extraction solvent droplets. After collection of the droplets, generally done by centrifugation, the extraction solvent can be used for further analysis.

Since DLLME does not require solid supports for the extraction solvent such as hollow membranes or capillary tubes, its automation using FT is straightforward. It has been coupled to FT using one of the following operation modes: (1) Injection of the solvent mixture in a sample flow and collection of the extraction solvent droplets on a hydrophobic column with later elution.[25−27](2) Injection of the solvent mixture into a sample-filled reaction chamber with passive phase separation due to an extraction solvent density >1 g cm^{-3} (i.e., sedimentation of the droplets).[28] (3) Filling a fraction of a syringe pump with the solvent mixture and fast injection of the watery phase with passive phase separation due to an extraction solvent density <1 g cm^{-3} (i.e., floating of the droplets at the top of the syringe).[29−31]

The last operation mode has the advantage that it can be performed relatively fast since fewer steps are cleaning and no elution steps are required. Thus, this technique was used for the complete automation of DLLME-based determination of Al^{3+} in seawater.

EXPERIMENTAL SECTION

Reagents and Solutions. All solutions were of analytical-reagent grade and doubly distilled water provided by a Milli-Q Direct-8 purification system (resistivity >18 MΩ cm, Millipore Iberica S.A.U., Spain) was used throughout. All glassware and polyethylene were previously soaked in 10% (v/v) HNO_3 and rinsed with Milli-Q water prior to use. The aluminum stock solution of 13.5 mg L^{-1} was prepared by diluting a commercial 1000 mg L^{-1} $Al(NO_3)_3 \cdot 9H_2O$ atomic absorption standard (Scharlab, Barcelona, Spain) in 0.5 mol L^{-1} HNO_3. For calibration purposes, Al^{3+} standard working solutions were prepared by appropriate dilution of the stock solution with Milli-Q water or artificial seawater acidified with HCl to pH 3.

Reagent 1 was an ammonium acetate buffer of 2 mol L^{-1} (pH 5.1) prepared by adding 3.3 mL of glacial acetic acid to 10.8 g of ammonium acetate and then diluted to 100 mL. Reagent 2 was a LMG stock solution of 1.5 mmol L^{-1}, prepared by dissolving 103 mg of LMG in 200 mL of Milli-Q water. *n*-Hexanol was used as an extraction solvent with ethanol used as a dispersing solvent. The extractant solution was prepared by mixing *n*-hexanol with ethanol in a 1:8 volumetric ratio, if not otherwise indicated. A reference material of trace elements in wastewater (SPS-WW2 Batch 106, Spectrapure Standards AS, Oslo, Norway) was also analyzed for evaluation of the accuracy of the developed method, as recommended by the National Institute of Standards and Technology. For interference studies, a standard solution of 10 mmol L^{-1} NaF (Panreac, Barcelona, Spain) and a standard solution of 25 μmol L^{-1} of $Fe(NO_3)_3 \cdot 9H_2O$ (Scharlab, Barcelona, Spain) were used.

For masking of interfering fluoride and ferric ions, a Be^{2+} solution of 2.5 mmol L^{-1} was prepared by the dilution of a commercial beryllium nitrate solution 35% w/w in H_2O (Sigma Aldrich, Spain) and a solution of *o*-phenanthroline (Acros organics, Geel, Belgium) of 15 μmol L^{-1}. All reagent solutions were stored in polyethylene bottles at 4 °C in the dark.

Synthetic seawater was used for most optimization experiments and preparation of the calibration standards. It was prepared according to standard recipe[32] by dissolving in Milli-Q water the following reagents to the final concentrations given in mg L^{-1}: 20 ($SrCl_2 \cdot 6H_2O$), 30 (H_3BO_3), 100 (KBr), 700 (KCl), 1470 ($CaCl_2 \cdot 2H_2O$), 4000 (Na_2SO_4), 10780 ($MgCl_2 \cdot 6H_2O$), 23500 (NaCl), 20 ($Na_2SiO_3 \cdot 9H_2O$), and 200 ($NaHCO_3$).

Sample Collection and Preparation. Different coastal seawater samples were collected in polyethylene flasks from different beaches from Mallorca collected in March of 2012. A map of locations can be found in the Supporting Information. The required amount of 1 mol L^{-1} HCl to reach a final pH of 3 was added immediately (approximately 1 mL/L). The samples were measured in the proposed analyzer system without any other previous treatment but sedimentation of coarse particles.

Manifold Configuration. The sequential injection analysis (SIA) system used in this work is schematically illustrated in Figure 1. It comprised a 5000-step syringe pump (SP) from Crison Instruments SA (Alella, Barcelona, Spain), a rotary 8-port multiposition valve (MPV) purchased from Sciware SL (Palma de Mallorca, Spain), and a homemade fluorescence detector described in detail in Detection Cell and Equipment. All tubing connecting the different components of the flow system was of polytetrafluoroethylene (PTFE) with either 0.8 mm or 1.5 mm inner diameter (i.d.).

The SP was equipped with a 5 mL syringe (S1) from Hamilton Bonaduz (Bonaduz, Switzerland). A three-way solenoid head valve (V1) allowed the connection of S1 to either the central port of the MPV (position ON) or the detection flow cell and further to waste (position OFF). The MPV was used for the handling of solutions required for DLLME and the cleaning procedures. Lateral ports were connected to waste (position 1) and reservoirs of a blank standard (2), H_2O (3), a standard or sample (4), reagent 1 (5), reagent 2 (6), and the extraction solvent mixture (8). At port 7, a dilution chamber (DC) was realized with a 5 mL pipet tip. For real-sample measurements, a 45-position rotary autosam-

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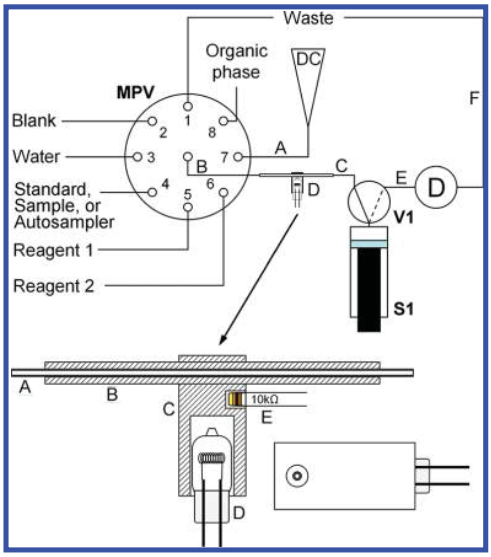

Figure 1. Sequential injection analysis (SIA) manifold used for DLLME of aluminum. Top: dilution chamber (DC), syringe pump (S1), MPV, 3-way solenoid valve (V1), connection tube A: 2 cm × 1.5 mm i.d., B and C: 15 cm × 0.8 mm i.d., D: heating device, E: 6 cm × 1.5 mm i.d., and F: 30 cm × 0.8 mm i.d. Bottom: A detailed representation of heating device as both a cross-sectional top view and side view. Elements include a glass capillary (A), a 10 cm × 4 mm o.d. × 2 mm i.d. brass tube (B), a 4 cm × 2 cm o.d. brass cylinder (C), a halogen light bulb (D), and an NTC resistance used as a sensor (E).

pler (AS) from Crison SA was connected to position 4 instead of the generally used supply of PTFE tube.

The central port of the MPV was connected to the head valve position ON of S1 by a holding coil (HC) consisting of two 10 cm PTFE tubes (0.8 mm i.d.) connected by a homemade heating device to accelerate the reaction. The heating device is shown in Figure 1. It consisted of a brass support for the tight insertion of a chemically inert 12 cm long glass capillary [1.5 mm i.d. × 2 mm outer diameter (o.d.)] used as a flow channel and a commercial halogen light bulb (12 V, 20 W) used as a heat source. Temperature control and bulb powering was done via a negative temperature coefficient (NTC) thermistor probe and a thermostat control circuit from CEBEK−Fadisel SL (Barcelona, Spain ref I-81), respectively. A temperature hysteresis of less than 1 K was achieved by increasing the value of the original feedback resistor on the operational amplifier of the thermostat circuit to 2 MΩ.

Detection Cell and Equipment. For fluorescence measurements, a specially made detection cell was used and is schematically shown in the Supporting Information. Shortly, it comprised a glass tube of 3 mm i.d. used as a detection cell flow channel. A bright green light-emitting diode (LED) of an emission wavelength of 500 nm powered by a mobile phone charger was used as an excitation light source and placed on top of a glass tube used as a detection flow cell (3, 3.5 cm × 5 mm o.d. × 3 mm i.d.) with a metal film bandpass interference filter between. The emission light was filtered by a long-path filter and detected with a photomultiplier tube. The photomultiplier tube (PMT) from Hamamatsu Phototonics K.K. (Hamamatsu, Japan, ref HS5784-04) was used for the detection of fluorescence emission and was mounted in a perpendicular position onto the glass tube.

An interference bandpass filter of 500 ± 10 nm (ref NT62-091) and a long-pass glass filter with a 580 nm cutoff wavelength (ref NT66-042) were purchased from Edmund Optics (Barrington, NJ, USA) and placed between the LED and glass tube and the glass tube and PMT, respectively. A control unit from Sciware SL was used for PMT supply and data readout. Spectra of the LED and filters used are given in the Supporting Information.

Software Control and Data Handling. The entire instrumentation used to perform the DLLME was controlled by AutoAnalysis 5.0 (Sciware SL) running on a commercial personal computer achieving complete automation of the analytical protocol (see Analytical Protocol and Flow Method).

The distinctive characteristic of this software is the possibility of using a single and versatile programming platform without further modification for whatever instrumentation, detection system, and data acquisition needed. Communication to the instrumentation assembly is based on individually loadable dynamic link libraries. The program is written in Delphi and C++ and allows the definition and execution of instruction protocols including the use of variables, loops, waiting steps, and operational procedures on a windows-based user interface. Detailed description can be found in the following papers.[33,34]

Analytical Protocol and Flow Method. The analytical procedure is given as Supporting Information. The instrument is initialized and the syringe is cleaned by the 5-fold aspiration of 0.2 mL of sample from the MPV and subsequent discharging to waste in V1 position OFF. Afterward, the sample followed by the buffer (R1) and LMG reagent (R2) were aspirated into the syringe and expulsed rapidly to the DC at MPV partition 7. For improved mixing, the content of the DC is aspirated again into S1 and then dispensed to the DC at a reduced flow rate to prolong the contact time between the liquid and the walls of the heating device, thus achieving efficient heating of the mixture and an enhanced reaction rate.

After a reaction time of 15 s, a small volume of the extraction solvent mixture is aspirated into S1 from the MPV position 8 followed by aspiration of the reaction mixture at the highest speed possible (30 mL min^{-1}). At this step, disruption of the organic solvent into small droplets of the extraction solvent is achieved at the rapid dissolution of the dispersing solvent into the aqueous sample.

After a waiting time for phase separation by the flotation and aggregation of the extraction solvent droplets at the top of the syringe, the syringe is emptied through the detection flow cell to the waste. This is done in two steps; the first step involves the slow passing of the organic phase containing the enriched reaction product for measurement with high time resolution, and the second step is the complete syringe evacuation at a flow rate of 15 mL min^{-1}.

■ RESULTS AND DISCUSSION

System Design and Preliminary Remarks. A simple system design was the aim. To minimize both dead volume and operation steps for saving time, the detection flow cell was mounted directly at the off position of V1. Syringe refilling was usually done in the head valve position; the off position was not required.

The detection flow cell was designed as a compromise between low dead volume in the range of the extraction solvent volume from former works (i.e., ca. 100 μL[29−31]) and detection sensitivity [i.e., maximum visible area for the PMT (65 mm^2)]. Using the described filters, a baseline of less than 2.5% of the working range was achieved. Due to the dependency of the

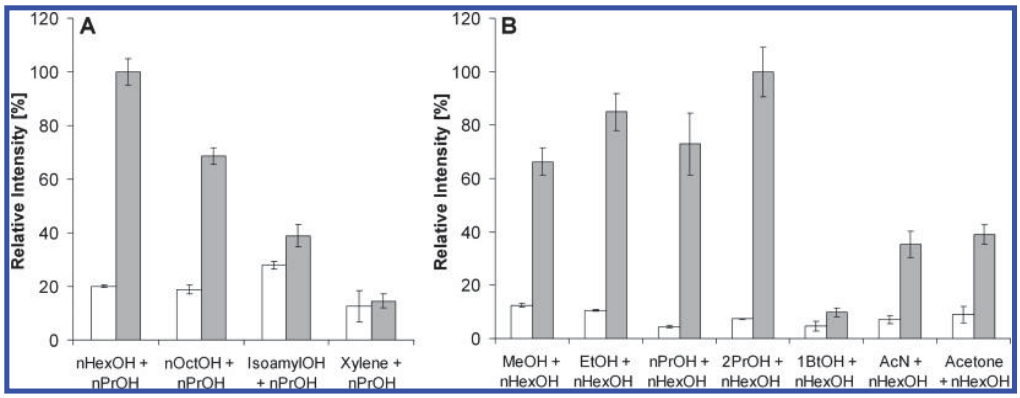

Figure 2. Study of the (A) extraction solvent using *n*-propanol as a dispersing solvent and (B) dispersing solvent using *n*-hexanol as an extraction solvent with the signal height obtained from the Milli-Q water blank samples and 500 nmol L^{-1} standard samples indicated. The standard deviation (n = 3) is indicated by error bars. Conditions and final concentrations: 15 μmol L^{-1} LMG, ammonium acetate 200 μmol L^{-1} (pH 5), 1 mL of solvent mixtures, a 1:10 ratio, and T = 20 °C.

sensitivity on both the PMT gain and the LED intensity, most results are given in the following as relative responses.

Excitation and emission spectra of the Al–LMG complex in *n*-hexanol as given elsewhere[10] have maxima at 500 and 580 nm, respectively. Thus, a LED with an emission spectrum of 500 nm was the optimal choice as the excitation light source. A higher sensitivity could have been achieved using an excitation bandpass filter of larger bandwidth and an emission filter of a shorter cutoff wavelength but to the cost of higher baseline level and noise.

Since the viscosity of *n*-hexanol used as an extraction solvent was sufficiently low, dilution after the extraction step as done in prior work was not required,[30] but measurement of the extraction solvent could be done directly after phase separation.

Sample heating was required due to the slow reaction kinetics between lumogallion and Al^{3+} as previously reported.[4] To avoid any contact with metals and due to the poor heat conductance of PTFE, a thin-walled glass capillary inserted in a heated brass support was used as a liquid guide. However, the heat transfer was inefficient at flow rates beyond 5 mL min^{-1} or a contact time of less than 5 s, respectively. Therefore, the heating step prior to the reaction was found to perform best at a flow rate of 5 mL min^{-1}.

All studies were done with both blank and standard solutions prepared with artificial seawater acidified to a pH of 3. The studies of the extraction and dispersion solvent type as well as the reaction time were performed with Milli-Q water.

Selection of Extraction and Dispersing Solvents. The main requirement of in-syringe DLLME is for an extraction solvent to be immiscible with water and of significantly lower density to allow efficient phase separation by floatation. Another characteristic of convenience is low viscosity and surface tension of the extraction solvent, since droplet fusion cannot be achieved by centrifugation but has to proceed spontaneously. Sticking to hydrophobic surfaces, such as the syringe piston head, is less pronounced for a less viscous solvent.

n-Hexanol, *n*-octanol, isoamyl alcohol, and a xylene isomeric mixture were tested as extraction solvents in 1:10 v/v percent mixtures with *n*-propanol. The results of repeated extractions of blank and standard solutions are shown in Figure 2A.

No significant extraction capacity of the complex was found for xylene. Since LMG is only moderately hydrophobic given the presence of hydroxyl groups and one sulfonate group of the molecule, the complex shows a higher affinity to slightly polar organic solvents than to nonpolar solvents.[10] Isoamyl alcohol gave an unacceptably high blank signal, on the order of the signal obtained with the standard solution. An additional problem is the high solubility of isoamyl alcohol in water.

n-Hexanol and *n*-octanol gave good extraction results with *n*-hexanol being superior to *n*-octanol with respect to both solvent characteristics discussed above and the standard signal, while blank signals did not differ significantly. Thus, *n*-hexanol was chosen for all further work.

In the next step, different dispersing solvents were tested in 1:10 v/v percent mixtures of *n*-hexanol with methanol, ethanol, *n*-propanol, 2-propanol, *n*-butanol, acetonitrile, and acetone. The results obtained from repeated extraction of Milli-Q water and standard samples are shown in Figure 2B. During the highly turbulent mixture of the organic and aqueous phases, the rapid dissolution of the dispersing solvent causes the disruption of the organic phase into small droplets of the extraction solvent, causing a nearly instantaneous multiplication of the effective extraction surface.

The relation between the standard and blank signals was found to decrease in the order *n*-propanol < 2-propanol < ethanol < methanol < acetonitrile < acetone < *n*-butanol. Hence, 2-propanol was chosen as the dispersing solvent for the study of reaction time and temperature. Although ethanol showed lower standard and higher blank signals, ethanol was chosen later since it did show much better performance when the aqueous-phase salinity was increased. This problem is explained in detail in Influence of Salinity.

Reaction Time and Temperature. It has been previously reported[4,8] that the chelating reaction of LMG with Al^{3+} is highly temperature and time dependent. Therefore, the effects of the temperature of the heating device and the reaction time in the dilution chamber on blank and standard signal heights were studied. Three different temperatures and four different reaction times were tested ranging from 20 to 42 °C and from 15 to 120 s, respectively, given as the final temperature of the reaction mixture in the dilution chamber. The results are given in the Supporting Information.

It was found that the blank signals did not show a significant dependency with time but increased slightly with temperature, which was probably due to a more efficient droplet formation at higher temperature because of a lower viscosity of the solvent. For the standard solution, strong dependency on the reaction

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temperature was found. While at 20 °C, the standard signal was about twice the blank signal and increasing slightly with time; a significant gain in sensitivity and a strong time dependency was found at a reaction temperature of 35 °C leading to stabilization for reaction times >60 s. For a reaction temperature of 42 °C, a further but less pronounced increase in sensitivity was found with no significant time dependency, indicating the steady state reaction conditions after only 15 s. Therefore, 15 s at 42 °C was chosen as the working conditions. Higher temperatures were not tested to avoid possible precipitation or incrustation in the heating device originating from seawater samples.

Influence of Salinity. Since this work was focused on the analysis of coastal seawater samples, the influence of sample salinity was studied using solutions prepared with NaCl in the range of 0−1 mol L^{-1}. A comparison between the use of ethanol and *n*-propanol as a dispersion solvent was performed with the results shown in the Figure 3.

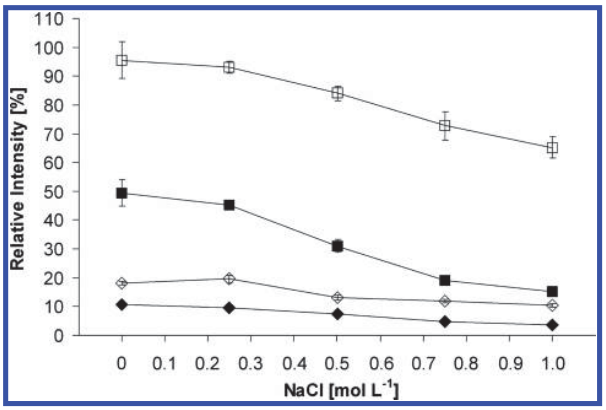

Figure 3. Influence of sample salinity on signal height of Milli-Q water blank sample (diamonds) and 250 nmol L^{-1} standard samples (squares) with the standard deviation (n = 3) indicated. Conditions as given in Figure 2, T = 42 °C, blank symbols for dispersion solvent *n*-propanol, and white symbols for dispersion solvent ethanol.

It was found that the signal decreased for both blank and standard solutions with increasing salinity with the major change observed from 0.25 mol L^{-1} to 0.75 mol L^{-1}. With ethanol used as the dispersion solvent, the signal decreased about 30% over the studied range, while *n*-propanol as the dispersion solvent made the signal decrease about 70%. The signal decrease was mainly related to the lower solubility of the dispersion solvent at a higher ionic strength of the aqueous phase, leading to less effective droplet formation and a higher content of dispersion solvent in the organic phase after extraction (i.e., dilution of the organic phase). Since the affinity of ethanol to water is much higher than it is for *n*-propanol, less dependency of the extraction efficiency on the salinity was found. For reasons of method robustness, ethanol was, therefore, chosen as the dispersion solvent, and artificial seawater was used for all further experiments.

Ratio and Volume of Extraction. Both the volumetric ratio of extraction and dispersing solvents and the absolute volume of the solvent mixture are known to affect the efficiency of DLLME.[22] Simultaneous optimization of both parameters was carried out following a 32 factorial design, with the levels of the volumetric ratio being 1:12, 1:8, and 1:6 and the levels of the solvent mixture volume being 0.6, 1.1, and 1.6 mL. Both the difference and the ratio of the standard and blank signals were used as response functions with an overall desirability ranging from 1 to 0.15 mL for the solvent mixture volume and 1 to 0.8 for the volumetric ratio (data not shown). The optima were found at a volumetric ratio of 1:8 and a volume of 0.95 mL. High robustness was achieved since a variation of 10% of both parameters did not change the overall desirability significantly.

Concentration of Lumogallion. The influence of the LMG concentration was studied in the range of 4−150 μmol L^{-1} given as the final concentration of the reaction mixture with the results given in Figure 4. It was found that the standard

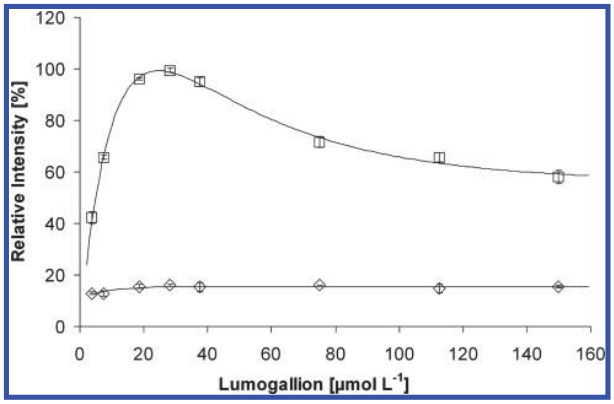

Figure 4. Influence of the concentration of LMG in the reaction mixture on the signal heights of artificial seawater blank samples (◇) and 250 nmol L^{-1} standard samples (□) with the standard deviations (n = 3) indicated. Conditions and final concentrations: 200 μmol L^{-1} buffer (pH 5.0), 0.95 mL of 1:8 ethanol/*n*-hexanol solvent mixture, and T = 42 °C.

signal increased drastically between 4 and 28 μmol L^{-1} and decreased for higher concentrations, stabilizing at a signal of half the maximum signal, found beyond 100 μmol L^{-1}. The bank signal maintained stable beyond 28 μmol L^{-1}.

While increasing the LMG concentration leads to enhanced reaction kinetics and thus an increase in complex formation, at higher LMG concentrations than 28 μmol L^{-1}, the capacity of extraction of the extraction solvent is overcome, indicated by the stabilization of the blank signal and decrease in the extraction efficiency of the Al−LMG complex.

pH and Concentration of Buffer Solution. The solubility of LMG, Al^{3+}, and its complex all depend on the pH of the aqueous phase. Since hydroxide formation of Al^{3+} is insignificant at a pH < 3.5, all samples and standard solutions were acidified to pH 3. However, this required the addition of an ammonium acetate buffer to guarantee optimal pH, which was reported to be pH 5.[9,10] Sample acidification of a pH < 3 was not done, so that the required amount of buffer could be minimized, and thus the volume of sample for the extraction procedure could be maximized. In order to avoid excessive dilution of the sample with the buffer addition, a buffer concentration of 5 mol L^{-1} was chosen.

The pH was studied in the range between 3.9 and 5.8. Sensitivity decreased significantly below pH 4.5 or above pH 5.5 with an optimum at pH 5.0 as expected, which was used further. Likewise, the final buffer concentration in the dilution chamber was studied in the range of 20−375 mmol L^{-1} acetate, with results shown in Figure 5. It was found that the sensitivity increased drastically from 20 to 150 mmol L^{-1} with a slow decrease beyond 150 mmol L^{-1}. Thus, 150 mmol L^{-1} (i.e., 300 μL) of a 2 mol L^{-1} ammonium acetate buffer was used further.

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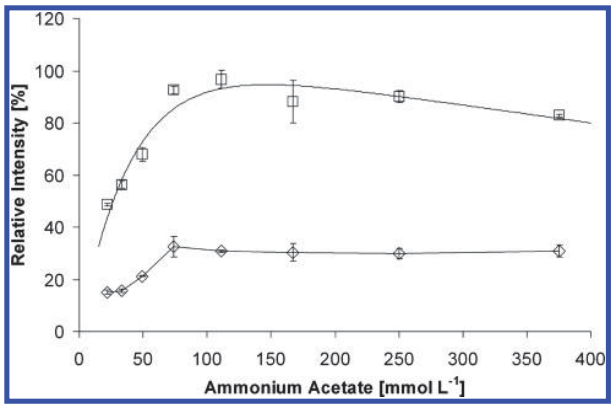

Figure 5. Influence of the ammonium acetate concentration in the final reaction mixture on the signal heights of artificial seawater blank samples (◇) and 250 nmol L^{-1} standard samples (□) with the standard deviations (n = 3) indicated. Conditions and final concentrations: 15 μmol L^{-1} LMG, 200 μmol L^{-1} buffer (pH 5.1), 0.95 mL of 1:8 ethanol/n-hexanol solvent mixture, and T = 42 °C.

At this time, the buffer solution and lumogallion were joined to one single reagent solution. It was proven that this did not affect the sensitivity or blank height of the method.

Chemical Interferences. Taking into account the results from prior works,[11,22] significant interferences of the given reaction were only found for the fluoride anion and ferric cation at typical concentrations in environmental water samples. The interference of fluoride is due to the fact that it forms a strong and poorly soluble complex with Al^{3+}, while Fe^{3+} competes with Al^{3+} over complex formation with LMG. Here, the interference of fluoride was most important due to its significant concentration in seawater in the range of 1.4 ppm.[35] The influence of fluoride can be reduced by masking it with a beryllium cation. The required Be^{2+} addition was studied by measuring a fluoride-free artificial seawater blank, standard, and standard with an addition of 2 ppm fluoride. From results shown in Figure 6, it became clear that an addition of 350 μmol L^{-1} Be^{2+} sufficiently compensates for about two-thirds of the fluoride interference but at the cost of a higher blank value, which increased slightly with a higher concentration of Be^{2+}.

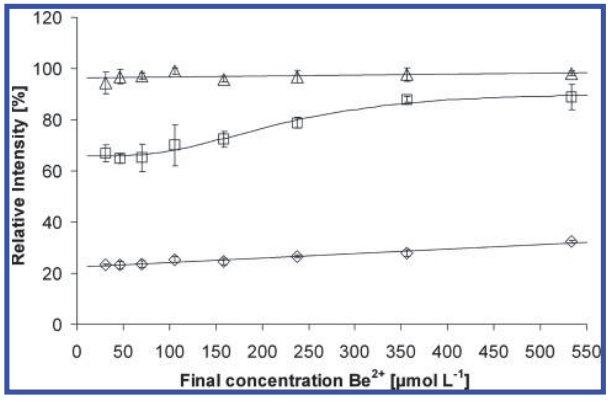

Figure 6. Influence of Be^{2+} on the signal height of artificial seawater blank samples (◇), 250 nmol L^{-1} standard samples (△), and 250 nmol L^{-1} standard samples with 2 ppm fluoride (□). The standard deviations (n = 3) are indicated. Conditions and final concentrations: 11 μmol L^{-1} LMG, 600 μmol L^{-1} ammonium acetate (pH 5.1), 0.95 mL of 1:8 ethanol/n-hexanol solvent mixture, and T = 42 °C.

The concentration of Fe^{3+} reported in coastal seawater ranges from 1 to 10 nmol L^{-1}.[36] In a former work, the addition of o-phenanthroline was used to suppress ferric cation interference in surface waters.[12] Signal heights obtained from artificial seawater without and with an addition of 11 nmol L^{-1} Fe^{3+} were compared; no significant difference was observed, and the addition of o-phenanthroline showed no significant effects. Thus, the reagent was modified only by the addition of beryllium to reach a final concentration of 350 μmol L^{-1} in the reaction mixture.

Method Performance. The proposed and optimized method was characterized by repeated calibrations proving a linear behavior of the signal height with increasing concentration up to 1000 nmol L^{-1}. A calibration example is given as Supporting Information. The calibration curve function, evaluated on 5 subsequent days, followed the equation: peak height = 1.76 ± 0.02 [L $nmol^{-1}$] · c [nmol L^{-1}] + 256 ± 8.7 (R^2= 0.999).

The system proved to be stable and robust over at least one week of operation, indicated by the low standard deviations of the blank and calibration curve slopes as well as the baseline stability, mainly influenced by fluctuations of the LED emission intensity and PMT gain. Limits of detection and quantification (LOD, LOQ) were calculated as the concentration yielding a peak height over the blank signal by a 3- and 10-fold standard deviation, respectively. An LOD of 8.0 ± 0.5 nmol L^{-1} and an LOQ of 26.7 ± 1.6 nmol L^{-1} were obtained, allowing determination of Al^{3+} in surface and coastal seawater samples. The LOD and LOQ were calculated according to the IUPAC recommendation.[37,38] The relative standard deviation (RSD) of repeated measurement was generally below 5% of the peak height. The RSD for eight replicate determinations of 200 nmol L^{-1} Al^{3+} was <1.5%.

In comparison with the former works employing lumogallion,[9,12] a lower sensitivity was achieved. The main reasons for this are most likely longer reaction times, higher incubation temperatures, more sensitive detection equipment, and additional purification of all used reagents in these studies.

The entire analytical procedure lasted about 262 s, enabling a measuring frequency of 13 h^{-1}. For each analysis, about 5 mL of sample including the required volume for syringe cleaning, 930 ng of LMG, and only 120 μL of n-hexanol and 830 μL of ethanol were required.

Validation and Real Sample Analysis. Coastal seawater samples were analyzed for evaluation of the applicability of the proposed analyzer system. For this, a rotary autosampler unit from Crison Instruments was connected to the MPV position 4 to analyze the samples rapidly one after another. The samples were acidified to pH 3 and not measured prior to at least 3 h to guarantee the total dissociation of the Al^{3+} without further treatment, sedimentation of particulate matter, and preservation at 4 °C until analysis. Sample spiking was done to evaluate the analyte recovery and matrix effects. The results are given in Table 1.

As can be seen, all samples showed concentrations in the range of 5-fold the LOQ, thus proving the suitability of the linear working range for coastal seawater samples. Standard addition gave analyte recoveries in the range from 97% to 113%, proving general applicability and adequateness of the analyzer system to real sample analysis. The results were in good agreement with the reported values for the surface seawater concentration of aluminum in the Mediterranean sea.[39] The trueness of the analytical method was proven by the

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Table 1. Results from Coastal Seawater Sample Analysis

sample	added (nmol L^{-1})	found[a] (nmol L^{-1})	recovery (%)	t_{exp}[b]
S-1	0	106 ± 12		
	100	219 ± 16	112.6	0.8
	200	318 ± 12	106.1	1.7
S-2	0	93 ± 16		
	100	197 ± 3	103.9	2.3
	200	297 ± 32	101.9	0.2
S-3	0	120 ± 20		
	100	232 ± 4	112.1	0.4
	200	322 ± 12	100.6	0.2
S-4	0	80 ± 9		
	100	192 ± 11	111.3	3.1
	200	274 ± 17	97.1	0.4
S-5	0	96 ± 12		
	100	200 ± 6	103.9	1.1
	200	292 ± 11	98.1	0.6
S-6	0	142 ± 11		
	100	245 ± 2	102.6	2.9
	200	346 ± 6	102.1	1.1
S-7	0	43 ± 6		
	100	146 ± 7	103.5	1.2
	200	239 ± 25	98.3	0.2

[a]Results are expressed as the mean value ± standard deviation ($n = 3$). [b]$t_{crit} = 4.3$.

student t test. The overall calculated values of t were ≤3.1 and given a critical value of 4.3 at the confidence level of 95%, the results did not show any significant differences from the expected concentration values.

To the best of our knowledge, no adequate reference material or commercial international seawater standards for aluminum are available. Thus, for testing the robustness and trueness of the proposed method, a dilution of the wastewater reference material SPS-WW2 was analyzed. The reference material was diluted 2000-fold to allow for the concentration to fit within the linear range of the method. The results are given in Table 2. *o*-Phenanthroline solution with a final concentration

Table 2. Results from the Measurement of Reference Material

sample	proposed method (μmol L^{-1})	certificated value (μmol L^{-1})	recovery (%)
SPS-WW2-B	254 ± 25	371 ± 2	68.6
SPS-WW2-A*	379 ± 15	371 ± 2	102.3

*To mask iron, 8 μmol L^{-1} *o*-phenanthroline was added.

of 8 μmol L^{-1} was added, as recommended elsewhere[10], to mask the considerable concentration of iron. The final concentration value calculated from the measured results was 379 ± 15 μmol L^{-1} and was compared using the student t-test to the given reference concentration value of 371 ± 2 μmol L^{-1}, with no significant difference found at a confidence level of 95%.

All calculations of the recovery percentages were made according to IUPAC.[40]

CONCLUSIONS

A novel method for the fully automated determination of aluminum in seawater using in-syringe DLLME of the Al–LMG complex and fluorimetric detection was developed. All implied chemical and physical parameters were thoroughly optimized, and the analyzer system was successfully applied to the determination of coastal seawater samples. The obtained analytical performance including limit of detection, reproducibility, repeatability, time of analysis, and data from the add-recovery test were well suited for field work application.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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5.6.1. Supplementary information - paper 1

Map S1. Maps of sampling locations. S1: Ses Illetes, 39º31'60N; 2º34'60E; S2: Platja de Palma, 39º33'47N; 2º38'26E; S3: Sant Elm, 39º34'37N; 2º21'15E; S4: Sa Rápita, 39º18'25N; 2º1'47E; S5: S'Arenal, 39º30'5N; 2º45'3E; S6: Platja de Muro, 39º47'15N; 3º7'47E; S7: Formentor, 39º55'41N; 3º8'6E

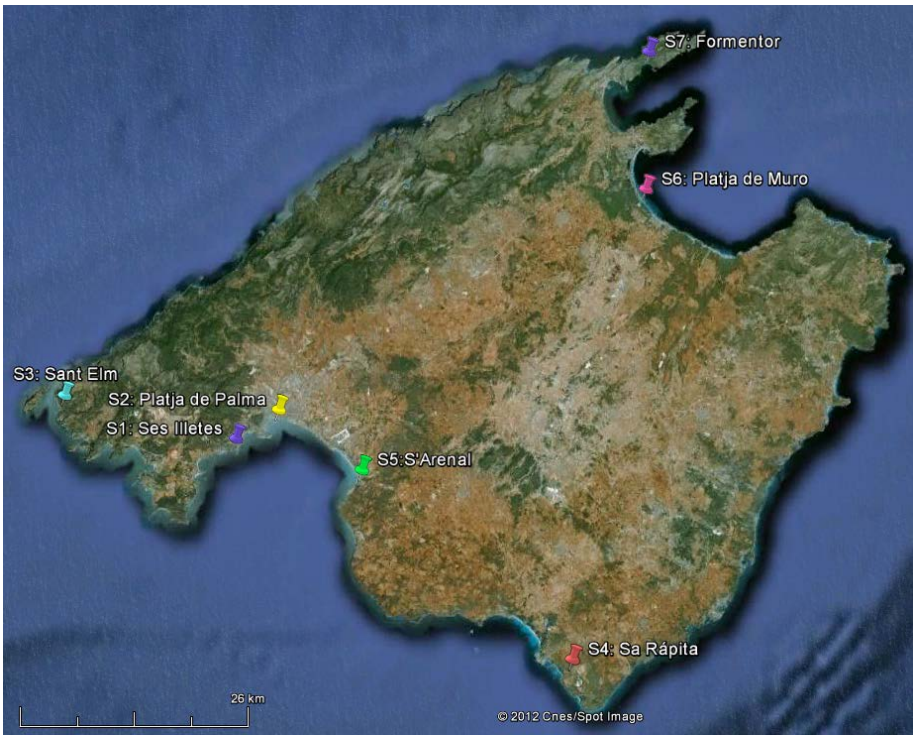


Figure S1. Schematic description of detection cell assembly: photomultiplier tube (1), long-path filter (2), detection flow cell (3), metal-film band-pass interference filter (4), green LED (5). Emission and transmission spectra are given in the left side diagram.

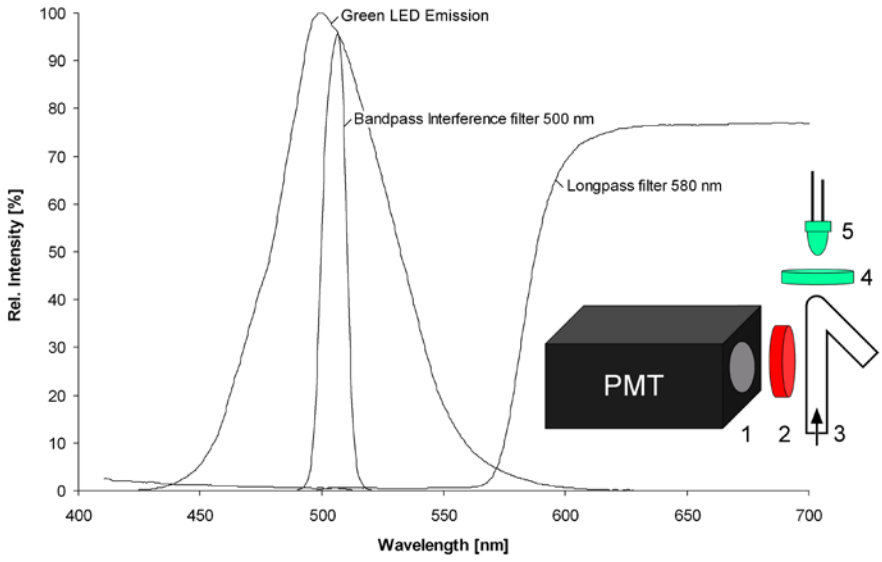


Table S3. Procedure for automated aluminium extraction

Instrument	Operation			Comment
Valve	Valve A move to position 2			Clean Syringe with Sample
Loop	START: Repeat 5 times			
SP	Pickup	0.200 mL	at 15 mL min^{-1} [On]	
SP	Dispense	0.200 mL	at 10 mL min^{-1} [Off]	
Loop	END			
Valve	Valve A move to 4			Aspiration of Sample
SP	Pickup	3.86 mL	at 12.5 mL min^{-1} [On]	
Wait	Wait 2 seconds			
Valve	Valve A move to position 5			Aspiration of reagent 1
SP	Pickup	100 µL	at 12.5 mL min^{-1} [On]	
Wait	Wait 1 seconds			
Valve	Valve A move to position 6			Aspiration of reagent 2
SP	Pickup	95 µL	at 12.5 mL min^{-1} [On]	
Wait	Wait 1 seconds			
Valve	Valve A move to position 7			Dispense to mixing chamber and re-aspiration for mixing
SP	Empty	Complete	at 12.5 mL min^{-1} [On]	
SP	Pickup	4.0 mL	at 12.5 mL min^{-1} [On]	
SP	Priming in dispense at 5.0 mL min^{-1} [On]			Slow dispense to mixing chamber for heating
SP	Heads: Off			
Wait	Wait 15 seconds			Reaction time
Valve	Valve A move to position 8			Aspirating of Organic Phase
SP	Pickup	0.95 mL	at 10 mL min^{-1} [On]	
Valve	Valve A move to position 7			Dispersive Liquid-Liquid Microextraction
SP	Pickup	4.0 mL	at 20 mL min^{-1} [On]	
Wait	Wait 5 seconds			
Wait	Wait 30 seconds			Phase separation
Detector	Measure every 0.2 s with 8 points to average			Discharge through detector to waste and measurement
SP	Dispense	0.800 mL	at 2.5 mL min^{-1} [Off]	
Detector	Stop measure			
SP	Empty	Complete	at 15 mL min^{-1} [Off]	

* SP: Syringe Pump

Figure S4. Study of effect of reaction time and temperature (42ºC black, 35ºC grey, and 20ºC white symbols). Signal height obtained for Milli-Q blank (diamonds) and 500 nmol·L^{-1} standard (squares) indicated. Standard deviation (n = 3) is indicated by error bars. Conditions and final concentrations: 15 µmol L^{-1} LMG, ammonium acetate 200 µmol·L^{-1}, pH 5, 1 mL of solvent mixtures, ratio of 1:10, T=20°C.

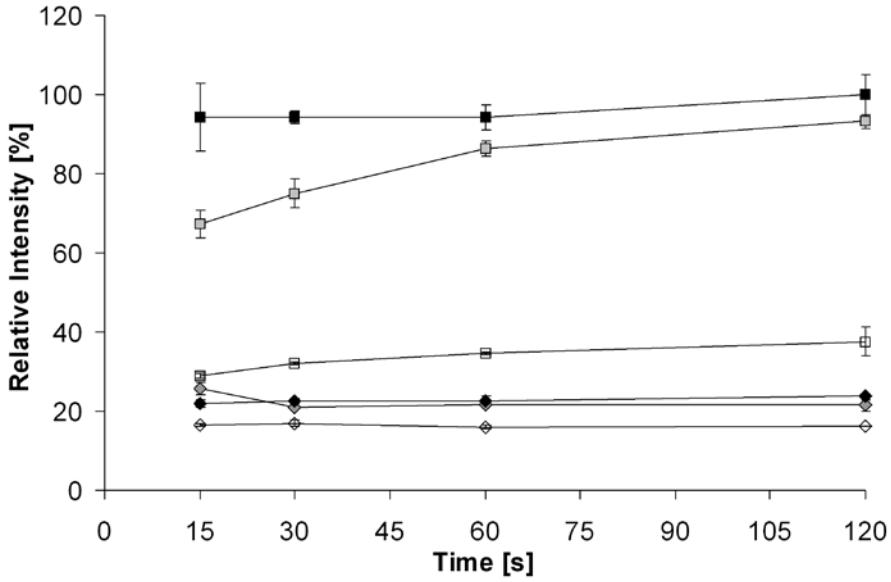
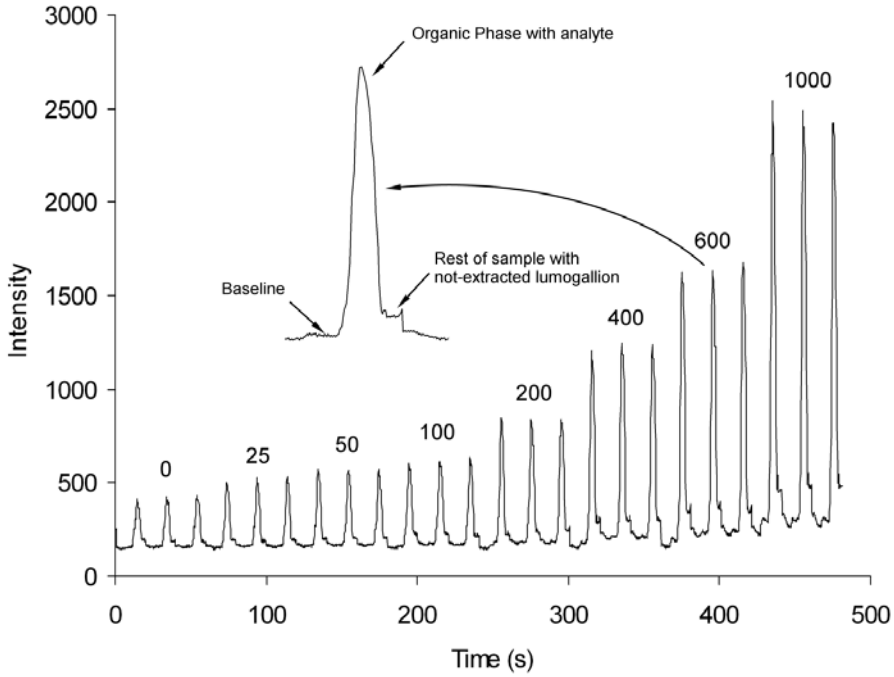


Figure 5S. Peak examples from standard calibration using spiked artificial seawater under optimized conditions. Concentrations are given in nanomol per liter.



5.7. Original paper 2

Title: In-syringe-stirring: a novel approach for magnetic stirring-assisted dispersive liquid-liquid microextraction

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In-syringe-stirring: A novel approach for magnetic stirring-assisted dispersive liquid–liquid microextraction

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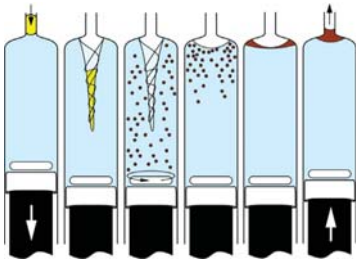
HIGHLIGHTS

- We propose a new automatic magnetic stirring assisted dispersive liquid–liquid microextraction.
- It allows the extraction of aluminum from seawater and freshwater samples within less than 4 min.
- The method was applicable to the natural samples.

GRAPHICAL ABSTRACT



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ABSTRACT

For the first time, the use of a magnetic stirrer within the syringe of an automated syringe pump and the resulting possible analytical applications are described. A simple instrumentation following roughly the one from sequential injection analyzer systems is used in combination with an adaptor, which is placed onto the barrel of a glass syringe. Swirling around the longitudinal axis of the syringe and holding two strong neodymium magnets, it causes a rotating magnetic field and serves as driver for a magnetic stirring bar placed inside of the syringe.

In a first study it was shown that this approach leads to a sealed but also automatically adaptable reaction vessel, the syringe, in which rapid and homogeneous mixing of sample with the required reagents within short time can be carried out.

In a second study in-a-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction (MSA-DLLME) was demonstrated by the application of the analyzer system to fluorimetric determination of aluminum in seawater samples using lumogallion.

A linear working range up to 1.1 μmol L^{-1} and a limit of detection of 6.1 nmol L^{-1} were found. An average recovery of 106.0% was achieved for coastal seawaters with a reproducibility of 4.4%. The procedure lasted 210 s including syringe cleaning and only 150 μL of hexanol and 4.1 mL of sample were required.

1. Introduction

Dispersive liquid–liquid microextraction (DLLME) has drawn a major interest from scientists from different analytical disciplines since its first description by Rezaee et al. in 2006 [1]. This is most likely due to the possibility of high extraction efficiencies and large enrichment factors with a simple and rapid procedure.

DLLME is based on the dispersion of the extraction solvent into fine droplets, which multiplies enormously its contact surface with the aqueous sample and by this, the extraction efficiency for the analyte of interest.

The original methodology requires a dispersion solvent as major component of the organic phase, which dissolves preferably in the aqueous phase at the rapid injection of the solvent mixture into the aqueous phase. Thus, a very small amount of extraction solvent is

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effectively dispersed into droplets, which afterwards are forced to coalesce by a centrifugation step. The organic phase is then transferred into the detector or used for chromatographic separation.

However, the dispersion solvent assisted DLLME has a few inconveniences. The additional solvent leads to increase waste production. The method requires additional optimization effort (dispersion solvent quantity and kind) and, the most important, the dispersion solvent increases the solubility of the analyte in the aqueous phase. Furthermore, the distribution of the dispersion solvent between both phases and by this, the final volume of the organic phase depends on the sample salinity.

Consequently, alternative DLLME methodologies have been developed, where extraction solvent dispersion is achieved by kinetic energy. Depending on the mode of achieving droplet formation or stabilization of the droplets in the aqueous phase, ultrasound-assisted DLLME [2], air-assisted DLLME [3], vortex-assisted DLLME [4], magnetic stirring-assisted DLLME (MSA-DLLME) [5], and surfactant assisted DLLME [6] can be distinguished among others. For details, the reader is referred to recent and extensive review articles on this topic [7–10]).

In spite of the high interest in the development and application of DLLME techniques, the potential of their automation using analytical flow techniques (FT) [11,12] such as Sequential Injection Analysis (SIA) [13–15] has been widely disregarded. Direct coupling with the intended detection technique, higher reproducibility, higher sample throughput, and automated cleaning of the extraction vessel are possible benefits of FT-based automation. These have been demonstrated successfully. However, only by three distinct automation modalities so far:

1. Extraction in flow by confluence of the aqueous sample and an organic solvent mixture with droplet collection on a hydrophobic material and subsequent elution to a detection flow cell requiring additional solvent [16–18].
2. Use of an extraction vessel as a batch approach of automation of the manual DLLME protocol. The solution handling is accomplished by two separate SIA systems [19].
3. In-syringe DLLME by aspiration of the organic solvent mixture followed by sample aspiration at very high flow rate that leads to DLLME. After floating and coalescing of the solvent droplets, the organic phase is expelled into the detection flow cell [20–24].

Up to date, there are hardly any works on FT-based automation of DLLME, in which the dispersion solvent was omitted leaving alone an automation approach of air-assisted DLLME [25]. To the best of our knowledge, the present work reports the first FT-based automation of MSA-DLLME. It is based on a novel approach using a magnetic stirring bar within the syringe pump of a SIA system. Hence, a sealed but adaptable reaction vessel is obtained, in which all solutions can be aspirated with high precision and mixed homogeneously and nearly instantaneous. If air and an extraction solvent lighter than water are used, vortex formation will allow the contact of the extraction solvent with the turning stirring bar and hereby, the dispersion of the solvent into fine droplets. Stopping the stirring allows then droplet floatation, coalescence, and expulsion of the extraction solvent into a detection flow cell.

The system was used for the extraction of aluminum (Al^{3+}) as lumogallion (LMG) complex from seawater samples. This also allowed a critical comparison with a similar application but based on in-syringe dispersion solvent-assisted DLLME, which was reported recently [23]. In both works, LMG was chosen as a very selective fluorescence reagent for aluminum [26]. In contrast to the often-used morin, the LMG-Al complex is extractable into moderately hydrophobic organic solvents. It further shows low interference from sample matrix or other cations and has therefore been successfully used in oceanographic research over about three decades [27–32].

Although aluminum is a non-essential element, its determination in seawater is of interest as concentration data allow the calculation of atmospheric deposition of dust particles on the ocean surface due to its presence in numerous minerals. Then, these calculations allow the estimation of the entry of essential trace nutrients such as iron originating from the dissolution of the dust particles and which are limitation factors for growth of algae.

Herein, in-syringe MSA-DLLME is presented. The improvement of existing analyzer systems for aluminum as well as the demonstration and application of a novel extraction technique was intended with the critical discussion of its shortcomings and potential benefits for future works.

2. Methods and materials

2.1. Reagents

All chemicals were of reagent grade for analysis and ultrapure water (resistivity >18 MΩ cm Millipore Iberica S.A.U., Madrid, Spain) was used throughout. All glassware and polyethylene bottles were previously soaked in 10% (v/v) HNO_3 and rinsed with ultrapure water prior to use. All working solutions were stored in polyethylene bottles at 4 °C in the dark when not used.

An aluminum stock solution of 13.5 mg L^{-1} was prepared by diluting a commercial 1000 mg L^{-1} $Al(NO_3)_3{\cdot}9H_2O$ atomic absorption standard (Scharlab, Barcelona, Spain) in 0.5 mol L^{-1} HNO_3. Synthetic seawater (SSW) prepared according to standard recipe as given elsewhere [33] was used for most optimization experiments and for standard preparation. To eliminate aluminum contamination of the SSW, the formed $Al(OH)_3$ at the slightly alkaline pH of the SSW (pH 8) was removed by filtration through a 0.45 μm membrane filter.

Acidification was done to avoid Al^{3+} hydrolysis and loss of Al^{3+} availability for the complex formation with LMG. Adjustment to a lower pH was impractical due to the later required adjustment to the optimal reaction pH of 5.0.

A reagent solution of 1.5 mmol L^{-1} lumogallion (4-chloro-6-(2,4-dihydroxyphenylazo)-1 phenol-2-sulfonic acid) and a buffer solution of 5 mol L^{-1} of ammonium acetate (NH_4Ac) buffer, adjusted with glacial acetic acid to pH 5.4, were prepared. For extraction, n-hexanol was used throughout.

For measurement of aluminum in seawater, the interference of fluoride anion has to be taken into consideration since aluminum fluoride formation competes with the formation of the LMG-complex. In a previous work, this interference was considerably reduced at the addition of beryllium. Therefore, 25 mmol L^{-1} of beryllium nitrate were added to the LMG reagent solution to yield a final concentration of 350 μmol L^{-1} Be^{2+}, which had been found to be the optimal value in our previous work [23].

A 1 mg L^{-1} rhodamine B solution was used for studies of in-syringe homogenization by stirred-assisted mixing.

2.2. Sample collection and preparation

Coastal seawater samples were collected at different bays of the island Mallorca to evaluate the methods applicability to surface seawater analysis. The samples were acidified to pH 3 at the time of collection. The samples were measured with the proposed analyzer system without any other previous treatment but allowing only the grouse particles to sediment. Likewise, two pond water samples were collected on different places on the Mallorca Island, acidified to pH 3, and measured under the same conditions. After acidification and before measurement, the samples were allowed

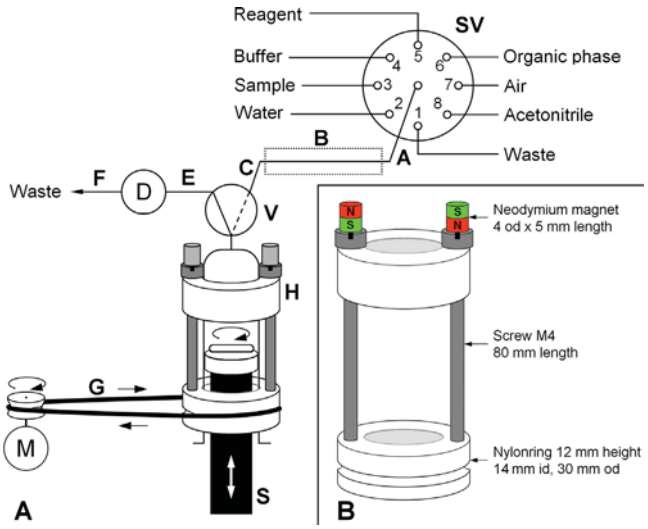

Fig. 1. (A) Analyzer manifold with selection valve (SV), syringe pump (S), solenoid 3-way head valve (V), detection flow cell (D), heating device integrated into the HC (B) and the magnetic stirring bar driver (H) placed onto the syringe barrel. A motor (M) is used to drive it via a rubber band (G). PTFE tubing (0.8 mm i.d.) of 15 cm (A, C), 10 cm (E), and 40 cm (F): 10 cm. (B) The magnetic stirring bar driver placed onto the syringe glass barrel shown in detail consisting of two nylon rings, two long iron screws and two neodymium magnets.

to stand for at least 3 h, both for sedimentation but also to ensure the dissolution of aluminum hydroxides.

2.3. Manifold configuration

The MSA-DLLME manifold is depicted in Fig. 1A with all tubing dimensions indicated. Polytetrafluoroethylene (PTFE) tubing of 0.8 mm inner diameter (id) was used for the entire manifold.

The computer controlled flow setup comprised a 5000-step multisyringe pump (Crison SL, Alella, Barcelona) and the rotary 8-port selection valve (SV, Crison SL, Alella, Barcelona) for liquid handling and distribution. The multisyringe pump was equipped with a sole glass syringe (S) of 5 mL purchased from Hamilton Bonaduz AG (Bonaduz, GR, Switzerland). A three-way solenoid head valve (V) on-top of the syringe enabled the connection to either the central port of the SV (position ON, activated) or to the detection cell and downstream located waste for quantification of the extracted analyte as well as for discharge during syringe cleaning (position OFF, deactivated).

Peripheral ports of SV were connected to reservoirs of waste (1), water (2), sample (3), buffer (4), lumogallion reagent (5), n-hexanol (6), air (7), and acetonitrile (8). Water and acetonitrile were used for cleaning of the detection flow cell or the syringe, which was routinely done daily.

The connection between the central port of the SV and the syringe head valve was done by a short holding coil (HC) consisting of two PTFE tubes of 15 cm in length holding a prior described heating device [23] in the middle. Heating was done to favor the slow reaction between LMG and Al^{3+}. Briefly, it consisted of a 12 cm long, 1.5 mm id glass tube inserted into a brass support, which was heated using a commercial halogen light bulb (12 V, 20 W). Temperature control with a hysteresis of <1 K was achieved using a control circuit from CEBEK Fadisel SL (Barcelona, Spain Ref. I-81).

2.4. Magnetic stirring bar driver

The entire analytical procedure was carried out in the syringe including sample mixing with reagents and extraction. To achieve homogeneous and rapid mixing without an additional mixing chamber as generally done [21,23,24], a magnetic Micro stirring bar (10 mm length, 3 mm diameter) was used within the syringe. This arrangement was done to the best of our knowledge for the very first time. The top position of the syringe piston was adjusted in such a way, that a gap less about 0.5 mm was left at emptying the syringe to avoid any damage.

To drive the stirring bar in the syringe, a commercial magnetic laboratory stirrer was impractical. Therefore, a rotating magnetic field was achieved by the use of a specially developed magnet driver, shown in Fig. 1B. Two rings made of nylon were used as bearings, which could be placed easily onto the syringe, with the bottom ring sliding on the flange of the syringe barrel. Two M4 steel screws of 80 mm in length were used as spacers and connection between both nylon rings. The so-obtained assembly could freely rotate around the syringe longitudinal axis.

By placing two neodymium magnets (5 mm × 4 mm o.d.) on top of the screws, the screws were magnetized and thus, a magnetic field in the syringe along its whole length was obtained. This magnetic force was sufficient to attract and, at turning the device to force the rotation of the stirring bar inside the syringe independently from the position of the syringe piston.

The bottom ring showed further a groove for the placement of a rubber band, which allowed propelling the driver with a low-cost DC motor. The DC motor was activated using a home-made relay and regulation circuit board by an auxiliary supply port of the multisyringe module. The circuit is given in Supplement material 1.

2.5. Detection equipment

A specially made detection cell was used for fluorescence measurements. A detailed description of the cell design can be found elsewhere [23]. Shortly, it comprised a glass tube of 3 mm id is used as detection cell flow channel. A bright green LED with an emission wavelength of 500 nm, powered by a mobile phone charger, was used as excitation light source and aligned with the glass tube. A photomultiplier tube (PMT) from Hamamatsu Phototonics K.K. (Hamamatsu, Japan, Ref.: HS5784-04) was used for detection of fluorescence emission and was mounted in perpendicular position onto the glass tube.

An interference band-pass filter of 500 ± 10 nm (Ref.: NT62-091) and a long-pass glass filter of 580 nm cut-off wavelength (Ref.: NT66-042) from Edmund Optics (Barrington, NJ, USA) were placed between LED and glass tube and glass tube and PMT, respectively. Spectra of the used LED and filters can be found in a previous work [23].

In addition, a polycarbonate collector lens (F 22 mm, Ø 22 mm) was placed onto the PMT to achieve higher sensitivity. A control unit from Sciware Systems, S.L. (Palma de Mallorca, Spain) was used for PMT supply and data readout. A gain of 18% was chosen for the PMT.

2.6. Software control and data handling

The software AutoAnalysis 5.0 (Sciware Systems, S.L., Palma de Mallorca, Spain) was used for operational control of the flow instrumentation as well as data acquisition from the detection equipment and data evaluation.

The program, written in Delphi and C++, allows the definition and execution of instruction protocols, including the use of variables, loops, waiting steps, and procedures on windows based user surface. Detailed descriptions of the software structure and features are given elsewhere [34].

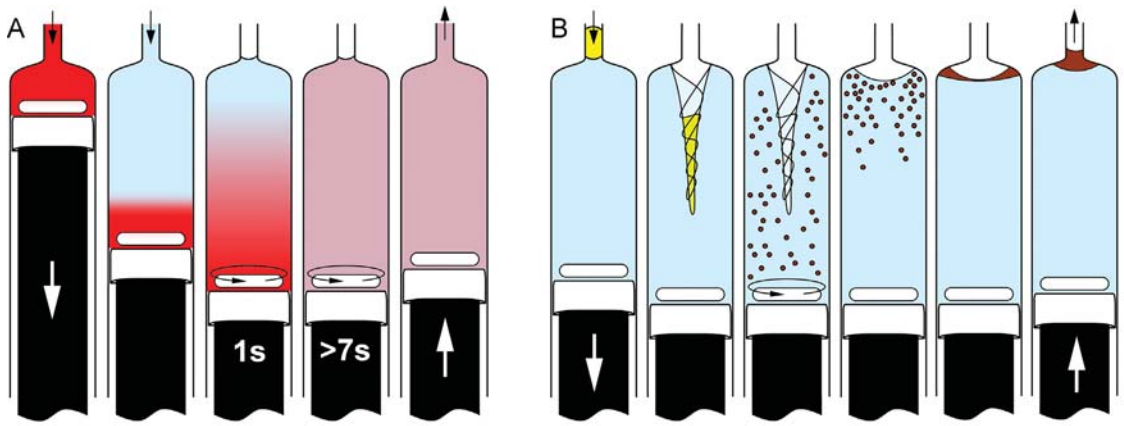

Fig. 2. Schemes of the both operation schemes tested in this work. (A) Mixing and homogenization of rhodamine B solution and water and (B) MSA-DLLME of LMG-Al complex with n-hexanol.

2.7. Analytical protocols and methods

The operation methods for testing in-syringe dilution and homogenization as well as MSA-DLLME are given schematically in Fig. 2. The operation method for MSA-DLLME is further available in Supplement material 2.

All analytical procedures required the cleaning of the syringe due to the unavoidable dead volume caused by the stirring bar. It was given by the syringe inner diameter of 10.5 mm and the height of the magnetic stirring bar of 3 mm minus its proper volumetric displacement of about 70 μL. However, the cleaning could be performed efficiently because the stirring allowed instantaneous homogenization of the dead volume in the syringe with the cleaning solution. So, three-fold aspiration of 0.8 mL of water (V in position ON, stirring activated) and discharge to waste (V in position OFF) was sufficient and allowed syringe cleaning in less than 30 s. In addition, procedures for cleaning of supply tubes on the SV and the detection cell were established.

In-syringe dilution and homogenization was studied using 1 mg L^{-1} rhodamine B solution and fluorimetric detection. Subsequently, 1 mL of rhodamine solution, 3 mL of ultrapure water, and 200 μL of air were aspirated into the syringe omitting stirring. Then, the syringe content was mixed by activation of the stirring for a defined time. Afterwards, the stirring was stopped and the syringe content was dispensed through the detection cell for the evaluation of the achieved homogenization.

MSA-DLLME was started by the aspiration of 240 μL buffer, 60 μL of LMG reagent, and 4.1 mL of sample into the syringe. Sample aspiration was done at a reduced flow rate of 4 mL min^{-1} to increase the heat transfer from the heating device to the sample and during repeated activation of the in-syringe stirring. Then, the stirring was deactivated and during a reaction time of 15 s, 150 μL of n-hexanol were aspirated into the HC to heating it up.

Afterwards, the stirring was started again and 400 μL of air were aspirated so that the n-hexanol in the HC and also part of the air could enter the syringe. The air allowed the formation of a vortex in the syringe (see Section 3.2.3). At contact of the organic phase with the stirring bar, it was dispersed into small droplets. The stirring was kept activated for 40 s to perform MSA-DLLME. The stirring speed was 2000 min^{-1}.

Afterwards, the stirring was stopped, which allowed the formed n-hexanol droplets to float and coalesce during 30 s at the brim of the concave liquid meniscus formed by the aqueous phase in the syringe. To improve droplet aggregation, the liquid surface was put in movement by a short movement of the piston (approx. 1 mm) by the instruction of complete filling just before the next step. The method was finalized by pushing the organic solvent, enriched with the LMG-Al complex, slowly through the detection cell to waste under continuous data evaluation. Finally, the remaining liquid was rapidly discharged from the syringe to waste.

3. Results and discussion

3.1. Study of in-syringe mixing

To evaluate the potential and characteristics of in-syringe magnetic stirring, the required mixing time for complete homogenization was studied for 1, 3, 7, 12, and 18 s. The experiment was done with an aqueous dye solution as well as with a dye solution prepared in a 20% (w/V) glycerol mixture. The later solution was used to simulate a sample of approximately twice the viscosity of water (about 12% higher after homogenization). The experiment was carried out in triplicate in order to evaluate the reproducibility of the mixing process. The operation scheme of this experiment is represented in Fig. 2A. The experimental conditions and the average measurements at each moment during syringe content expulsion and respective standard deviations are represented in Fig. 3.

For a mixing time of 1 s, the difference between using aqueous or 20% (w/V) glycerol dye solution was easily discernible while for 3 s, the behavior was similar. Besides, the mixing pattern after 3 s could be described as reproducible as the standard deviation decreased considerably. After 7 s of stirring, the dye gradient in the syringe was less than 5% and after 12 s, complete homogenization was achieved for both dye solutions.

It should be pointed out that later experiments showed that homogenization can be achieved even faster if a small volume of air is aspirated into the syringe permitting the formation of a vortex or evidently, if the aspiration is already initiated during aspiration of the solutions. In conclusion, in-syringe stirring permits homogeneous mixing of large volumes within a few seconds and, within the studied range, independently from the viscosity of the sample, which is in contrast to non-segmented FT.

3.2. In-syringe magnetic stirring assisted DLLME

3.2.1. Preliminary remarks

The main challenge was the study and optimization of the physical parameters related to the extraction. For these first experiments, the same reagent as optimized during a previous work was used, which seemed justified since it was based on the same chemical reaction and detection technique but on another different extraction technique [23].

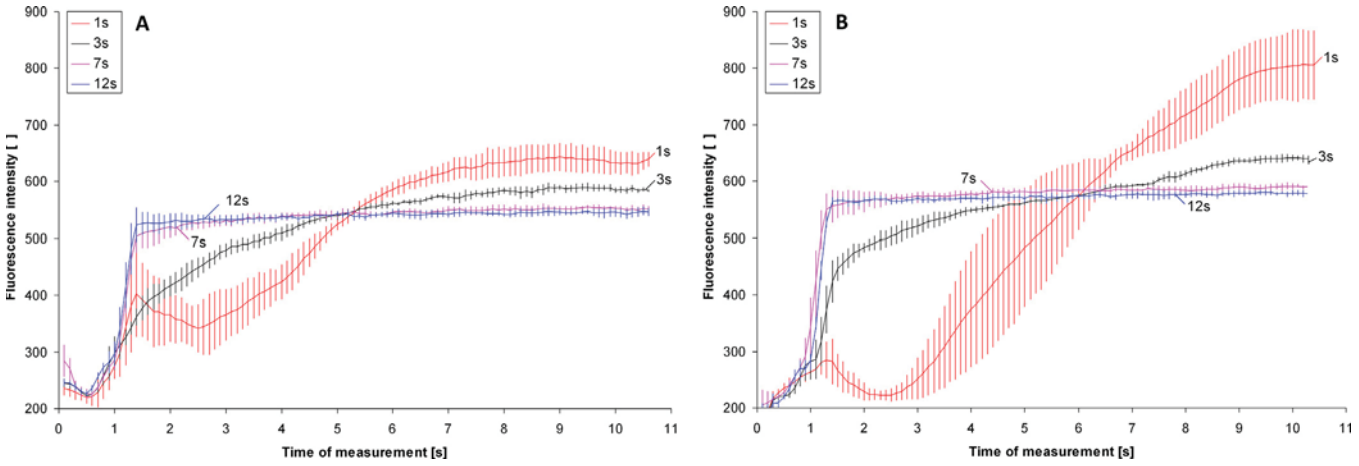

Fig. 3. Results from the study on the required time for homogenization of 1 mL of 1 mg L^{-1} rhodamine B solution with 3 mL of ultrapure water. (A) Dye solution prepared with water and (B) dye solution prepared with 20% (w/V) glycerol–water.

Likewise, n-hexanol was used for the extraction of the LMG-Al complex since it has fulfilled all requirements in the previous work. These were a lower density than water, a low solubility in water, and the best extraction capacity found of all tested solvents for the LMG-Al complex [23,29].

Therefore, only n-octanol was tested as alternative extraction solvent but discarded due to the observed sticking to the hydrophobic surfaces of the piston head and stirring bar, slow coalescence of the droplets after DLLME, and lower signal reproducibility. Other typical extraction solvents of lower polarity or lighter alcohols showed to be impractical.

3.2.2. Voltage of motor and stirring rotation speed

The stirring during the aspiration of n-hexanol into the syringe causes the disruption of the solvent into fine droplets and thus favors the mass transfer of the LMG-Al complex over a larger boundary surface increases the extraction efficiency. The influence of the voltage of the motor used for driving the stirring bar driver was studied in the range of 3.6 (ca. 1500 rpm) to 6.3 V (ca. 2600 rpm). The results are given in Supplement material 3A. The signal height escalated by factor of 2 between voltages of 4.6 V and 5.2 V. For lower voltages than 4.6 V or higher voltage than 5.2 V, the effect of voltage change on the signal height was little. A voltage of 5 V corresponded to a revolution speed of the stirrer in the syringe of about 2000 rpm. Only for higher voltages than 5 V, i.e. higher rotation speeds than 2000 rpm, a pronounced vortex was formed in the syringe and only then, the n-hexanol was drawn efficiently into the vortex and disrupted into small droplets. Therefore, a voltage of 5.5 V was chosen for all further experiments corresponding to a rotation speed of approximately 2300 rpm.

3.2.3. Volume of air

After the aspiration of n-hexanol, a volume of air was aspirated into the HC to aspirate the n-hexanol volume completely into the syringe. Vortex formation causing n-hexanol dispersion as well as droplet recombination after MSA-DLLME was improved if the volume of this air was larger than the HC inner volume (approximately 200 μL), i.e. when the head space of the syringe was partly filled with air.

The first observation is due to the fact that a vortex can be formed only on an open liquid surface in the syringe and the work required for the formation of the vortex is smaller if the surface is larger. Without air in the syringe, the extraction mode would mimic single drop extraction. However, preliminary experiments showed that this mode requires considerably longer extraction times to yield similar efficiencies, i.e. several minutes. This is in well accordance to the extraction times reported for non-automated single drop extractions [35]. The second observation is due to the formed meniscus of the aqueous phase and resulting prominence of the liquid surface on the syringe walls. Consequently, the floating n-hexanol droplets are forced to accumulate in this limited area achieving faster droplet coalescence.

The influence of the air volume on the signal height was studied in a range of 300–500 μL with conditions and results given in Supplement material 3B. It was observed, that the signal increased about 12% with a larger volume of air and also the reproducibility of measurement improved slightly from 6.6% to 2.3% RSD. On the other hand, a plus of air in the syringe also reduced the usable liquid volume in the syringe and by this the amount of sample, which could be used for the extraction procedure. Taking this into account, the effective signal increase is only 7%. Thus, a volume of 400 μL air was finally chosen.

3.2.4. Reaction and extraction times

The reaction time t_R and the extraction time t_E were optimized following a central composite experimental design. The studied range, experimental conditions, and results are given as Supplement material 4 and 5. Here, t_R is defined as the time between in-syringe mixing of sample and reagents and the addition of n-hexanol. The parameter t_E is defined as the stirring time in the presence of n-hexanol.

Two approaches were tested. In the first, n-hexanol and air are aspirated after t_R, i.e. the n-hexanol is only in short contact time with the heating device and enters the syringe nearly at ambient temperature. In the second, n-hexanol is aspirated during t_R into the HC while the air is aspirated after t_R, i.e. the solvent is allowed to heat up during t_R in the HC.

From the results it becomes clear that n-hexanol heating was highly favorable. This was due to the lower viscosity of hexanol at higher temperature and thus disruption into smaller droplets and improved extraction efficiency. Using the first approach, i.e. n-hexanol at ambient temperature, the best results were predicted for a minimal t_R, most likely because the mixture of sample and reagents in the syringe is at its maximal temperature, which decreases during t_R.

Using the second approach, i.e. pre-heated hexanol, signal heights were doubled. Longer t_R were advantageous due to proceeded reaction yield with a predicted maximum at 20 s. Simultaneously, shorter t_E were required than in the first approach where maximization of t_E was predicted as optimal in order to

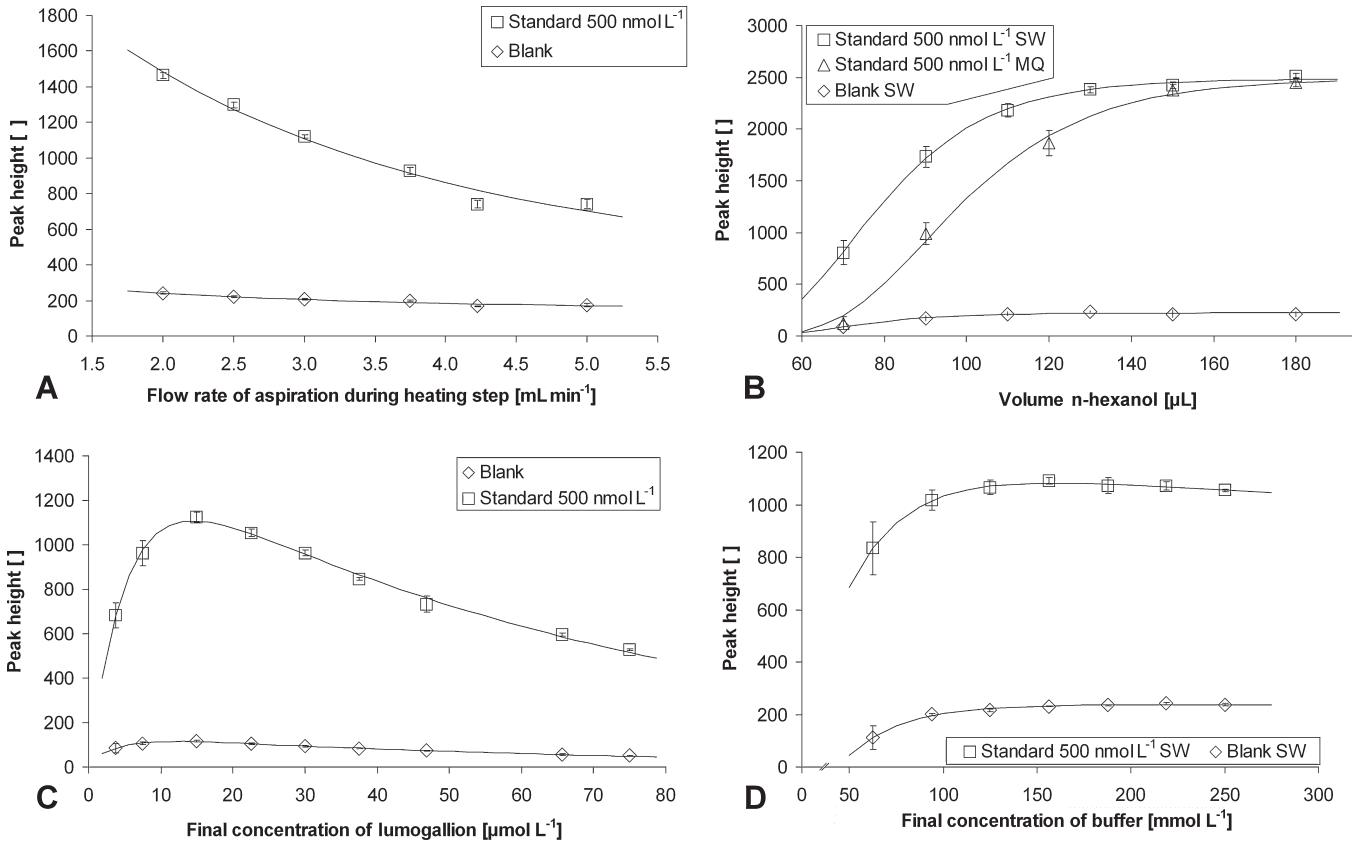

Fig. 4. Influence of aspiration flow rate (A), the volume of n-hexanol (B), the concentration of lumogallion (C), and NH4Ac buffer (D) in the final mixture. Conditions 500 nmol L^{-1} aluminum ultrapure standard (triangles), SSW standard (squares) and SSW blank (diamonds), motor voltage 5.5 V, 400 µL air, reaction temperature 45 °C, 15 s reaction time, 40 s extraction time. Further: (A) with 150 µL n-hexanol, 120 µL of 365 µmol L^{-1} LMG and 5 mol L^{-1} NH_4Ac pH 5.1. (B) as (A) with aspiration flow rate 4 mL min^{-1}, (C) as (B) with 150 µL n-hexanol, (D) as (C) with 60 µL 1.5 mmol L^{-1} LMG reagent.

compensate the shorter reaction time. On the base of the found results and optimization, 20 s of t_R and t_E of 40 s applying the second approach of heated n-hexanol were used further.

3.2.5. Volume of the extraction solvent

The volume of n-hexanol was studied in the range of 70–180 µL for blank SSW and for two 500 nmol L^{-1} Al^{3+} standards, one prepared with SSW, the other one prepared with ultrapure water. The results and experimental conditions are given in Fig. 4A.

It was found that for the SSW blank and SSW standard, the signals increased up to 130 µL reaching a stable level beyond. A similar behavior was observed for the signal obtained with the ultrapure standard. Here, the same signal level was reached but an about 20 µL larger volume of n-hexanol was required to achieve comparable results, being the result of the higher solubility of n-hexanol in ultrapure water compared to saltwater.

Visual inspection and the obtained results allowed the following conclusions. First, a small portion of the n-hexanol was not disrupted into droplets but remains floating at the surface explaining why the DLLME was less efficient at n-hexanol volumes lower than 100 µL. Second, the ionic strength of the sample did not affect the signal height as long as the solubility of n-hexanol in the sample [<6 µL mL^{-1}] is taken into account. The signal heights obtained with ultrapure and SSW standards with 150 µL of n-hexanol did not differ significantly (3% found), i.e. the dependency of the signal height on the ion strength of the sample solution was minimal. As consequence, a volume of 150 µL of n-hexanol was chosen for all further work. Increasing the n-hexanol volume beyond the studied range would probably have led to decreasing signal heights due to dilution of the extracted LMG-Al complex in the solvent.

3.2.6. Flow rate for sample aspiration

The flow rate for sample aspiration into the syringe was of high interest since it determined the contact time of the sample with the heating device integrated in the HC. A lower flow rate would favor a faster reaction rate and higher yield as reported elsewhere [23,28,29] but prolong the method execution time. A higher sample temperature could further improve the extraction efficiency due to lower viscosity, while the antagonistic effect of a higher solubility of n-hexanol and the LMG-Al complex in the aqueous phase has to be considered.

The effect of the flow rate on the signal height was studied in the range of 2–5 mL min^{-1}. The experimental conditions and results are represented in Fig. 4B. It was found that the signals of both SSW blank and standard followed an exponential decrease with higher flow rates. As a compromise between sensitivity and time of analysis, a flow rate of 4 mL min^{-1} was chosen.

At the adjusted temperature of the heating device of 65 °C, the sample entered the syringe at the chosen flow rate with about 45 °C. A higher heating device temperature was discarded to avoid bubble formation.

3.2.7. Concentration of lumogallion

After optimization of the physical parameters, the reagent composition, chosen from the previous work [23], was re-evaluated. First, the influence of LMG quantity in the aqueous phase before extraction was studied in the range of 3.8–75 µmol L^{-1} final concentration using a 500 nmol L^{-1} SSW standard and SSW blank. The results and experimental conditions are given in Fig. 4C.

It was observed that both the standard and blank signals increased rapidly up to a maximum at 15 µmol L^{-1} with a less

Table 1
Results from the analysis of costal seawater samples and natural water samples and with one spiked concentration. Conditions as in Fig. 4 D.

Type	Added concentration [nmol L^{-1}]	Signal ($n = 3$)	Found concentration [nmol L^{-1}]	Recovery
Seawater 1	0	504.2 ± 73.1	104.5	
	50	598.1 ± 20.2	153.1	97.3%
Seawater 2	0	1070.1 ± 51.5	398.8	
	100	1281.5 ± 105.1	508.7	109.9%
Seawater 3	0	496.0 ± 15.9	100.5	
	100	701.7 ± 27.3	207.4	106.8%
Seawater 4	0	378.2 ± 11.5	39.3	
	200	838.0 ± 39.1	278.2	119.4%
Seawater 5	0	475.3 ± 1.5	89.7	
	100	674.9 ± 8.7	193.4	103.7%
Pond water 1	0	581.1 ± 42.8	144.7	
	200	962.6 ± 59.2	342.9	99.1%
Pond water 2	0	400.3 ± 9.7	50.8	
	200	838.8 ± 39.4	262.9	106.1%

pronounced decrease for higher concentrations. This behavior was most likely due to the moderate solubility of the LMG-Al complex in water and thus retention of aluminum in the aqueous phase at high LMG concentration. Therefore, a LMG concentration of 15 μmol L^{-1} was chosen yielding the maximal signals.

3.2.8. Concentration of buffer

NH_4Ac buffer was used to adjust the optimal reaction pH and to increase the ionic strength of the aqueous phase to improve the extraction efficiency and to decrease its solubility for n-hexanol. A larger volume of buffer favors the method's robustness in respect of the pH and ionic strength of the proper sample. However, a larger volume of buffer also implies a smaller volume available for sample in the syringe and so, a lower possible preconcentration factor.

The effect of the final buffer concentration in the aqueous phase was studied in the range of 60–250 mmol L^{-1}. The NH_4Ac buffer solution was prepared highly concentrated (5 mol L^{-1}). At this concentration, the measurement of the pH value with a commercial pH meter is not reliable, so that the buffer was adjusted to pH 5.4, which yielded the reported optimal reaction pH value of 5.0 [23,29] at a 50-fold dilution with ultrapure water. Results and experimental conditions are given in Fig. 4D.

While the blank signal increased with the buffer concentration up to 150 mmol L^{-1} and remained stable beyond, a clear signal maximum was found at 150 mmol L^{-1} NH_4Ac for a 500 nmol L^{-1} acidified SSW standard. Therefore, this concentration was chosen as optimal.

3.2.9. Phase separation time

The time of phase separation by droplet flotation and aggregation was tested for 20, 30, and 40 s using a 500 nmol L^{-1} aluminum SSW standard. Average signal heights of 3 subsequent extractions yielded 1771 ± 82, 1915 ± 33, and 1949 ± 13 units for 20, 30, and 40 s, respectively. As expected, the signal height increased and the signal reproducibility improved with longer times but less pronounced from 30 to 40 s than from 20 to 30 s. To minimize the execution time, a phase separation time of 30 s was chosen for all further work.

3.2.10. Real sample analysis and analytical performance

Calibration was done with standards up from to 1.9 μmol L^{-1} and found to be linear up to at least 1.1 μmol L^{-1}. At 1.4 μmol L^{-1}, the deviation from the extrapolated calibration curve was still only about 6%. The calibration curve followed the function signal height = 1.925 [L $nmol^{-1}$] · c [nmol L^{-1}] + 302.6, $r^2 = 0.998$. The limit of detection and the limit of quantification were calculated from the triple and 10-fold standard deviation of the blank and the calibration curve slope yielding 6.1 nmol L^{-1} and 20.2 nmol L^{-1}, respectively.

The preconcentration factor was calculated from the used sample volume and the final volume of hexanol (ca. 125 μL) to be about 33. The extraction efficiency can be estimated to be higher than 95% since the baseline found for the aqueous phase after the extraction was negligible.

To evaluate the applicability of the proposed automated MSA-DLLME method, five coastal surface seawater samples and two pond-water samples were measured with the described analyzer system. All samples were further spiked with aluminum standards in a similar concentration range as the natural concentration. The same calibration prepared with synthetic seawater water was used for the evaluation of all samples. The results are summarized in Table 1.

The sample concentrations were all within the linear working range. An average recovery of 106.0% ± 7.3% was achieved, 102.6% ± 5.4% omitting seawater sample 4, for which the recovery values exceeded the acceptable range for unknown reason.

The method showed to be applicable to the determination of aluminum in various water samples. The positive deviation of most recovery values was most likely due to the fact that spiking was done directly before analysis without aging time. Since in this work, similar conditions of the chemical parameters have been applied as in our previous work, and the reaction is well-known to be highly selective, a study of single components as potential interferences was not repeated.

An average repeatability of 3.3% ($n = 4$) was found for calibrations standards while for real samples, the average value was slightly higher with 4.4% ($n = 4$). The entire analytical procedure including initial syringe cleaning took 210 s, allowing a measuring frequency of 17 h^{-1}. In contrast to MSA-DLLME based on manual operation [4], the proposed system achieved efficient extraction in 40 s instead of several minutes and under fully automated conditions. Thus, the analytical performance was adequate for the determination of aluminum in all tested matrices.

In comparison with a former work using the same reaction and instrumentation but based on dispersion solvent assisted DLLME [23], about 25% lower LOD and LOQ values, a 20% shorter time of analysis and an 8% higher sensitivity were achieved by simultaneous reduction of organic solvents from 950 μL to 150 μL. The repeatability and linear working range were comparable.

Another improvement over the former work was a higher robustness in respect of the sample salinity due to the omission of the dispersion solvent under the optimized conditions, i.e. a n-hexanol volume of 150 μL as discussed in Section 3.2.5. This was also demonstrated by the fact that the same calibration with standards prepared with SSW was adequate for both freshwater and seawater samples and the recovery values found both sample types were comparable.

Table 2
Comparison with prior methods for the determination aluminum using solvent emulsification or DLLME, respectively.

Extraction solvent [μL]	RSD [%]	LOD [ppb]	ULR [ppb]	Sample [mL]	Time [min]	Extraction	Detection	Ref.
600	1.7	0.05	–15	25	<10	IL-DLLME	FL	[40]
132	4.5	0.8	–250	20	>8	DLLME-SFO	ICP-OES	[44]
48	2.6–5.3	0.6–0.9	–1000	10	>11	US-DLLME	ICP-OES	[42]
75	3.2	1.7	n.g.	10	>15	USILDLLME	UV–vis	[43]
98	1.87	0.13	–1000	10	>10	US-DLLME	ICP-OES	[41]
950	<5	0.22	–27	3.9	4.4	In-syringe DSA-DLLME	FL	[23]
150	3.3–4.4	0.16	–33	4.1	3.5	In-syringe MSA-DLLME	FL	This work

Abbreviations: DSA, dispersion solvent assisted; DLLME, dispersive liquid–liquid mixroextraction; FL, fluorescence; IL, ionic-liquid based; IS-MSA, in-syringe magnetic stirring assisted; n.g., not given; SE, surfactant enhanced; SFO, solidification of organic drop; US, ultrasound assisted; ULR, upper linear working range limit; UV–vis, spectrophotometry.

These improvements are considered to be related to the possibility to perform the whole procedure including reagent and sample mixing in the syringe, i.e. faster, and due to the omission of dispersion solvent, which permitted the use of a larger volume of sample (4.1 mL), and a high and reliable extraction efficiency, which is not affected by the mixture of dispersion solvent and aqueous phase.

3.2.11. Comparison and outlook on further potential and applications

A comparison with prior reported methods based on emulsification of extraction solvent and sample or dispersive liquid–liquid microextraction for the determination of aluminum is given in Table 2. Apart from this and our former work [23], all other works reported manual procedures. The proposed work was found comparable or better in respect of most characteristics to the prior reported applications whereas the preconcentration factor and the linear working range were smaller. Nevertheless, the working range could be extended by in-syringe dilution of the sample with ultrapure water. In respect to the time of analysis, the presented work was clearly superior. However, it has to be taken into account that manual procedures allow treating several samples in parallel and consequently allow increasing the effective sample frequency.

The presented work is the first application of in-syringe MSA-DLLME and was done with the intention to demonstrate the potential of in-syringe stirring in comparison with dispersion solvent assisted DLLME [23]. The usefulness of in-syringe stirring when dealing with samples of distinct viscosities was further proven.

As a disadvantage, the dead volume produced by the stirring bar has to be addressed. However, syringe cleaning can be performed fast and, due to the stirring, very efficient. It should be pointed out, that no memory effect was observed and cleaning with water was sufficient to avoid cross-over of analyte at the change of sample or standard solution.

On the other side, the use of external extraction chambers elsewhere proposed [19] implies even more time for cleaning since the entire extraction chamber needs to be filled with the cleaning solution and then emptied. If only one pump is used, this takes an additional step to re-aspirate the cleaning solution from the extraction chamber before discharging it to waste. Using in-syringe extraction, the inner walls of the "extraction chamber" (the syringe) are wiped by the syringe piston and the required volume to clean is reduced to the small dead volume, so that not the entire syringe has to be cleaned.

Temperature turned out to be one of the most important parameters of this work due to its influence on both the liquid viscosity and the reaction rate. A more efficient heating device could therefore improve the achieved performance. Another improvement might be possible by fluorimetric measurement of the organic phase directly in the syringe following the recently proposed methodology of Lab-In-A-Syringe [22]. Such combination could also enable in-syringe titrations with the interesting characteristic that the titration vessel can be adapted in volume without the interference of air.

The proposed instrumentation could further be used to carry out classical analytical protocols, i.e. the step-wise addition and mixing of small volumes of reagents to a large volume of sample such as done in "batch automation" [36–38]. Finally, the proposed system could be coupled to liquid or gas chromatography to carry out sample clean-up and analyte pre-concentration.

In comparison with former in-syringe automation in syringe extraction but posterior derivatization in a reaction vial [39]. Here, both, the reaction and the extraction were carried out in-syringe, since the stirring action enabled complete mixing of all solutions. Nevertheless, fully automation could only be achieved, if standard preparation and sample provision using an autosampler would be enabled.

4. Conclusions

A stirring bar placed into the syringe of a computer controlled syringe pump was used for the first time for magnetic stirring-assisted dispersive liquid–liquid microextraction (DLLME). The optimized method enabled efficient DLLME within a comparably short time and is based on the disruption of the extraction solvent by the kinetic energy of the swirling stirring bar. Better or similar analytical performance than in previous works based on DLLME was achieved and the method's applicability to the determination of aluminum in surface seawater and freshwater samples was proven. Dependency of the analytical performance on the sample salinity and viscosity was demonstrated to be widely overcome. In-syringe stirring can enable novel protocols for sample preparation, analyte pre-concentration, and complex analytical applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.05.049.

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5.7.1. Supplementary information - paper 2

Figure S1. Voltage regulator for the stirring DC motor

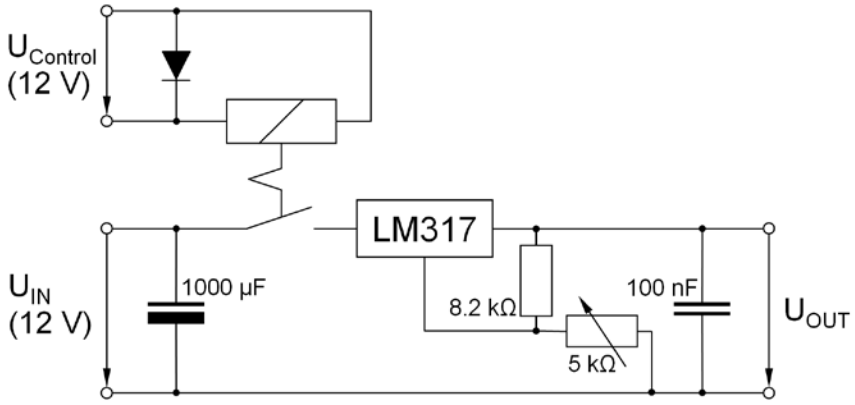


Table S2. Procedure for in-syringe stirring assisted dispersive liquid-liquid microextraction of aluminium

No	Instrument*	Operation*			Comment
1	SV	Valve A move to position 3			
2	Loop	START: Repeat 3 times			
3	S	Pickup	0.800 mL	at 15 mL min^{-1} V [On] after 0.400 mL activate agitation	Clean syringe with sample
4	S	Dispense	0.800 mL	at 10 mL min^{-1} V [Off] without agitation	
5	Loop	END			
6	SV	Valve A move to 4			
7	S	Pickup	240 µL	at 7.5 mL min^{-1} V [On]	Aspiration of buffer
8	Wait	Wait 2 seconds			
9	SV	Valve A move to position 5			
10	S	Pickup	60 µL	at 7.5 mL min^{-1} V [On]	Aspiration of reagent
11	Wait	Wait 1 seconds			
12	SV	Valve A move to 3			
13	S	Pickup	4.1 mL	at 4 mL min^{-1} V [On] and activate and deactivate agitation each 0.200 mL	Aspiration of sample
14	Wait	Wait 2 seconds			
15	S	Deactivate agitation			
16	SV	Valve A move to position 6			Aspirating of HexOH
17	S	Pickup	0.150 mL	at 2.5 mL min^{-1} V [On]	into the holding coil
18	SV	Wait 1 seconds			for heating up
18	Wait	Wait 2 seconds			Reaction time
19	SV	Valve A move to position 7			
20	S	Pickup	0.400 mL	at 2.5 mL min^{-1} V [On] and activate agitation	Aspirating of air
21	Wait	Wait 40 seconds			MSA-DLLME
22	S	Deactivate agitation			Phase separation
23	Wait	Wait 30 seconds			and droplet
24	S	Fill	Complete	at 15 mL min^{-1} V [Off]	aggregation
25	D	Measure every 0.2 s with 8 points to average			Discharge through
26	S	Dispense	0.750 mL	at 3 mL min^{-1} V [Off]	detector to waste
27	D	Stop measure			and measurement
28	SP	Empty	Complete	at 15 mL min^{-1} V [Off]	Empty syringe rapidly to waste

* D: Detector, S: Syringe Pump, SV: Selection valve, V: Solenoid syringe head valve

Table S-4. Data of experimental design results for reaction time and extraction time for both cold and warmed-up n-hexanol.

Reaction time [s]	Extraction time [s]	Sum time [s]	Mean ± SD Cold hexanol	Mean ± SD Hot hexanol
3	40	43	683 ± 62	1746 ± 114
5	15	20	404 ± 9	1616 ± 22
5	65	70	1045 ± 135	1818 ± 18
15	5	20	266 ± 12	1429 ± 35
15	40	55	802 ± 122	1860 ± 79
15	40	55	757 ± 71	1843 ± 39
15	75	90	851 ± 102	1835 ± 45
25	15	40	415 ± 43	1809 ± 96
25	65	90	785 ± 22	1814 ± 128
30	40	70	591 ± 26	1827 ± 86

* Conditions: 500 nmol L^{-1} aluminium synthetic seawater standard, n = 3

Figure S5. Representation of experimental design results for reaction time and extraction time for both cold and warmed-up n-hexanol. Conditions: Motor voltage 5.5 V, 150 µL HexOH, 4.2 mL SSW with 500 nmol L^{-1} Al^{3+}, 120 µL of 365 µmol L^{-1} LMG and 5 mol L^{-1} NH_4Ac pH 5.1, reaction temperature 45°C.

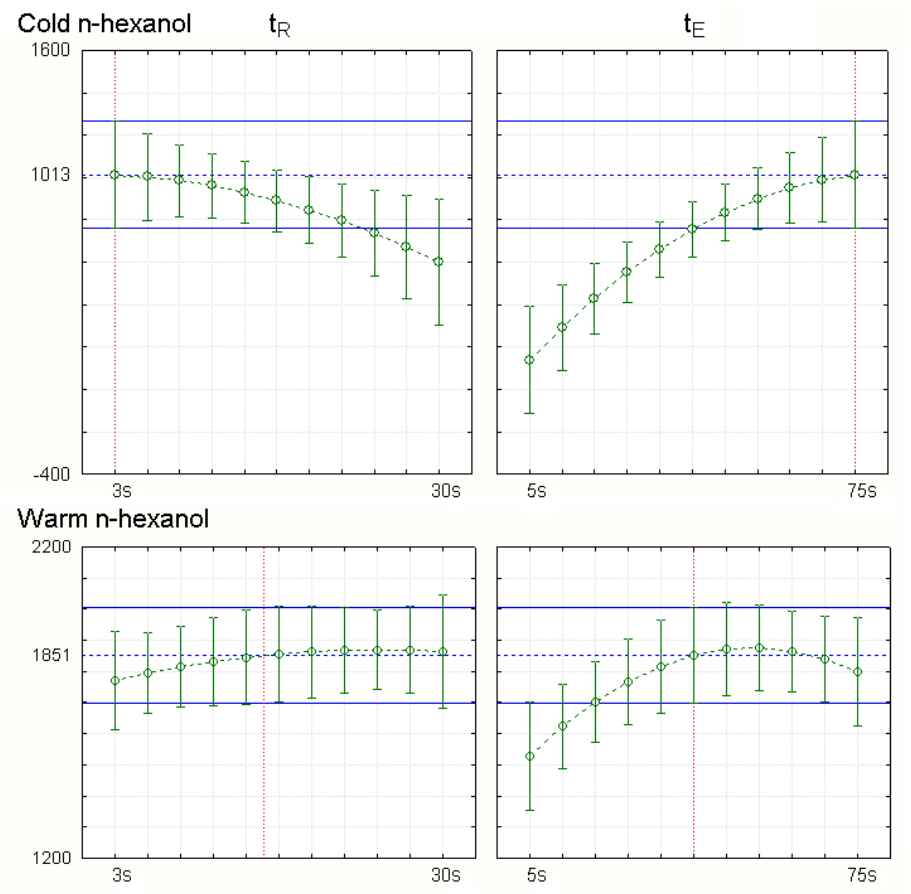
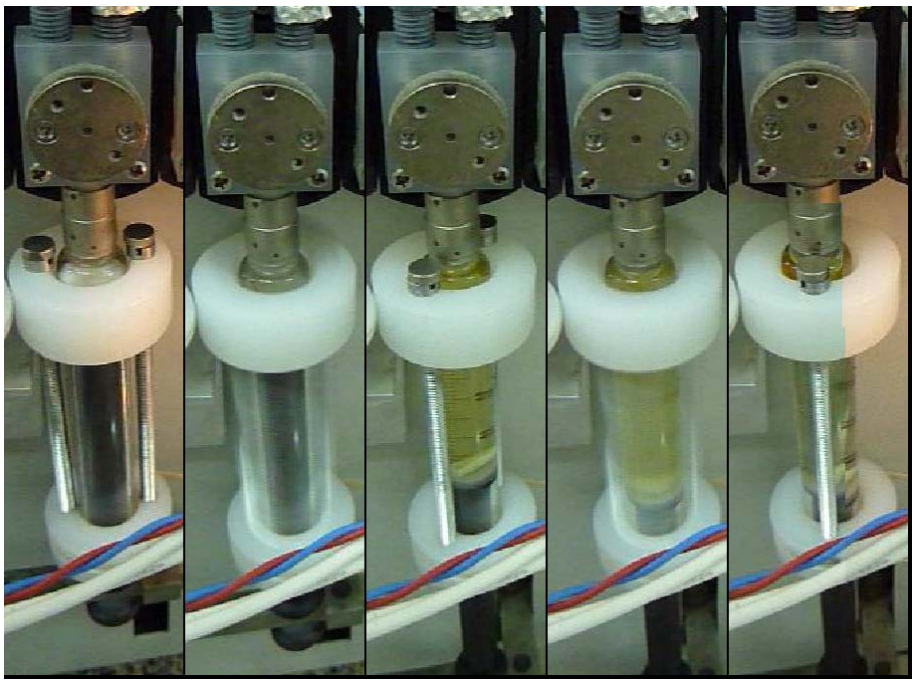


Figure S6. Photo documentation of the different stages of the in-syringe magnetic stirring assisted DLLME procedure. For better visualization of the organic phase, a 10 µmol L^{-1} aluminium standard was used. Numbering from left to right 1: Start of procedure with stirring bar visible, 2: Stirring during cleaning, 3: Reaction mixture in the syringe with syringe bar visible, 4: Stirring for formation of hexanol droplets for dispersive liquid-liquid microextraction, 5: Phase separation with visible accumulation of the hexanol droplets as enriched organic phase at the top of the syringe.



CHAPTER 6

IN-SYRINGE DLLME METHODS FOR SURFACTANTS DETERMINATION

6. In-syringe dispersive liquid-liquid microextraction methods for surfactants determination

6.1. General remarks about surfactants

Surface Active Agents (SAAs), also denoted as surfactants, are a broad group of chemicals that play a major role in a great variety of fields [1]. SAAs have a typical molecular constitution essentially linear and asymmetric, containing a hydrophobic long-chain (tail) and a hydrophilic polar group (head) in their structures. The hydrophobic part is an aliphatic, straight or branched chain, containing in general between 10 and 20 carbons. The hydrophilic part is the responsible for their water solubility, being a polar acidic group such as sulfate, sulfonate or carboxylate, or basic group such as amine, quaternary ammonium salt, pyridinium ion and nonionic polar group.

In addition, owing to the amphiphilic structure of SAAs, they have specific physicochemical properties like ability to form micelles, adsorption at phase boundaries and reduction of the surface tension. SAAs are usually classified according to the charge of their ionic group. Four types of surfactants are attained following this criterion: anionic surfactants (AS), cationic surfactants (CS), nonionic surfactants and amphoteric surfactants [1] as can be seen in Table 6.1. Their main characteristics are:

- AS are characterized by having a negatively charged hydrophilic group. AS constitute the most important group in terms of consumption on a worldwide basis [2].
- CS have a positively charged basic hydrophilic group.
- Non-ionic surfactants have a no ionized hydrophilic group. These are capable of forming salts and are characterized by usually having ethylene oxide units.
- Amphoteric surfactants are those simultaneously having acidic and basic groups. They can behave as anionic and cationic surfactants, depending on pH.

Table 6.1 Classification of surfactants

Category	Typical example
Anionic surfactants	Soaps Alkylbenzensulfonates Alkysulfonates α-Olefin sulfonates Alkil sulphates Alkyl ether sulphates
Cationic surfactants	Alkyltrimethylammonium salts Tetraalkylammonium salts Alkyldimethylbenzylammonium salts Alkylpyridinium salts.
Nonionic surfactants	Alcohol polyethoxylates Alkylphenol ethoxylates
Amphoteric surfactants	Alkil betaines Alkil sulfo betaines

Nowadays, SAAs consumption has exponentially increased, since these are applied in formulation of agents used in the industry, household and personal-care products, due to their specific properties, e.g. washing, wetting, emulsifying and dispersing [3]. The total amount of surfactants (without soaps) consumed in Western Europe in 2010 was 2.94 Mt, 1.39 Mt of which were non-ionic, 1.19 Mt were anionic, 0.25 Mt were cationic and 0.09 Mt were amphoteric, according statistics published by the European Committee of Surfactants and their Organic Intermediates (CESIO) [4].

The nature of their uses, and the fact that SAAs are water-soluble, results in their release into the environment being able to harm both humans and aquatic environment [5]. For example SAAs can affect humans, e.g. irritate eyes and skin and can also cause dermal necrosis and hypotension. Regarding aquatic organisms, these can inhibit biological activity. Depending on their physicochemical and biological properties these chemicals may eventually reach different environmental compartments like rivers, sediments and soils. In Figure 6.1 is represented the distribution and mobility of surfactants in the environment.

Although they are quite biodegradable [6] and the efficiency of waste water treatment plants (WWTPs) to remove them is generally high, surfactants still remain in various aquatic ecosystems where they are subject to a variety of physical and chemical changes. SAAs can be adsorbed on the surface of solid particles or in water vapor droplets, which leads to their occurrence in the atmosphere in aerosol form. In addition, the amphiphilic properties of surfactants and wet deposition facilitate the presence of

these compounds in wet and dry atmospheric precipitation [7]. Moreover, the volatility of some surfactants enables them to evaporate into the atmospheric air. SAAs are then transported with the air and eventually deposited and bioaccumulated in organisms [8].

Thus, contamination of the environment by surfactants arising from their widespread domestic and industrial use is of public concern. Intending to prevent water pollution, governmental agencies involved with control of water quality have established normative indicating the maximum allowed concentrations. In this sense, it has become necessary to monitor their levels and impact on the environment in order to assure that the surfactants concentration does not surpass the tolerated limits [9]. Therefore, the development of efficient methods for surfactants determination is of great relevance. In this thesis we have focused in the development of automated methods for anionic and cationic surfactants.

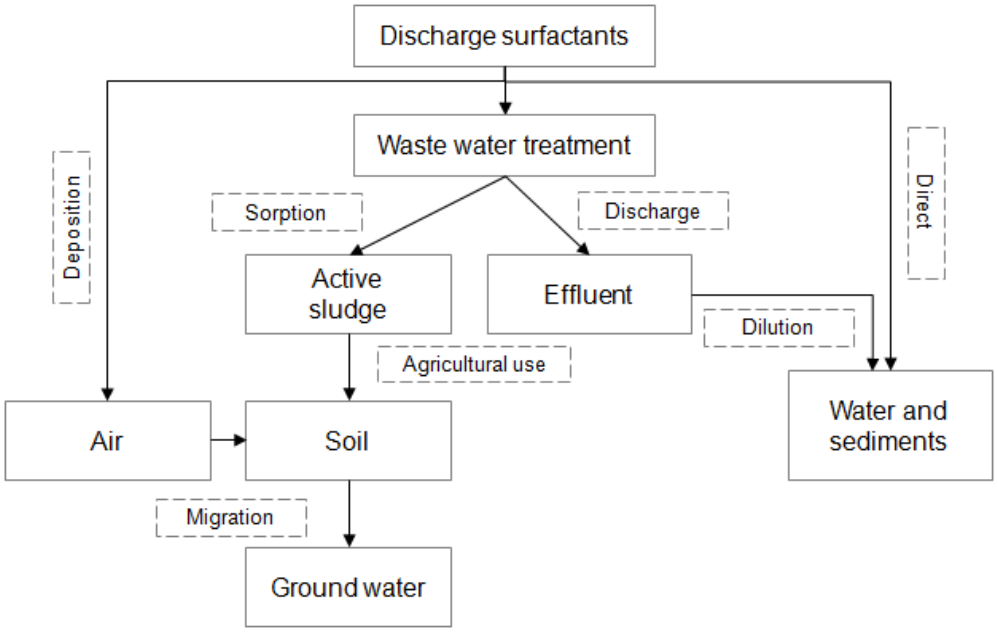


Figure 6.1 Distribution of surfactants in the environment.

6.2. Detection techniques for surfactants

During the last years, different techniques have been used for the determination of the total amount of surfactants and of individual compounds. Most commonly used techniques to measure total content of SAAs in environmental samples are: spectrophotometry [10, 11], potentiometric titration [12], and tensammetry [13]. On the other side, the techniques applied to measure individual content of surfactants are usually HPLC-MS [14, 15] and GC-MS [16, 17].

Chromatographic techniques allow the characterization of individual homologs of SAAs in different environmental samples with low LODs. However, from the point of view of industrial analysis, the most common demanded information is usually referred to the less specific total content. Thus, for routine determination of total concentration of ionic surfactants the most commonly used detection technique is spectrophotometry based on the formation of ion-pair with dyes.

For AS the most commonly used dyes are e.g. methylene blue (MB), ethyl violet (EV), rhodamine 6G, brilliant green and methyl orange (MO) [18, 19]. While for cationic surfactants the most commonly used dyes are: disulfine blue, bromophenol blue, orange II, pyrocatecol violet and Chrome Azurol S (CAS) [20, 21].

6.3. Surfactants extraction and preconcentration techniques

In environmental samples the SAAs are usually at trace levels, usually below the LOD of most analytical techniques. Thus, sample preparation techniques are often necessary for enrichment and sample clean-up prior detection. Most common techniques used to isolate surfactants from liquid environmental samples are LLE [22, 23], SPE [14, 24], and from solid samples, solid–liquid extraction (SLE) [25, 26].

The commonly reference methods used to determine the concentration of AS and CS are those based on the formation of Methylene Blue Active Substances (MBAS) [27] and Disulfine Blue Active Substances (DBAS) [21], respectively. These methods are based on the formation of ion-pair between the surfactant and a dye followed by LLE into chloroform involving several sample processing steps carried out manually and determination of the extracted complex by UV-VIS spectrophotometry. However, both reference methods are not only long and tedious but also present some setbacks such

as large consumption of sample and chloroform a toxic organic solvent. Thus, the major drawback of these reference methods, i.e. the use of large amounts of highly toxic solvents, has been minimized by the use of new sample pretreatment techniques focused on reducing solvent consumption, which have been successfully applied to extract surfactants from aqueous samples, e.g. SPME [28], hollow fiber - liquid phase microextraction (HF-LPME) [29] and DLLME [30].

6.4. Automation of the DLLME methods for surfactants determination

As mentioned above, the reference methods used to determine AS and CS are based on the formation of ion-pair between the surfactant and a dye involving long analysis time and a large consumption of sample and chloroform. These drawbacks have been battled using flow analysis techniques methods such as FIA [20, 31], SIA [10] and MCFIA [23]. Therefore, by automating these determinations exploiting flow analysis techniques, remarkably decrease of analysis time and volume of sample and reagents are accomplished. Thus, flow analysis techniques play a major role in surfactants determination. Hence, the aim of the following presented works was to develop a simplification of the MBAS and DBAS methods based on their automation by in-syringe MSA-DLLME. Since chloroform was used as extractant, for the first time the multisyringe burette was placed up-side down to have the organic fraction after extraction at the head of the syringe. Thus, a new and simplified magnetic driver was designed due to the stirring bar is always at the same positions, i.e. just above the inlet. This is described in more detail in section 3.1.2.2.

Thus, on the one hand, a simple and rapid method for the determination of anionic surfactants was developed overcoming the disadvantages of the official analytical method. The method for the determination of anionic surfactants followed the standard protocol of ion-pair formation with methylene blue and extraction into chloroform. The developed in-syringe MSA-DLLME system permitted the reduction of chloroform in 250 folds (only 200 µL), the sample volume reduction (4 mL) and a huge increase of the analysis frequency since the entire procedure, including sample mixing with reagents, extraction, phase separation, detection and system cleaning, involved just 345 s. A linear working range up to 0.3 mg/L was established. The LOD and LOQ were 7.0 µg/L and 22 µg/L, respectively, as can be seen in Table 6.2. The RSD was below 3 %. Moreover, the applicability of the developed method for MBSA index determination was demonstrated by the analysis of water samples by the cited method and the reference

method, finding concentrations of AS in the range of 0.032–0.213 mg/L and not encountering significant differences between both methods. In addition, standard additions gave analyte recoveries between 95 % and 106 %.

On the other hand, an automated in-syringe MSA-DLLME simple analyzer for the extraction of CS as an ion-pair with disulfine blue dye was developed. The new system configuration further enabled two procedures, a simplified one with direct analyte extraction and a second one including automated in-syringe washing of the organic phase with water and barium acetate solution to minimize interferences. As can be seen in Table 6.2, good repeatability was achieved in both methods. A great reduction of the organic solvents involved and of the sample volume was achieved in comparison to manual approaches by the complete automation and miniaturization of the extraction procedure extracted form DBAS protocol. The simple extraction method is faster, i.e. only 240 s, and was satisfactorily applied for the analysis of DBAS as cetyltrimethylammonium bromide in tap water samples. However, when dealing with more complex matrices the recoveries attained weren't satisfactory. Thus, the procedure 2 including organic solvent washing improved the applicability of the present method, giving satisfactory results when analyzing well water, mineral water and lixiviates. The interference of AS was considerably reduced by organic solvent washing with water, disulfine blue and barium acetate solutions. However, typical AS interference wasn't totally overcome. If high AS interference is present a previous extraction using anionic exchange will be required.

To sum up, the concentration ranges are appropriate for AS and CS determination in water samples as can be seen in Table 6.2. Furthermore, reagent consumption and waste generation are greatly reduced in comparison to previously manually published studies. Thus, the proposed methods are useful tools which could be used to monitor surfactants in water samples.

Table 6.2 Performance of the proposed methods for anionic and cationic surfactants determination.

	Anionic surfactants in-syringe MSA-DLLME method	**Cationic surfactants in-syringe MSA-DLLME method**	
Reagent consumption per assay		Procedure 1	Procedure 2 (including washing step)
Chloroform (µL)	200	220	260
Sample (mL)	4	4	4
Determination rate (h^{-1})	10	15	6.6
Dynamic range (mg/L)	0.022-0.3	0.019-0.29	0.015-0.29
LOD (µg/L)	7.0	5.8	4.4
Repeatability (RSD %)	3.0	3.3	3.5
Samples	Waste, tap and natural waters	Lixiviates, tap and natural waters	

More detailed information is given below in two original research papers result of these investigations which were published in scientific international journals with high impact factor.

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6.6. Original paper 1

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In-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction for automation and downscaling of methylene blue active substances assay

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ABSTRACT

A simple and rapid method for the determination of the methylene blue active substances assay based on in-syringe automation of magnetic stirring-assisted dispersive liquid–liquid microextraction was developed. The proposed method proved to be valid for the determination of anionic surfactant in waste, pond, well, tap, and drinking water samples.

Sample mixing with reagents, extraction and phase separation were performed within the syringe of an automated syringe pump containing a magnetic stirring bar for homogenization and solvent dispersion. The syringe module was used upside-down to enable the use of chloroform as an extraction solvent of higher density than water.

The calibration was found to be linear up to 0.3 mg/L using only 200 μL of solvent and 4 mL of sample. The limits of detection (3σ) and quantification (10σ) were 7.0 μg/L and 22 μg/L, respectively. The relative standard deviation for 10 replicate determinations of 0.1 mg/L SBDS was below 3%. Concentrations of anionic surfactants in natural water samples were in the range of 0.032–0.213 mg/L and no significant differences towards the standard method were found. Standard additions gave analyte recoveries between 95% and 106% proving the general applicability and adequateness of the system to MBSA index determination. Compared to the tedious standard method requiring up to 50 mL of chloroform, the entire procedure took only 345 s using 250-times less solvent.

1. Introduction

Anionic surfactants (AS) [1] are the most common surfactant group used in industrial detergent formulation, cosmetics, and household cleaners [2] and their consumption of AS is steadily increasing due to the raise of population. Although AS are biodegradable [3] it is well known that high concentrations of anionic surfactants in water can harm aquatic organisms [4,5]. Because of the quantity originated from wastewater treatments plants effluents and untreated urban wastewater discharges [6] is high, many aquatic ecosystems receive large quantities of AS. So that AS can also be found in surface and groundwater endangering the quality of drinking water. Hence, determining AS is of interest for environmental and health studies [7,8] as well as quality and safety control. The European environmental regulations established a maximum tolerated limit of 0.2 mg/L for AS in water supplies for human consumption [9].

The most commonly reference method used to determine AS as sum parameter in water is the methylene blue active substance index (MBAS) [10]. This method consists in the formation of ion-pairs between AS and the cationic dye methylene blue (MB) followed by their extraction into chloroform and determination of the extracted complexes by spectrophotometry. However, the reference method is not only long and tedious but also presents a series of drawbacks such as consumption of large volumes of sample and chloroform being a toxic organic solvent. To address these drawbacks, a number of studies were focused on the development of miniaturized and environmentally benign methods based on liquid–liquid extraction (LLE) automated using analytical flow techniques (FT). In Table 1, an overview and comparison of these methods is given. FT-based LLE was first proposed by Karlberg and Thelander [22] and Bergamin et al. [23] who demonstrated minimization of sample and reagent consumption, risk of sample contamination, and operator's intervention as well as enhanced sampling throughput. The determination of AS based on the coupling of LLE and FT was reported for the first time by Kawase et al. [11] in 1978. Analytical procedures used for the determination of AS are reviewed elsewhere [24]. In 2006, a new

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Table 1
Comparison of various flow methods for determination of MBAS index in water samples with developed method.

Flow technique	Extraction technique	Solvent volume (μL)	Dynamic range (mg/L)	LOD (mg/L)	RSD %	DR (h^{-1})	Refs.
FIA	MLLE	1770	<360	4	1.5	80	[11]
FIA	LLE	490	0.1–4	–	3.0	50	[12]
FIA	LLE		0.04–3.5	0.04	1.2	20	[13]
FIA	LLE	200	0.1–1	0.07	6.7	20	[14]
FIA	DBALLME	2	<5.0	0.4	5.0	15	[15]
FIA	MLLE	–	0.02–5	–	–	–	[16]
FIA	–	500	<6	–	4.6	10	[17]
FIA	MMLLE	–	70–700	35	1.8	50	[18]
SIA	LLE	300	1–10	0.5	5.0	5	[19]
MCFA	MM	700	0.2–1.7	0.008	5.9	20	[1]
FIA	LLME	50	0.03–0.3	0.02	2.4	240	[20]
MCFA	LLME	44	0.05–2.0	0.02	1.5	18	[21]
SIA	MSA-DLLME	220	0.025–0.3	0.007	3	10	This work

Abbreviations: DBALLME, drop-based automated liquid–liquid extraction; DLLME, dispersive liquid–liquid microextraction; DR, determination rate; FIA, flow injection analysis; MCFA, multicommuted flow analysis; LLE, liquid–liquid extraction; LLME, liquid–liquid microextraction; LOD, limit of detection; MLLE, membrane liquid–liquid extraction; MM, multicommutated; MMLLE, microporous membrane liquid–liquid extraction; MSA-DLLME, Magnetic stirring-assisted dispersive liquid–liquid microextraction; RSD, Relative standard deviation; SIA, sequential injection analysis.

concept of miniaturization of LLE was proposed by Rezaee et al. [25] denoted dispersive liquid–liquid microextraction (DLLME). A mixture of an extraction solvent and a dispersion solvent with high miscibility in water is rapidly injected into an aqueous sample to form a cloudy component emulsion. By centrifugation, the extraction solvent containing the enriched analytes can be separated and then injected into an appropriated analytical instrument. The advantages of DLLME are its simplicity of operation, rapidity, low cost, high-recovery, high enrichment factor, and minimal waste generation [26]. However, the distribution coefficient of the analyte between organic and aqueous phase could be altered by the dispersion solvent making a comparison with standard protocols based on classical LLE difficult. Besides, method optimization requires finding a suitable dispersion solvent as well as an optimal mixing ratio with the extraction solvent. The alternative to tackle these problems was the replacement of the dispersion solvent by kinetic energy leading to air-assisted [27], vortex-assisted [28], ultrasound-assisted [29], magnetic-stirring-assisted (MSA) dispersion [30]. More recently, the concepts of DLLME and FT automation were combined [31–33]. Here, in-syringe DLLME has demonstrated to be a specially promising technique for automated DLLME, [34–37] with the late report of automated in-syringe MSA-DLLME [38,39] due to its simplicity and versatility. The aim of the present work was to develop a simplification of the MBAS method based on in-syringe MSA-DLLME with the novel modification that the syringe was used upside down in order to use chloroform as extraction solvent to achieve comparability towards the standard procedure for MBSA determination.

2. Material and methods

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals from Scharlab SA (Barcelona, Spain) unless otherwise indicated and bi-distilled quality water provided by a Milli-Q Direct-8 purification system (resistivity >18 MΩ cm, Millipore Iberica S.A.U., Spain) was used throughout. All material were previously soaked for at least 24 h in 10% (v/v) HNO_3 and rinsed with water before used. A stock solution of 10 mg/L sodium dodecyl benzene sulphonate (SDBS) (Sigma Aldrich, Steinheim, Germany) was used as standard solutions of anionic surfactants. For calibration, SDBS standard working solutions were prepared daily by appropriate dilution. A stock solution of 700 mg/L methylene blue (MB) (Panreac SA, Barcelona, Spain) was prepared by dissolution of an appropriate amount of the reagent in Milli-Q water. A solution of 127 mmol/L sodium hydrogen phosphate and 100 mmol/L H_2SO_4 were used for in-syringe buffer preparation. To accelerate phase separation, a 648 mmol/L Na_2SO_4 solution was used as additional reagent. Chloroform was used as extraction solvent without any previous treatment. All reagent solutions were kept in glass bottles at 4 °C.

For the reference procedure, the following solutions were used as recommended [10]: MB solution: 30 mg/L MB in sulfuric acid–sodium phosphate buffer (concentrations 0.123 mol/L and 0.362 mol/L, respectively) and washing solution being the same buffer but without MB.

Solutions used in interference studies were prepared from $CaCl_2$, $MgCl_2 \cdot 2H_2O$, NH_4Cl, $AlCl_3 \cdot 6H_2O$, $Pb(NO_3)_2$, $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot H_2O$, $NaNO_3$, $NaNO_2$, NaCl,$NaHCO_3$, Triton X-100, humic acid and CTAB. The substances were chosen in agreement with former interference studies [20,21]. In order to study the influence of water hardness on the extraction process, artificial freshwaters of different hardness grades were prepared according to standard recipes for “very hard water”, “hard water” and “moderately hard water” [10].

2.2. Sample collection and preparation

Different natural water samples were collected and analyzed: drinking water, pond water, well water, and tap water from different places on Mallorca and wastewater from entrance and effluent of a local biological treatment plant. Samples were collected in polyethylene bottles and stored at 4 °C until analysis. Wastewater samples and pond water were paper-filtered to remove suspended particles.

2.3. Manifold configuration

The system used in this work is depicted in Fig. 1 and follows a prior designs [38,39]. It comprised a 5000-step syringe pump (SP) from Crison SL (Alella, Barcelona, Spain) with a 5 mL glass syringe (S) and a rotary 8-port multiposition valve (MPV) from Sciware System SL (Palma de Mallorca, Spain). PTFE tubing of 0.8 mm inner diameter (id) was used for the entire manifold. A short PTFE tube was placed into the syringe inlet to minimize the dead volume. A three-way solenoid head-valve (V) on top of the syringe enabled the connection to either the central port of the MPV (position ON, activated) or to a detection cell and downstream located waste for quantification of the extracted analyte and discharge during syringe cleaning (position OFF, deactivated). Peripheral ports of the MPV were connected to reservoirs of waste (1), water (2), sample (3), MB (4), NaH_2PO_4 (5), chloroform (6), air (7), H_2SO_4 (8), and Na_2SO_4 (9). The connection between the common port of the MPV and the syringe head-valve was done by a holding coil (HC) of 26 cm in length. For sample measurements, a 15-position rotary autosampler from Crison SA was used. For dispersion of the extraction solvent, a magnetic stirring bar (10 mm × 3 mm in diameter) was placed inside the syringe.

In this work, given the fact that the extraction solvent had a higher density than water and thus accumulated at the bottom, the syringe module was used upside-down.

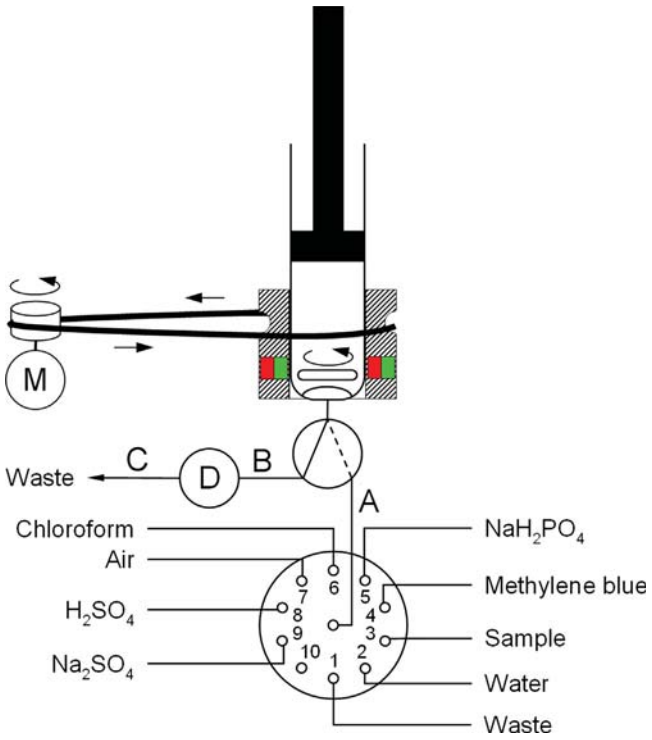

Fig. 1. Schematic manifold used for in-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction (MSA-DLLME) MBAS determination. The manifold was composed of a multiposition valve (MPV), syringe pump (S) with a magnetic stirring bar inside, solenoid 3-way head valve (V), detection flow cell (D), and a DC motor (M), which is used to drive it via a rubber band. PFTE tubing were in length: 26 cm ((A), HC), 5 cm (B), and 50 cm (C).

As detection system, a USB 2000 CCD spectrometer, a deuterium-halogen light source (DH-2000-BAL), and optical fiber of 400 μm core diameter (all purchased from Ocean Optics, Dunedin, FL, USA) were used. A flow cuvette of 1 cm optical path length and 1.5 mm flow channel diameter from Hellma Analytics (Müllheim, Germany) and a fiber-optics cuvette support from Ocean Optics was used throughout. The cell was connected via a 10 cm long PTFE tube of 0.8 mm id to the OFF position of V. Furthermore, to improve the wettability of the cuvette for the organic phase, one-time silanization was done by flushing the cuvette subsequently with piranha solution (3:1 mixture of concentrated H_2SO_4 and 30% hydrogen peroxide), 2 mol/L of NaOH, water-free methanol, and water-free toluene. Then, the cuvette was blown dry by nitrogen flow and a 1:10 mixture of dichlorodimethylsilane in water-free toluene was let react with the free hydroxyl-groups of the wall surface for 10 min. Finally, the cuvette was flushed with methanol. It should be pointed out that preparation and handling of the solutions for silanization should be done with great care, under fume hood, and in the minimum amount possible (here <5 mL). Piranha solution is an extremely strong oxidizing and unstable reagent tending to decompose at the presence of smallest amounts of catalysts.

2.4. Magnetic stirring bar driver

The prior described magnetic stirring device to generate a rotating magnetic field [38] was simplified since the stirring bar remains at same position at the bottom of the syringe. It was made of a Deldrin® tube of 20 mm × 25 mm in diameter, which fitted snugly over the syringe glass barrel (14 mm inner diameter). It held two small neodymium magnets (5 mm × 4 mm in diameter), which were strong enough to levitate the stirring bar inside the syringe. Turning the driver device by a DC motor connected via a rubber band forced the stirring bar to rotate at equal speed. The motor was activated using a homemade relay and regulation circuit board enabling two different stirring speeds by employing two auxiliary supply ports of the syringe pump. The stirring speeds were approximately 1000 rpm and 2000 rpm for, slow mixing and solvent dispersion for DLLME, respectively. The circuit to control the motor is given elsewhere [38].

2.5. Data acquisition and evaluation

Absorbance measurements of the chloroform phase were done at 656 nm, corrected at a wavelength of 710 nm where MB did not show any significant absorbance, allowing the correction of analyte unspecific intensity variations. The instrumentation was controlled by the software package AutoAnalysis 5.0 (Sciware Systems SL) achieving complete automation of the analytical protocol (see Section 2.6) as well as data acquisition and processing [40,41]. Design of experiments and result evaluation were done with the software package STATISTICA 8.0. The difference of the absorbance between standard and blank signal was used as analytical response.

2.6. Analytical protocol and flow method

The method for MSA-DLLME is given as Supplementary material S-1. Additionally, the analytical protocol is given schematically in Fig. 2. First, the syringe was cleaned by three-fold aspiration of 0.5 mL of water (stirring activated) and discharge to waste. Then, the following solutions (for concentrations see Section 2.1.) were subsequently aspirated into the syringe: 200 μL of $NaSO_4$, 130 μL of H_2SO_4, 200 μL of NaH_2PO_4, 100 μL of MB, and 3.7 mL of sample., under low-speed stirring for mixing the syringe content. Then, 200 μL of chloroform were aspirated followed by 350 μL of air to drive all chloroform into the syringe. During air-aspiration and the following 80 s, rapid-speed stirring was activated. At contact of the chloroform with the stirring bar, the solvent was dispersed into small droplets, thus enabling DLLME. During the last five seconds, the stirring speed was decreased, which favored the coalescence of the fine chloroform droplets. Afterwards, during a phase separation time of 30 s, the enriched droplets were accumulated at the bottom of the syringe. In the following, the organic phase enriched with the analyte-MB ion-pairs was slowly propelled through the flow cuvette under

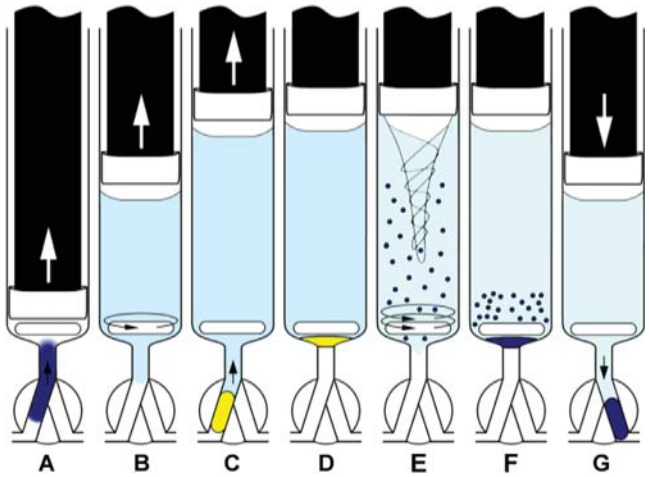

Fig. 2. Performed operation scheme for in-syringe MSA-DLLME of anionic surfactants into chloroform as MB ion-pairs. (A) Aspiration of MB, (B) Aspiration of sample and mixture with reagents, (C+D) Aspiration of chloroform, (E) DLLME, (F) Phase separation, (G) Propulsion of enriched organic phase to detector.

continuous data evaluation. All experiments were performed in triplicate.

2.7. Reference method

In order to evaluate the accuracy of the developed MSA-DLLME method, results were compared with those obtained by a simplified reference method derived from APHA 5540C [10]. 25 mL of sample were transferred into a separating funnel and containing 2.5 mL of MB solution (see Section 2.1) and 10 mL of chloroform were added. After extraction for 30 min, phases were let separate. Then, the organic layer was collected and the extraction was repeated twice with additional 10 mL of chloroform each. The extracts were combined and aliquot of 10 mL was washed twice with 50 mL of the recommended washing solution. Finally, the absorbance of washed extract was measured at 652 nm.

3. Results and discussion

3.1. Preliminary remarks

To achieve that the droplets would accumulate at the inlet of the syringe, the syringe pump was used up-side down implying several particular changes in the operation, here firstly described. Most importantly, it was unavoidable that air bubbles would accumulate in the syringe causing that each solution displacement required additional time. For example, dispensing caused the compression of the air cushion before the liquid in the HC would start to move. Likewise, the stored air pressure still caused liquid displacement over a few seconds while the syringe operation already had stopped. So, operation steps which required high reproducibility were followed by a waiting time of 2 s.

Due to the fact that any air, which surpasses the remaining dead volume inside the syringe, was expulsed towards the detection cell and waste at emptying the syringe during cleaning, the volume of air inside the syringe was reproducible and equal to the syringe dead volume. Consequently, all liquid was expulsed apart from adhered liquid films on the surfaces at emptying the syringe, so cleaning was more efficient than in the previous works [38,39]. An additional advantage was that the stirring bar was not displaced by the syringe piston, so that a much simpler device as described in Section 2.4 could be used for the creation of the rotating magnetic field.

The configuration allowed to use chloroform as denser solvent than water. An initial attempt to use *n*-hexanol as less harmful solvent and the prior system configuration, i.e. normal syringe orientation, was rejected due to the blank values resulted unacceptably high since solubility of MB itself in *n*-hexanol is significant.

Despite of the recommendation from the standard procedure of doing a washing step to eliminate some interferences by back-extraction, in this work a simple extraction was carried out. Several applications of direct extractions have demonstrated that the interference level at this simplified mode is equally low and the comparability with the standard method is given [13,21]. Therefore, it was opted for the simple extraction to minimize the sample manipulation and analysis time.

3.2. Phase separation time

First of all, the required time for the separation of both phases, chloroform and aqueous sample, was studied in the range of 5 to 40 s using both a blank (water) and 0.4 mg/L SBDS standard. While the blank signal remained constant over the studied range, the standard signal increased rapidly up to a maximum at 30 s with constant signals for longer times (data are not shown). Therefore, 30 s of phase separation time were applied further on.

3.3. Multivariate optimization of experimental conditions

A two-level fractional factorial design (2^{6-2}) was selected to screen the relevance of the the concentrations of H_2SO_4 (A), of MB (B), of Na_2SO_4 (C), and of NaH_2PO_4 (D) as well as the volume of chloroform (E) and the extraction time (F) in the method.

Triplicate measurements of the centre point were also added to evaluate the potential curvature and the significance of the result variability, using standard solutions (0.4 mg/L). The range of variables (data are presented in Supplementary material S-2) affecting the extraction and the results were obtained with variance (ANOVA) with 95% probably. The data are presented in Supplementary material S-3 and S-4. According to ANOVA table and Pareto chart results, the most significant factors were the extraction time (positive dependency) the volume of chloroform (negative dependency). Moreover, the interaction between H_2SO_4 and NaH_2PO_4 was statistically significant, while the concentration of Na_2SO_4 had no significant impact on the extraction recovery and thus was fixed.

Based on the screening study results, a face-centered central composite design (CCD) with a total number of 27 experiments was made to estimate the critical values of the variables to be significant plus the concentration of MB to achieve minimal consumption while the volume of chloroform was fixed to 200 μL for CCD and studied posteriori. Taking into account the results of screening, the ranges of four variables (A, B, D and F) were modified to achieve the highest extraction efficiency (data are presented in Supplementary material S-5). The quality of the fit of the linear-quadratic model was explained by the coefficient of determination and the lack of fit value ($p > 0.05$). A regression coefficient of $r^2 = 0.980$ (adjusted $r^2 = 0.957$) indicated a good relationship between the experimental data and the fitted model. The histogram of residuals and predicted vs. observed values showed satisfactory distributions. Therefore, critical values were obtained using the desirability function (data are presented in Supplementary material S-6). Thus, optimum conditions are used in all further experiments: 200 μL of 648 mmol/L $NaSO_4$, 130 μL of 100 mmol/L H_2SO_4, 200 μL of 127 mmol/L NaH_2PO_4 and 100 μL of 700 mg/L MB, and 30 s of separation time.

3.4. Volume of the extraction solvent

According to the reference method, chloroform was chosen as extraction solvent. The volume of chloroform used in the procedure is highly important since a larger volume could yield higher extraction efficiency while a smaller volume could yield a higher concentration factor and thus a higher sensitivity and minimize the environmental impact and costs of the method. This study was performed by the comparison of the sensitivity of four different calibration curves in a range from 100 to 250 μL of chloroform (data are not shown). Using 100 μL and 150 μL of chloroform, the repeatability was not acceptable with RSD values higher than 10%. For volumes larger than 200 μL, the signal height was reducing hence losing sensibility. Thus, 200 μL was chosen as best value to establish a compromise between the sensitivity and repeatability.

3.5. Extraction time

In LLE, the aim is to transfer a maximum amount of the analyte from one liquid donor phase to an immiscible acceptor phase. The extraction rate will decrease as the system approaches the steady-state expressed by the partition coefficient. Furthermore, DLLME and related techniques such as used in this work require very short extraction times, as the contact surface between both phases

is enormously increased by droplet dispersion. The effect of the stirring time on the absorbance was studied in the range of 50 to 110 s for a blank solution and for a 0.2 mg/L SBDS standard. It was observed that the absorbance of the blank remained constant over time while the standard signals increased nearly linearly with the extraction time up to 80 s, reaching a stable level and RSD values about 2% beyond. Thus, a pre-concentration time of 80 s was chosen for the method in order to minimize the analysis time.

3.6. Study of possible interferences

As only moderately soluble in water, MB can form extractable ion-pairs with other anions, which then act as positive interferences of the procedure. On the other hand, especially organic and large cations can compete with MB, leading to negative interference. The effect of potentially species on the proposed procedure in concentrations similar or higher than reported for surface water [9]. Standards of 0.100 mg/L of SBDS including the potentially interfering compounds were prepared from stock solutions (see Section 2.1) and assessed with the developed MSA-DLLME method. The percentage of found interference of each ion is given as Supplementary material S-7. Mostly, the interference level was well-below 10%, even for even higher concentration as normally found in natural waters.

Slight negative interferences were observed from aluminium and CTAB. However, it should be pointed out that these interferences are common for the MBAS method and were former reported to similar or even higher extent [20,21].

Further it was found that the observed effects of chloride and nitrate as typical interfering anions of the MBAS assay [10] were very low even at the studied concentration exceeding typical concentrations in surface waters. Moreover, similar observance was made by other researchers [13–21]. Thus, it was decided to omit the step of extraction washing.

On the other side, it was noted that the method could not be applied to seawater since the signals of both blank and standard solutions increased linear with the chloride concentration for concentrations beyond 600 mg/L chloride.

The effect of water hardness in the extraction process was also studied owing to the high concentration of carbonate in freshwaters on Mallorca Island. Five calibrations using SBDS standard prepared with Milli-Q water and artificial “moderately hard water”, “hard water”, and “very hard water” (see Section 2.1) were measured and compared. The results showed that there was no significant effect of the water hardness on the method sensitivity with 95% confidence intervals for no hardness added and the maximum hardness value. The results led to the conclusion that the selectivity of the method was appropriate for MBAS determination.

3.7. Method performance

Under the optimized experimental conditions, the proposed method was characterized by repeated calibrations proving a linear behavior of the signal height up to 0.300 mg/L. The calibration curve, evaluated on 5 subsequent days, followed the equation: peak height$=(2.9\pm0.04)$ [SBDS mg/L]$+(0.042\pm0.04)$ ($R^2=0.994$). Limits of detection and quantification (LOD, LOQ) were calculated as the concentration yielding a peak height passing the blank signal by it triple and ten-fold standard deviation, respectively. A LOD of 7 μg/L and a LOQ of 22 μg/L were obtained for SBDS in water samples. The relative standard deviation (RSD) of repeated measurement was generally below 4% of peak height. The RSD value for ten-fold determinations of a 0.100 mg/L SBDS standard was $<3\%$.

In contrast to MSA-DLLME based on manual operation [10], the entire procedure (i.e. mixing of sample and reagents, extraction, phase separation, measurement, and system cleaning) took about 345 s allowing a measuring frequency of 10 h^{-1}. In addition, using an autosampler, the proposed system operated completely automated.

The pre-concentration factor can be estimated from the ratio between the volume of the sample (3.69 mL) and the volume of the solvent (200 μL) to be 18.5.

3.8. Method validation and application to water samples

For sample analysis, a rotary autosampler unit was connected to MPV position 3 so as to analyze the samples rapidly one-after-one and overnight. In order to assess the accuracy, water samples and spiked water samples were analyzed by the reference procedure, MBAS reference method (C_{LLE}) and with the proposed MSA-DLLME method ($C_{MSA\text{-}DLLME}$) and results obtained were compared. The found linear relationship followed the equation $C_{MSA\text{-}DLLME}=1.059\ (\pm0.135)\times C_{LLE}-0.005\ (\pm0.018)$ where the values in parenthesis are 95% confidence limits. Since the estimated slope and intercept did not differ statistically from values 1 and 0, no evidence of systematic differences between the two sets of results was given.

In order to evaluate the applicability of the proposed automated MSA-DLLME method, seven water samples were measured

Table 2
Analysis of SBDS in different water samples including the results of addition-recovery tests: G, conductance; SD, standard deviation.

Sample	pH	G	Added	Found[a]			Recovery			t_{exp}[b]
		(mS/cm)	(mg/L)	(mg/L)		SD	%		SD	
Waste water T3	7.9	3	0.00	0.113	±	0.002				
			0.05	0.160	±	0.003	97.4	±	2.9	0.5
Waste water T2	8.1	3	0.00	0.144	±	0.010				
			0.05	0.201	±	0.003	105	±	2	0.4
Well water 1	7.2	0.9	0.00	0.153	±	0.013				
			0.05	0.234	±	0.009	105	±	5	2.2
Well water 2	7.5	0.7	0.00	0.178	±	0.008				
			0.05	0.240	±	0.008	107	±	4	0.8
Tap water	7.0	0.5	0.00	0.058	±	0.005				
			0.05	0.105	±	0.005	93.8	±	9.2	0.4
Pond water	8.5	1.4	0.00	0.213	±	0.007				
			0.05	0.254	±	0.014	95.8	±	6.5	0.4
Drinking water	6.3	0.1	0.00	0.032	±	0.004				
			0.05	0.081	±	0.002	99.5	±	6.4	0.3

G: Conductivity.

[a] Results are expressed as the mean value ± standard deviation ($n=3$).

[b] t_{crit}: 4.3.

with the proposed analyzer system. All samples were further spiked with SBDS standard to evaluate the analyte recovery and matrix effects. The results are given in Table 2. All samples showed natural concentrations of MBAS in the range of 0.032–0.213 mg/L, thus proving the suitability of the linear working range for samples. Standard addition of SDBS gave analyte recoveries in the range from 95% to 113% proving the general applicability and adequateness of the analyzer system to real sample analysis. The trueness of the analytical method was proven by student *t*-test. The overall calculated values of t were ≤ 1.55 and given a critical value of 4.3 at the confidence level of 95%, the results did not show any significant differences from the expected concentration values.

3.9. Discussion on system performance and operation

In this work, we firstly used in-syringe magnetic stirring-assisted DLLME in combination with a solvent denser than water, i.e. chloroform. This had led to the requirement to use the syringe upside down to facilitate droplet coalescence at the conical part of the syringe inlet and to allow the heavier organic phase or "the analytical fraction" to be pushed out completely from the syringe and through the detection cell before the sample.

The possibility to use halogenated solvents in-syringe in combination with stirring allows the direct transfer of standard extraction procedures, which employ these solvents, with the possibility of using only a fraction of these solvents in the future and achieving environmental friendlier methods.

The proposed configuration included also the possibility to expel practically all liquid from the syringe by the cushion and lower therefore the dead volume to be cleaned. This feature can be of high advantage when the organic content should be kept for a second in-syringe operation but with prior and complete elimination of the rest of sample. By example, it would allow repeated sample preparation with following extractions into the same volume of solvent to increase the method's sensitivity. By this work, we therefore hope to widen the versatility and applicability of this recent technique for automation of liquid–liquid extraction and sample preparation.

4. Conclusions

In this work, a novel method for the determination of the MBAS index based on in-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction was presented. For the first time, a solvent denser than water was used in combination with this technique. Using multivariate optimization strategy enabled successful determination of the optimum conditions for the main experimental parameters taken into consideration during DLLME. Moreover, the developed system showed to be a robust and reliable alternative to existing methods for the spectrophotometric determination of anionic surfactants as sum parameter. The method proved to be selective with very low interference in spite of simplification of direct extraction from the acidified sample was done. A better sensitivity than in former works was achieved. The proposed method was successfully applied to the analysis of the MBAS index in a variety of water samples.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.06.063.

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6.6.1. Supplementary information – paper 1

Table S1. Procedure for in-syringe stirring assisted dispersive liquid-liquid microextraction of anionic surfactants

No	Instrument*	Operation*			Comment
1	MPV	Valve A move to position 3			Clean syringe with water
2	Loop	START: Repeat 3 times			
3	S	Pickup [On]	0.500 mL	at 15 mL/min V after 0.400 mL activate agitation	
4	S	Dispense [Off]	0.500 mL	at 10 mL/min V without agitation	
5	Loop	END			
6	MPV	Valve A move to 9			Aspiration of $NaSO_4$
7	S	Pickup [On]	200 µL	at 7.5 mL/min V	
8	Wait	Wait 2 seconds			
9	MPV	Valve A move to position 8			Aspiration of H_2SO_4
10	S	Pickup [On]	103 µL	at 7.5 mL/min V	
11	Wait	Wait 1 seconds			
12	MPV	Valve A move to 5			Aspiration of Na_2HPO_4
13	S	Pickup [On]	200 µL	at 7.5 mL/min V	
14	Wait	Wait 2 seconds			
15	MPV	Valve A move to 4			Aspiration of MB
16		Pickup [On]	100 µL	at 7.5 mL/min V	
17	Wait	Wait 2 seconds			
18	MPV	Valve A move to 3			Aspiration of sample
19	S	Pickup and activate and deactivate agitation each 0.200 mL	3.7 mL	at 5 mL/min V [On]	
20	Wait	Wait 2 seconds			
21	S	Deactivate agitation			
22	MPV	Valve A move to position 6			Aspirating of chloroform
23	S	Pickup [On]	0.200 mL	at 2.5 mL/min V	
24	SV	Wait 2 seconds			
25	MPV	Valve A move to position 7			Aspirating of air
25	S	Pickup [On] and activate agitation	0.350 mL	at 2.5 mL/min V	
26	Wait	Wait 80 seconds			MSA-DLLME
27	S	Deactivate agitation			Phase separation and droplet aggregation
28	Wait	Wait 30 seconds			
29	S	Pickup	0.080 mL	at 4 mL/min V [On]	
30	Wait	Wait 2 seconds			
31	D	Measure every 0.1 s with 12 points to average			Discharge through detector to waste and measurement
32	S	Dispense	0.420 mL	at 1 mL/min V [Off]	
33	D	Stop measure			
34	SP	Empty	Complete	at 15 mL/min V [Off]	Empty syringe rapidly to waste

Table S2. Factors and low and high levels in design matrix.

Variable	Levels	
	Low (-)	High (+)
(A) H_2SO_4 (mmol/L)	0.3	30
(B) MB (mg/L)	1.9	18.5
(C) Na_2SO_4 (mmol/L)	5	30
(D) NaH_2PO_4 (mmol/L)	1.39	23.15
(E) Chloroform (µL)	100	250
(F) Extraction time (s)	15	55

Table S3. Analysis of variance (ANOVA) for screening design

Factor	Sum of Squares	Df [a]	Mean Square	F-Ratio[b]	P-Value
Curvature	0.017442	1	0.017442	22.1817	0.042246
(A)[H_2SO_4]	0.006716	1	0.006716	8.541	0.099853
(B)[MB]	0.004665	1	0.004665	5.9332	0.135191
(C)[Na_2SO_4]	0.000349	1	0.000349	0.444	0.573759
(D)[NaH_2PO_4]	0.000008	1	0.000008	0.0105	0.92786
(E)Vol. Chloroform	0.181661	1	0.181661	231.0224	0.004301
(F)Extraction time	0.173753	1	0.173753	220.9658	0.004495
A by B	0.002182	1	0.002182	2.7743	0.237708
A by C	0.000158	1	0.000158	0.2015	0.697446
A by D	0.026806	1	0.026806	34.0894	0.028104
A by E	0.001757	1	0.001757	2.2351	0.273535
A by F	0.001054	1	0.001054	1.3399	0.366614
B by D	0.009855	1	0.009855	12.5323	0.071358
B by F	0.008096	1	0.008096	10.296	0.084934
A*B*D	0.00355	1	0.00355	4.5147	0.167532
A*B*F	0.006768	1	0.006768	8.6073	0.099194
Pure Error	0.001573	2	0.000786		
Total SS	0.446422	18			

[a] Df. Degrees of freedom
[b] Test for comparing model variance with residual (error) variance
R^2 = 0.99648
R^2 adjusted = 0.96829

Table S4. Standardized main pareto chart from the screening design. Vertical line in the chart defines 95% of confidence level.

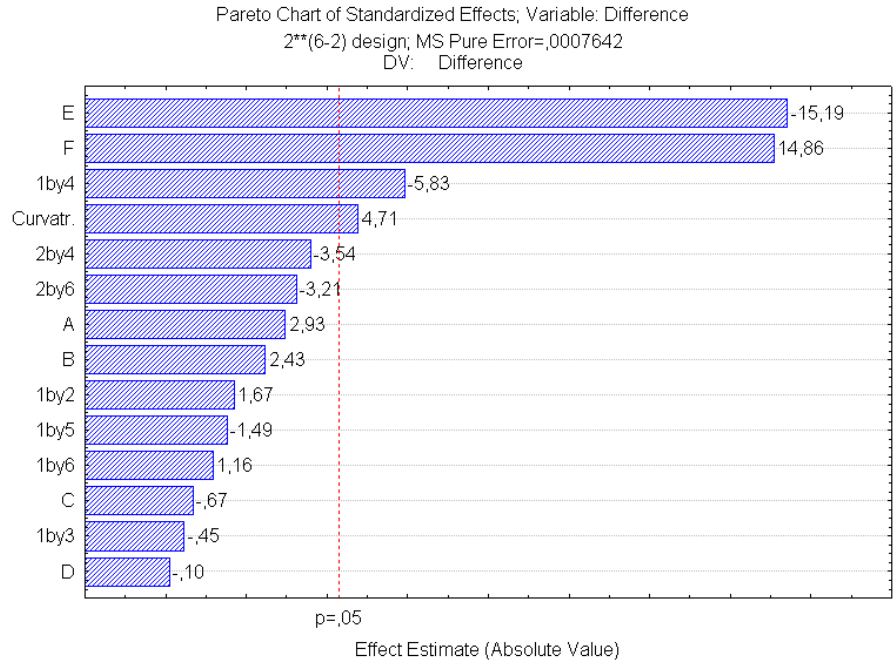

Table S-5. Factors, and low and high levels in design matrix.

Variable	Levels	
	Low (-)	**High (+)**
(A) H_2SO_4 (mol/L)	0.003	0.1
(B) MB (mg/L)	0.93	16.20
(D) NaH_2PO_4 (mmol/L)	0.23	11.57
(F) Extraction time (s)	20	80

Figure S-6. Desirability chart used to obtain the critical values of sulfuric acid (A), methylene blue (B), sodium dihydrogenphosphate (D) and extraction time (F). Rows correspond to 1: Blank signal, 2 Standard signal, 3 difference signal and 4 desirability.

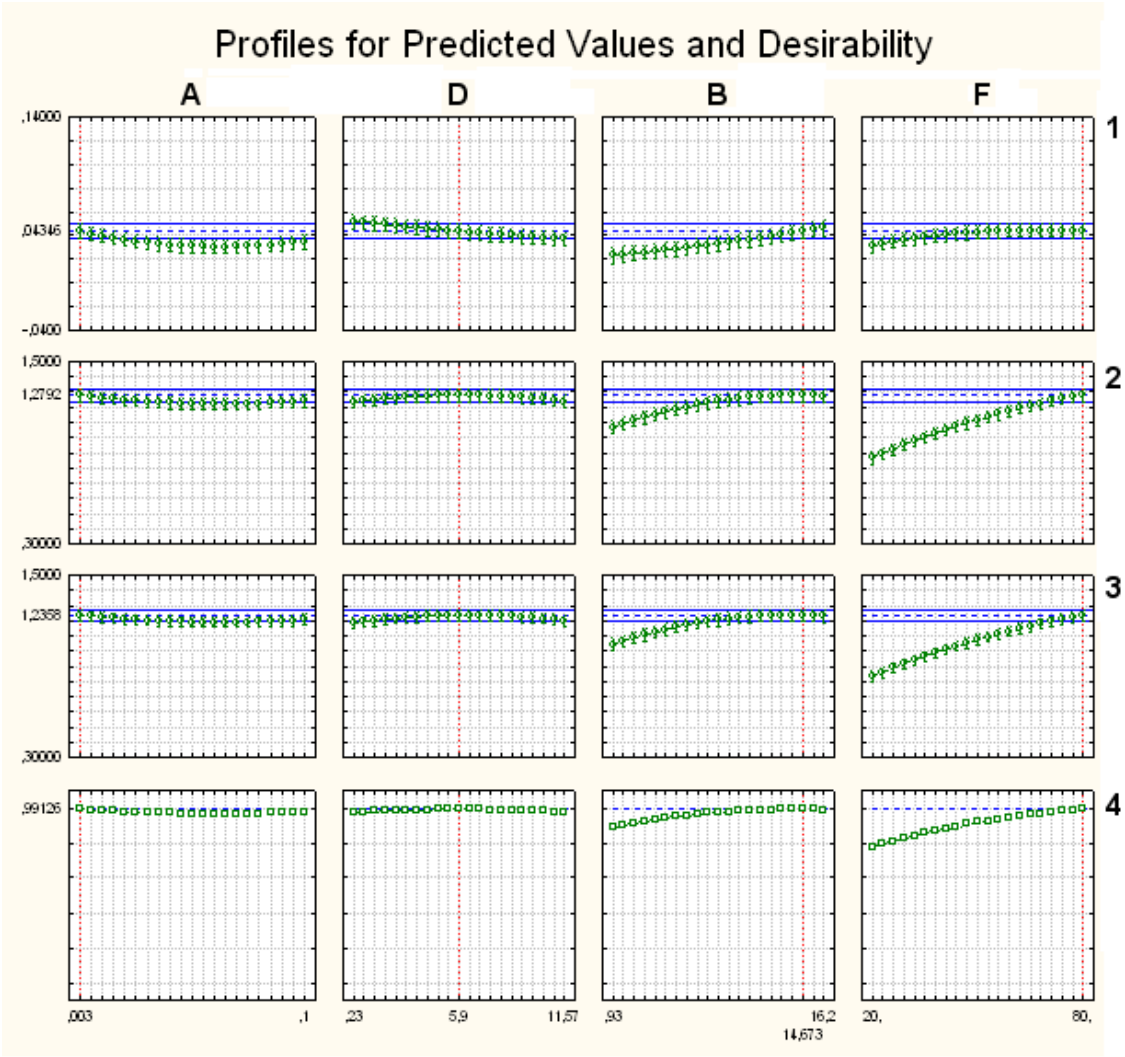

Table S-7. Respective percentage of signal interference for tested substances used in 0.100 mg SBDS/L standard solution.

Tested compound in standard	**Added as**	**Concentration of interfering ion[a] (mg/L)**	**Interference (%)**
Ca^{2+}	$CaCl_2$	50	1.1 %
Na^{+}	NaCl	500	6.2 %
Mg^{2+}	$MgCl_2 \cdot 2\ H_2O$	50	-4.1 %
NH_4^{+}	NH_4Cl	50	3.3 %
Al^{3+}	$AlCl_3 \cdot 6\ H_2O$	25	-9.3 %
Pb^{2+}	$Pb(NO_3)_2$	5	-1.1 %
Cu^{2+}	$CuSO_4 \cdot 5\ H_2O$	5	-4.5 %
Fe^{3+}	$FeCl_3 \cdot H_2O$	1	3.1 %
NO_3^{-}	$NaNO_3$	50	7.7 %
NO_2^{-}	$NaNO_2$	0.5	1.3 %
Cl^{-}	NaCl	500	6.2 %
Triton X-100	-	0.05	-3.2 %
Humic acid	-	1	1.1 %
CTAB	-	0.04	-11.9 %
	-	0.03	-7.9%

[a] Tested concentration of interfering ion in a standard 0.100 mg/L SBDS

6.7. Original paper 2

Title: In-syringe magnetic stirring-assisted dispersive liquid-liquid microextraction with solvent washing for fully automated determination of cationic surfactants

Authors: Burkhard Horstkotte, Ruth Suárez, Petr Solich and Víctor Cerdà

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In-syringe magnetic stirring assisted dispersive liquid–liquid micro-extraction with solvent washing for fully automated determination of cationic surfactants†

Burkhard Horstkotte,[a] Ruth Suárez,[b] Petr Solich[a] and Víctor Cerdà*[b]

An automated simple analyzer system for the extraction of cationic surfactants as an ion-pair with disulfine blue dye is described based on the technique in-syringe magnetic stirring-assisted dispersive liquid–liquid micro-extraction. The use of chloroform as an extraction solvent denser than water required the operation of the syringe pump upside-down. The remaining air cushion inside the syringe allowed emptying the syringe completely and reducing the dead volume significantly compared to previous studies. Since the stirring bar placed inside the syringe to obtain a closed yet size-adaptable mixing chamber remains at the same position, the former magnetic stirring bar driver was simplified. The new system configuration further enabled automated in-syringe washing of the organic phase with water and barium acetate solution to minimize interference. High signal repeatability with <5% RSD was achieved both for extraction as well as for double organic phase washing. Only 220 μL of the extraction solvent and 4 mL of the sample were required for simple extraction achieving a detection limit below 30 nmol L^{-1} and a linear response up to 1 μmol L^{-1} of cetyltrimethylammonium bromide. The time of analysis was 240 s for simple extraction. Considerable reduction of interference was achieved by extract washing up to 545 s. Analyte recovery in real water samples was 95.6 ± 7.0% on applying extract washing.

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1. Introduction

Flow techniques (FT) comprise different methodologies of sample treatment in flow in a tubing manifold and, unlike chromatography, without gradual separation. FT differ in the way of sample introduction and flow patterns as well as in the configuration and operation of specific analyzers, but have in common the automation of classical laboratory procedures including sample metering (aspiration or injection), handling (transport, splitting, *etc.*), modification (dilution, filtration, clean-up, and concentration), performing of chemical reactions (reproducible mixing with reagent and heating), and measurement.

FT are powerful tools to achieve minimization of solution consumption and to improve the reproducibility of analytical procedures. In contrast to other automation approaches (*e.g.* robotic systems), FT are self-cleaning, *i.e.* the manifold is flushed by carrier flow, which allows stand-alone operation while on the other hand, analyses are performed sequentially.

In 1990, the flow technique Sequential Injection Analysis (SIA)[1] originated from the idea of performing different flow procedures in one universal analyzer, which does not require manual re-configuration but which enables computer-controlled choice of the operation parameters such as timing, mixing patterns, and used volumes of samples and reagents.

The basic operation is a sequential aspiration of samples and further required solutions from the ports of a selection valve (SV) into a tube, denoted as the holding coil (HC), which connects the central common valve port to a bidirectional pump, generally of syringe type. Then, the flow is reversed and

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† Electronic supplementary information (ESI) available: ESI 1: control circuit for the DC motor used for in-syringe stirring. ESI 2: procedure 1 for automated in-syringe stirring-assisted DLLME of cationic surfactants without organic phase washing. ESI 3: procedure 2 for automated in-syringe stirring-assisted DLLME of cationic surfactants with double organic phase washing. ESI 4: photo documentation of the operation scheme of the simple extraction procedure 2. ESI 5: example of peak signals of calibrations with both procedures. ESI 6: Box–Behnken experimental design for the optimization of the volumes of buffer and DSB stock solutions and extraction time. ESI 7: optimization of parameters for simple extraction being the stirring time (a), volume of acetate buffer solution (b), and the volume of DSB solution (c). ESI 8: Box–Behnken experimental design for the optimization of the volumes of barium acetate and DSB stock solutions for extraction solvent washing. ESI 9: optimisation of parameters for extract washing being the stirring time (a), volume of DSB solution (b), and the volume of barium acetate solution (c). See DOI: 10.1039/c4ay01695e

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the stacked solutions are pushed through one lateral port of the SV to a detection flow cell. The reaction product is formed where the samples and reagent solutions penetrate each other by dispersion during aspiration and flow reversal. Since the procedure is exactly reproduced, quantification is possible even prior to reaching the reaction steady-state.

To date, hundreds of reported SIA applications have demonstrated the great potential of this technique and scientists' appreciation of its prominent features, such as simplicity of instrumentation, versatility of operation, and robustness. Comprehensive reviews and technical treatises on SIA can be found elsewhere.[2–5]

In SIA, the only solution ever allowed to enter the syringe pump is the carrier solution, generally water. Consequently, the HC has to be long enough to avoid syringe contamination by any solution aspirated from the SV. Otherwise, pump cleaning after each analysis would be required with an unacceptable share of the whole time of the procedure.

However, mixing large with small volumes of solutions in a HC of typically 0.8 to 1.5 mm inner diameter (id) is limited by the small contact area and imperfect penetration of solutions. Hence, when large volume ratios are favorable, such as to perform dilutions or liquid–liquid extractions (LLE), a mixing chamber connected to one lateral port of the SV is often used.[6–8] Nevertheless, cleaning of such a chamber also requires considerable time. First, the chamber has to be emptied, and then completely filled with a cleaning solution, followed by the re-aspiration of the chamber's content, and its final discharge.

An ingenious approach from the GlobalFIA company (Fox Island, WA, USA, http://www.globalfia.com) is a mixing chamber, which is shaken by a computer-controlled motor. Only one fraction of cleaning solution is required and standard extraction procedures can be patterned exactly while being sped-up and miniaturized.

In the last two years, the idea of using a syringe as a mixing and reaction vessel for SIA has been revisited. In 2012, Maya *et al.*[9] demonstrated in-syringe dispersive liquid–liquid microextraction (DLLME[10]) of benzo(*a*)pyrene from the water sample on a multi-syringe flow system. For this, a mixture of 1 : 9 parts of octanol and acetonitrile was aspirated into the syringe followed by rapid aspiration of the sample, which causes the disruption of the solvent mixture into fine droplets with later coalescence of the enriched octanol at the top of the syringe.

The special feature of a syringe as a reaction and extraction vessel is its size-adaptability facilitating the separation of organic and aqueous phases as well as posterior cleaning, since only a part of the syringe has to be filled with cleaning solution.

In the following work analytical reactions prior to in-syringe DLLME were included.[11–13] However, to achieve the mixing of the large volume of the sample with reagents, an additional external mixing chamber had still to be used. Therefore, using a magnetic stirrer inside the syringe[14,15] was a break-through for the "Lab-In-A-Syringe" technique since homogeneous and, more importantly, reproducible mixing is achieved within seconds.[16] The kinetic energy from the stirrer further enables efficient in-syringe stirring-assisted DLLME.[17] Detailed synopsis of DLLME and related techniques can be found elsewhere.[18,19]

An important drawback of this approach is the dead volume inside the syringe (to allow rotation of the magnetic stirring bar) and the HC, which therefore is made as short as possible. Besides, straightforward automation of standard extraction protocols should also allow the use of typical extraction solvents denser than water such as chloroform ($CHCl_3$) to improve comparability of methods. $CHCl_3$ has an over ten-times lower viscosity compared to previously used octanol and hexanol[9,11–16] and a greater difference in density towards water, which bears the potential of faster phase separation and droplet coalescence after DLLME.

In this work, we demonstrate the use of $CHCl_3$ for in-syringe stirring assisted DLLME for the determination of disulfine blue active substances (DBAS). Hereby, the syringe pump had to be used up-side down, which implied that air will accumulate in the syringe.

This resulted in the welcome benefit that all liquid could by expulsed from the syringe, which in turn facilitated automation of secondary procedure steps such as washing of the extraction solvent.

The DBAS index is the standard procedure for evaluation of the concentrations of quaternary ammonium cations (quats), which can be extracted as an ion pair with disulfine blue (DSB) into $CHCl_3$.[21] Quats are widely used as disinfectants, cationic surfactants (CS), or softeners and show in part microorganism toxicity.[22,23] Environmental accumulation can be due to adsorption on negatively charged surfaces such as clay particles. Control of waste-water effluents and better understanding of their environmental behavior have driven over decades the development of a new analytical procedure for their determination.

As a sum parameter, quats are mostly measured as an ion-pair with acidic dyes after LLE, where the DBSA index seems the most accepted one but with the costs of a large consumption of harmful $CHCl_3$.[22] Using FT, either LLE downscaling including the use of alternative anionic dyes to DSB[24,25] or alternative procedures even omitting LLE have been proposed, taking advantage of complex formations and absorbance enhancement during ion-pair formation.[26–28]

A relevant problem is the presence of anionic species, especially anionic surfactants (AS), which compete in on-pair formation and lead to analytical underestimations. Combination with or sole use of solid phase extraction has therefore been reported as useful to suppress and is also part of the sample preparation of the DBSA index.[21,28] Titration and membrane-based extraction protocols have been proposed further.[29–31] A synopsis about the determination of surfactants on HPLC but including a comprehensive section about sample pretreatment is further given elsewhere.[32]

In this work, we studied extract washing to decrease the overall interference of the procedure. Compared to the standard procedure, miniaturization and considerable reduction of the required volumes of the solvent and sample in combination with a large pre-concentration factor was demonstrated.

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Analytical Methods

2. Methods and materials

2.1. Reagents

All reagents were of "pro analysis" grade and bidistilled quality water (resistivity > 18 MΩ cm) was used throughout for solution preparation. All glassware and polyethylene bottles used were rinsed with water prior to use.

Stock solutions of 2 mmol L^{-1} cetyltrimethylammonium bromide (CTAB) in 20 mmol L^{-1} NaOH and 5 mmol L^{-1} sodium dodecylsulfonate (SDS) in water were prepared. Working standards were prepared daily by appropriate dilution. Sodium acetate buffer of 2 mol L^{-1} was prepared and adjusted with acetic acid to pH 5.0 and used as reagent 1. A stock solution of 10 mmol L^{-1} DSB (acid blue I) was prepared in 50% v/v ethanol. A 1 : 10 dilution was then used as reagent 2 for all experiments.

A barium acetate solution of 200 mmol L^{-1} was used as reagent 3 to decrease the interference of AS. Stock solutions of 200 μmol L^{-1} of other quaternary ammonium compounds were prepared for comparative studies given in Table 1. Didodecyldimethyl ammonium bromide, tetradecyltrimethyl ammonium bromide, tetradecyl trimethyl ammonium bromide, tetraethylammonium iodide, tetrabutylammonium hydroxide, tetramethylammonium iodide, and *N*-dodecyl-*N*-methylephedrinium bromide were purchased from Sigma Aldrich (Prague, Czech Republic). Carbethopendecinium bromide was purchased from Dr Kulich Phrama (Hradec Králové, Czech Republic). Dodecyl isochinolinium bromide and dodecylpyridinium bromide were products from synthesis as described elsewhere.[33,34]

The following compounds were used for interference studies with concentrations given in Table 2 being NaCl, KCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, $Pb(NO_3)_2$, $AlCl_3$, $CuSO_4 \cdot 7H_2O$, $MnCl_2$, $ZnCl_2$, NaH_2PO_4, NH_4NO_3, $NaHCO_3$, and Na_2SO_4.

Water-free methanol, toluene, and dichlorodimethylsilane were used for silanization of the detection flow cell described in Section 2.4. A mixture of 5% v/v *n*-hexanol in $CHCl_3$ was used as the extraction solvent unless not stated otherwise.

For method characterization, well, tap, mineral, and lixiviate water samples were collected in 1 L polyethylene flasks. Particles were let to sediment before aliquots were taken for analysis.

Table 1 Relative response of different quaternary ammonium compounds compared to CTAB at a concentration level of 600 nmol L^{-1} using procedure 1. All solutions were prepared with ultrapure water

Compound	Rel. response to CTAB [%]
Didodecyldimethylammonium bromide	55.1 ± 2.9
Tetradecyltrimethylammoniumbromide	110 ± 3.4
Tetrabutylammonium hydroxide	3.39 ± 0.3
Tetraethylammonium iodide	1.55 ± 0.1
Tetramethylammonium iodide	3.56 ± 0.3
Carbethopendecinium bromide	3.95 ± 0.1
N-Dodecyl-*N*-methylephedrinium bromide	134 ± 4.5
Dodecylisochinolinium bromide	59.0 ± 0.6
Dodecylpyridinium bromide	58.4 ± 1.1

Table 2 Results of study of interference. To a CTAB standard of 1.2 μmol L^{-1}, the listed compounds at the given concentration level were added. Procedure 1 refers to simple extraction, procedure 2 refers to extraction plus organic solvent washing with water and subsequent with barium acetate and DSB. Relative response values compared to a CTAB standard prepared with ultrapure water of equal concentration are given

Compound	Concentration [mmol L^{-1}]	Rel. response (procedure 1)	Rel. response (procedure 2)
NaCl	100	139%	106%
KCl	50	133%	105%
$MgCl_2$	5	147%	119%
$CaCl_2$	2	142%	109%
Fe^{3+}, Pb^{2+}, Al^{3+}, Cu^{2+}, Mn^{2+}, Zn^{2+}	Each 50×10^{-3}	421%	136%
NaH_2PO_4, NH_4NO_3	Each 0.1	121%	101%
$NaHCO_3$	10	91%	103%
SDS	0.6×10^{-3}	14%	23%
Na_2SO_4	10	97%	98%

2.2. Manifold configuration

The manifold is depicted in Fig. 1a with tubing dimensions indicated. PTFE tubing of 0.8 mm inner diameter (id) was used for the entire manifold.

The computer controlled flow setup comprised a 16 000-step multisyringe pump (MS) and the rotary 8-port SV (Sciware Systems SL, Palma de Mallorca, Spain) for liquid handling and distribution. For sample measurement and interference studies, a rotary autosampler from the same company was used. The MS was equipped with one glass syringe of 5 mL purchased from Hamilton Bonaduz AG (Bonaduz, GR, Switzerland, Model 1005 TLL-SAL SYR). A three-way solenoid head valve (V) on-top of the syringe enabled the connection to either the central port of the SV (position ON, activated) or to the detection cell and downstream located waste for quantification of the extracted analyte as well as for discharge during syringe cleaning (position OFF, deactivated). Peripheral ports of SV were connected to reservoirs of waste (1), air (2), water (3), sample (4), reagent 1 (5), reagent 2 (6), $CHCl_3$ (7), and reagent 3 (8). A HC of 35 cm

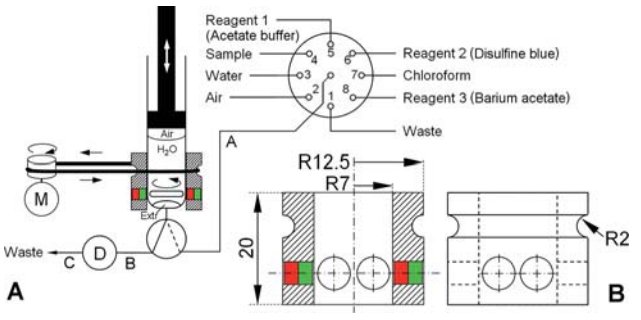

Fig. 1 (A): analyzer manifold with the selection valve (SV), syringe (S), solenoid 3-way head valve (V), detection flow cell (D), and DC motor (M). PTFE tubing (0.8 mm id) A: 35 cm, B: 10 cm, and C: 40 cm. (B): the magnetic stirring bar driver design given in detail consisting of a Delrin® tube and two neodymium magnets.

connected the central port of the SV to the syringe head valve in position ON.

A magnetic stirring bar (10 mm × 3 mm) was placed inside the syringe allowing homogeneous solution mixing and dispersion of the extraction solvent. The position of the syringe piston was adjusted to leave a gap of 4 mm on complete emptying, so that the stirring bar could freely rotate.

The syringe module was used upside-down to use an extraction solvent of higher density than water. This implied the advantage that an air cushion would remain inside the syringe, which displaced all liquid from the syringe on emptying and by this it reduced the dead volume to be cleaned between two analyses.

2.3. Stirring bar driver

Due to the fact that the stirring bar would remain at the same position inside the syringe, *i.e.* just above the inlet, the magnetic stirring bar driver used in previous studies could be simplified.[14,15] It consisted of a Delrin® tube of 20 mm in height, 25 mm outer diameter (od), and 14 mm id, which was placed over the syringe glass barrel and could rotate freely around the syringe in a longitudinal axis. Additional holes permitted the observation of the stirring bar inside the syringe.

As shown in Fig. 1b, the device held two oppositely faced neodymium magnets (5 mm × 4 mm od) and a groove for an elastic rubber band to impel the driver with a direct current (DC) motor (see Fig. 1a). The magnets were strong enough to levitate the stirring bar inside the syringe, so that the friction force was low, and to assure that even at high rotation speeds, the stirring bar would not gambol.

The DC motor was supplied *via* a homemade relay and a regulation circuit board (ESI1†). It enabled the choice of two different stirring speeds by activation of either two auxiliary analog supply ports (control voltages U1 and U2) of the MS module. The lower stirring velocity (U1 and U2 in ON) was adjusted to allow homogenization of the liquid content inside the syringe without vortex formation (*ca.* 1000 rpm). A higher speed (U1 in ON, U2 in OFF) was applied for DLLME to disrupt the organic solvent into fine droplets (*ca.* 3000 rpm).

2.4. Detection equipment and parameters

The software AutoAnalysis 5.0 (Sciware Systems SL) was used for operation control of the flow instrumentation as well as data acquisition from detection equipment and later data treatment. The program, written in Delphi and C++, allows the definition and execution of instruction protocols, including the use of variables, loops, waiting steps, and procedures on windows based user surface. A detailed description of the software structure and features is given elsewhere.[35]

A flow cuvette of 1 cm optical path length and 1.5 mm flow channel diameter from Hellma Analytics (Müllheim, Germany) was used throughout. The cell was connected *via* a 10 cm long PTFE tube of 0.8 mm id to the syringe head valve in position OFF. Downstream, a 50 cm long PTFE tube allowed solution discharge to waste.

The flow cuvette was placed in a CUV-UV fiber optic cuvette holder including collimating lenses and connected directly to a miniature USB2000 spectrometer, both from OceanOptics (Dunedin, FL, USA). A Vio High Power White LED from GE Lighting was used as a stable light source of wide emission spectrum (400 nm to 700 nm), and supplied by a constant current source (Sciware Systems SL).[36]

Absorbance measurements were performed at an analytical wavelength of 638 nm and corrected against the absorbance value measured at a reference wavelength of 550 nm on which DSB does not show any significant absorbance.

To improve the wettability of the cuvette walls by the organic phase and by this to obtain low baseline noise, silanization of the cuvette was done. For this, methanol and toluene were dried by the addition of water-free Na_2SO_4. The cuvette was cleaned with Piranha solution and then left to stand filled with 2 mol of NaOH for 1 h. Following, the solution was flushed subsequently with water, methanol, and toluene. Then, the cuvette was blown dry by nitrogen flow and a 1 : 10 mixture of dichlorodimethylsilane and dried toluene was let to react with the surface hydroxyl-groups for 10 min. Finally, the cuvette was flushed with methanol.

2.5. Analytical protocols and methods

Two different procedures were used: firstly, direct analyte extraction (procedure 1) and secondly including extract washing with water and subsequently with barium acetate and DSB (procedure 2). The procedures are given as ESI 2 and 3.† The operation scheme and photo documentation are further given in Fig. 2 and ESI 4,† respectively. Both procedures started with the cleaning of the syringe by threefold aspiration of 0.6 mL of the sample or the respective standard solution from the SV under high speed stirring and dispensed through the head valve position OFF to waste.

Then, buffer, DSB solution, and the sample were aspirated into the syringe under low speed stirring for homogenization. Then, the required volume of the organic phase was aspirated followed by a volume of air being large enough to fill the HC, so that the organic phase entered the syringe completely. High speed stirring was done for 35 s for DLLME. Here, it was found

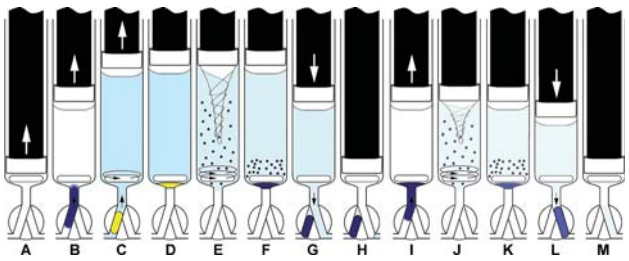

Fig. 2 Operation scheme of extraction with simple extract washing. Aspiration of the sample, buffer, and DSB (a & b), mixing (c) and aspiration of ExtrS and air (d), MSA-DLLME (e), sedimentation of ExtrS (f), saving ExtrS in HC and discharge of the aqueous phase to waste (g & h), aspiration of DSB, barium acetate, and water (i), washing of ExtrS by MSA-DLLME (j), sedimentation of ExtrS (k), propelling ExtrS to detector (l), syringe content discharge to waste (m).

advantageous to start and end with 5 s of stirring at lower speed to overcome the inertia of the solution at starting and to improve posterior droplet coalescence, respectively.

After phase separation and droplet coalescence, either the organic phase was pushed slowly through the detection cell followed by emptying the syringe completely at a high speed (procedure 1) or, for extract washing, the organic phase was pushed into the HC, and then, the remaining liquid was dispensed through the detection cell to waste (procedure 2).

In procedure 2, the extract was re-aspirated into the syringe together with water, barium acetate, and DSB solution, followed by another DLLME step, phase separation, and then measurement. An additional washing step with pure water was done equally before performing the extraction step with barium acetate. A 40 μL larger volume of the organic solvent was required for procedure 2 since a part of the organic phase would dissolve in the aqueous sample and washing solutions.

3. Results and discussion

3.1. Preliminary considerations on system design and extraction solvent

In contrast to the first applications,[14,15] in the present work, in-syringe magnetic stirring assisted dispersive liquid–liquid microextraction (IS-MSA-DLLME) was studied with the syringe placed up-side down. This approach is similar to recently described piston-propelled flow-batch but uses the commercially available and instrumentation of a simple SIA system, *i.e.* a syringe pump and SV.[37,38] The approach implied several changes in the operation characteristics but also offers new potentials and possible applications.

First, trapping of air bubbles in the syringe had to be taken into account. To keep this process reproducible, the remaining dead volume when the syringe piston is in down position, given by the space required for free rotation of the magnetic stirring bar, was allowed to be air.

Consequently, the syringe could be emptied nearly completely, leaving alone any adhered liquid film on the surfaces. So, the syringe and HC cleaning required less than half the time and sample volume than in the previous studies.[14,15] On the other hand, any solution handling required a posterior waiting time of 2 s due to the compressibility of the air inside the syringe and consequently delayed solution movement.

Second, the stirring bar is always located in the same position in the syringe, as it is not moved by the syringe piston. This fact allowed using a much simpler stirring bar driver than in the previous studies and only two small neodymium magnets were sufficient to levitate the stirring bar inside the syringe, minimizing any friction.[14,15]

Third, the chosen syringe orientation required the use of a halogenated solvent of density higher than water. While solvents lighter than water as used previously are less toxic than halogenated ones,[10,12–16] $CHCl_3$ shows some important advantages. Firstly, $CHCl_3$ is used as an extraction solvent in many standard procedures as well as for DBAS, so it is likely to yield good comparability. Secondly, the present automated procedure allows reducing the required volume of $CHCl_3$ greatly and by this the environmental impact compared to standard procedures. Finally, $CHCl_3$ has a ten-times lower viscosity than the previously used 1-hexanol,[12–15] while the relative density difference to water is larger than for 1-hexanol, accelerating phase separation and solvent droplet coalescence after DLLME.

3.2. Preliminary experiments

Using pure $CHCl_3$ as the extraction solvent, the signals were irregular and did not show the expected rectangular shape. It was proven, that this was not due to inhomogeneity of the organic phase after droplet coalescence but due to an insufficient wetting of the flow cell inner walls with the organic solvent. Therefore, cell silanization was done to yield higher hydrophobicity (see Section 2.4.).

Since signal improvement was not sufficient, the addition of *n*-hexanol to $CHCl_3$ was tested as a "sticky" additive. It was found, that a plateau-like signal shape was obtained for hexanol concentrations between 2.5% v/v and 10% v/v with best reproducibility found for 5% v/v, which was used as an additive further on.

By a stepwise increase of the volume of solvent it was found that a volume of 220 μL was required for efficient droplet formation. Also, for smaller volumes, signal reproducibility decreased and especially droplet coalescence was incomplete, so that a small amount of the organic phase could remain in the syringe. A 40 μL larger volume was required when organic phase washing was done as about 20 μL were lost by dissolution in the aqueous phase in each washing step. A larger volume of the organic phase would have required a larger holding coil (undesired increase of the system's dead volume) and have led to a signal decrease (dilution of the organic phase).

A typical peak sequence under optimised conditions is given for both procedures in ESI 5.† It can be seen, that with a higher analyte concentration, the signal plateau shows more and more inclination. This is due to the fact that a small volume of water remains in the cuvette from the initial syringe cleaning, which causes that the signal is initially lower until the water is pushed out by the solvent.

The phase separation time was tested over the range of 15 to 35 s using a 500 nmol L^{-1} CTAB standard. The signal did not change significantly but the reproducibility was significantly better for 35 s compared to shorter times (data not shown). Therefore, 35 s for phase separation was used in all the following experiments.

3.3. Optimization of simple extraction (procedure 1)

A Box–Behnken experimental design was chosen for the optimisation of the volumes of the sodium acetate buffer and DSB stock solution as well as the extraction time in the ranges of 50–250 μL (40 to 190 mmol L^{-1} acetate), 50–250 μL (12.5 to 62.5 μmol L^{-1}), and 15 to 45 s, respectively. A 1 μmol L^{-1} CTAB solution (4.1 mL) with the addition of 0.2 μmol L^{-1} SDS was used to favour conditions under which the selectivity against the interference of AS would be improved. As desirabilities, the reproducibility and the difference between standard signal to

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blank signal (water) were used. The results and conditions are given in ESI 6.† A positive dependency was found for all parameters, but was most pronounced for the extraction time.

In the following, univariant studies were done to optimize all parameters, starting with the extraction time as the parameter with the largest effect and using the adapted concentrations of buffer and DSB. The results and experimental conditions for each study are given in ESI 7a–c.†

First, it was found, that the extraction time had no significant effect on the blank signal while the signal for the standard increased from 15 to 50 s but following a saturation behavior and did not change significantly for times longer than 40 s. As a compromise between time of analysis and signal height, 35 s were chosen for further work.

For the final buffer concentration, the signal height increased for low concentrations but did not change significantly beyond 200 μL, while the blank value decreased slightly and in an approximation linearly with higher buffer concentrations. A volume of 250 μL corresponding to a concentration of 190 mmol L^{-1} was therefore chosen for further work.

Finally, the blank signal increased linearly with higher concentrations of DSB while for the standard, a clear maximum was found. A stock solution volume of 150 corresponding to 36.6 μmol L^{-1} DSB in the final mixture was therefore chosen. To sum up, the univariant studies confirmed the results from the prior experimental design.

3.4. Optimization of extraction with extract washing (procedure 2)

The standard procedure for DBAS demands for AS and anion separation on an anionic exchange resin with subsequent elution of potentially retained CS with methanol, eluate reduction by evaporation, and finally carrying out the ion-pair extraction with DSB.[21] In this work, washing of the organic phase was done to reduce the interference level. Ba^{2+} was tested as a promising cation to complex interfering anions and to decrease their interaction with the analyte. For this, the syringe was emptied with the organic phase stored in the HC, and then washed inside the syringe with a mixture of barium acetate and DSB solution. For extract washing, certain volumes of DSB and barium acetate stock solutions were mixed with 2 mL inside the syringe, denoted as the washing mixture in the following.

For the optimisation of the volume of barium acetate and DSB stock solutions, again a Box–Behnken design was chosen in the ranges of 30–150 μL (2.8 to 13 mmol L^{-1}) and 50 to 250 μL^{-1} (23.5 to 107 μmol L^{-1}), respectively. The results and experimental conditions are given in ESI 8.†

For this and later univariant studies of the parameters, a standard of 500 nmol L^{-1} CTAB plus 250 nmol L^{-1} SDS were used. The height of the signal obtained with this solution was taken as desirability. For both parameters, optima were found within the working domain, which were then used for univariant studies.

The experimental conditions and results of the univariant studies for the procedure of organic solvent washing are given in ESI 9a–c.† A linear signal increase for a standard of 500 nmol L^{-1} CTAB plus 250 nmol L^{-1} SDS with the washing time was found, while the influence on the blank signal was insignificant. This proves that a longer extraction time decreases, while only slightly, the SDS interference. As a compromise between the time of analysis and the signal height, 50 s were chosen for organic phase washing.

For the amount of DSB, a linear signal increase was found for the blank while a saturation curve was found for the standard signal. For volumes below 250 μL, the standard signal increase was larger than for the blank, indicating that the influence of not using DSB would have led to the loss of the analyte. Therefore, a volume of 200 μL corresponding to a final DSB concentration of 88 μmol L^{-1} was used in the following. The effect of barium acetate on the blank signal was not significant, thus, extraction of an ion-pair between Ba^{2+} and DSB did not occur. However, addition of barium acetate to the washing mixture yielded an up to 33% increase of the standard signal with a slight signal decrease for concentrations beyond 13 mmol L^{-1}. Hence, this concentration, *i.e.* 150 μL of the stock solution, was chosen for future work.

Although the system configuration allowed emptying the syringe completely, it was noticed, that a minimum amount of sample would remain as a liquid film on the surfaces. To avoid carry-over of sulfates or carbonates, which could lead to precipitation with Ba^{2+} and interfere the determination, an additional washing step of the syringe with water but under low speed stirring was included.

3.5. Response to other quats and interference study

For characterization of the method's response to different quaternary ammonium compounds, other quats, mostly CS, were tested. Solutions of 600 nmol L^{-1} were prepared for each single compound with ultrapure water and their respective extraction efficiency evaluated by comparing the responses with the one obtained with a CTAB standard solution of equal concentration. The results are given in Table 1.

Most compounds gave less signal than CTAB and in tendency, the extraction efficiency decreased, as expected, with shorter alkyl-chain length. In a former work, equal molar responses were achieved for different CS but careful adjustment of methanol as an additive to the aqueous phase had to be made, which also would be a significant variation from the standard procedure.[24]

To study the interferences, the two procedures were tested on standard solutions including compounds in concentrations equal or higher than found in natural water samples. The results are given in Table 2. It can be seen that, using procedure 1 pattering the DBAS standard procedure, *i.e.* simple extraction, most tested compounds showed a strong interference while applying extract washing and the interference level was considerably reduced. The most notable interference was still observed from SDS, which suppressed the signal significantly by competing in the ion-pair formation with DSB. However, a considerable improvement, *i.e.* a signal increase, of about 60% was achieved by extract washing with water and barium acetate.

As expected, the interference from larger and higher charged cations and especially the transition metal cations – well-known

to form stable complexes with many organic reagents – was significantly larger even at a lower concentration level than for the well-soluble alkali halogen salts NaCl and KCl. Extract washing with barium acetate solution especially decreased the interference of hydrogen phosphate and hydrogen-carbonate most-likely due to the formation of insoluble precipitates, while for the cations the washing effect or "leaching" of the extraction solvent by the washing solution is supposed to be the cause of interference decrease.

A possible approach to improve the method could be the use of a less hydrophilic dye and thus a stronger ion pairing reagent such as Erythrosine B.[31]

Recently we found in a work using in-syringe DLLME for the determination of AS based on ion-pairing with methylene blue that the relationship between the NaCl concentration and the blank signal was linear. It is therefore reasonable to assume that for lower concentrations than the used ones in this study, a proportional decrease of the interference level would be observed.[20]

3.6. Analytical performance and sample analysis

The finally chosen parameters and evaluated analytical performance are summarized in Table 3. Important benefits of the proposed system and method were the complete automation and miniaturization of the extraction procedure adopted from DBAS protocol. Only 220 μL of the solvent mixture and 4 mL of the sample were required for the simple extraction procedure, while the standard procedure requires several tens of milliliters of chloroform. In addition, using an automated system, open handling of harmful chloroform, sample transfer, or cleaning of the glass material are avoided.

Performing organic solvent washing, the method towards the sample matrix was considerably improved although to the cost of a prolonged time of analysis, a 40 μL larger volume of chloroform, and about 20% lower sensitivity (calculated from calibration slopes).

The method was highly sensitive with limits of detection below 20 nmol L^{-1} for both procedures. The procedure repeatability was 4% and a linear working range up to 800 nmol L^{-1} was achieved. An extension is straightforward by simply using a smaller volume of the sample and carrying out in-syringe sample dilution with water.

The results of the analysis are given in Table 4. It can be seen, that the DBAS index expressed as the concentration of the CTAB surfactant in the untreated samples was generally in the range of the LOQ. Using both procedures, the blank values decreased with organic solvent washing while for samples spiked with a CTAB standard, the signal and analyte recovery increased throughout.

The analyte recovery with procedure 2 was generally within acceptable limits, *i.e.* 90–104%, however, a recovery value of 85% was found for the lixiviate. Lower recovery values were most-likely related to analyte adsorption to particulate organic matter, clay particles, or due to interference of present AS.

An extraction efficiency of >95% and a preconcentration factor of 22.7 for a 4 mL sample (17 for 3 mL) were achieved. The final solvent volume (175 μL) was calculated from the flow rate during the measurement step, peak width (7 s), the sensitivity (slope), the used volume of sample, and the molar extinction coefficient of DSB of about 47 000 AU L mol^{-1}.[39]

In comparison with prior indicated applications using FT for the determination of CS, the excellent sensitivity and low detection limit of 12 nmol L^{-1} (4.4 ppb) should be pointed out, which were found to be superior to the former studies. On the other hand, one analysis required a significantly longer time due to batch-wise operation and employing both analyte extraction and extract washing.

Non-extractive methods can operate with higher repeatability and at measurement frequencies at 60 h^{-1} to 140 h^{-1} but to the cost of much lower sensitivity.[24,26,27] A similar performance in respect of time and sensitivity was achieved by Lindgren and Dasgupta (1992) while an interference study was missing in this work.[25] It should be pointed out that none of the given methods followed the standard procedure for the determination of DBAS, which could make a comparison of the results for complex matrices rather difficult.

Table 3 Optimized conditions and analytical performance of the proposed procedures for the determination of DBAS. Organic solvent composition was 5% v/v *n*-hexanol in chloroform

Parameter	Procedure 1	Procedure 2
Organic solvent consumption	220 μL	260 μL
Sample volume[a]	4 mL	4 mL
Sodium acetate (3.1 mol L^{-1})	250 μL	250 μL
Disulfine blue (1 mmol L^{-1})	150 μL	150 μL + 250 μL
Barium acetate (200 mmol L^{-1})	—	150 μL
Time of analysis	240 s	545 s
Sample frequency	15 h^{-1}	6.6 h^{-1}
Average repeatability	3.3% RSD	3.5% RSD
Limit of detection	16 nmol L^{-1}	12 nmol L^{-1}
Limit of quantification	52 nmol L^{-1}	41 nmol L^{-1}
Linear working range[a]	Up to 0.8 μmol L^{-1}	
Calibration function (3 mL sample)	750 mAU L $μmol^{-1}$ c + 47.5 mAU	622 mAU L $μmol^{-1}$ c + 91.3 mAU

[a] Due to in-syringe stirring, in-system sample dilution with water can be carried out to extend the linear working range. For this, the possible 4 mL are put together from sample and water.

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Table 4 Results from sample analysis using simple extraction (procedure 1) and extraction with organic solvent washing with water and subsequent with barium acetate (procedure 2) under the optimized conditions given in Table 3[a]

P	Sample	Addition CTAB [μmol L^{-1}]	Found CTAB [μmol L^{-1}]	Rel. Recovery [%]
1	Well water 1	—	0.028	73.6%
		0.500	0.396	
2	Well water 1	—	0.036	92.1%
		0.500	0.496	
1	Well water 2	—	0.094	82.2%
		0.600	0.587	
2	Well water 2	—	0.077	97.6%
		0.600	0.662	
2	Well water 3	—	0.034	99.1%
		0.500	0.529	
1	Lixiviate	—	0.127	62.8%
		0.600	0.504	
2	Lixiviate	—	0.077	84.8%
		0.600	0.589	
1	Tap water 1	—	0.084	94.2%
		0.600	0.649	
2	Tap water 1	—	0.064	104%
		0.600	0.688	
2	Tap water 2	—	0.031	102%
		0.250	0.285	
2	Mineral water	—	0.039	89.7%
		0.250	0.263	

[a] P procedure.

In conclusion, the method proved to be applicable to water samples when extraction solvent washing is carried out. It could not overcome the typical AS interference and likewise require prior elimination of AS by anion exchange. However, due to the achieved miniaturization, the required amount of resin, operation time, and the volume of the solvent could be reduced and due to the high method sensitivity and possibility to perform in-system dilution of the sample with water, even solvent evaporation as part of the pretreatment step could be avoided.

4. Conclusions

An automated method for the determination of CS from water samples was reported based on a novel configuration of in-syringe analysis, in which a denser solvent than water can be applied. In-system washing of the organic solvent was facilitated by the proposed analyzer configuration and a significant reduction of interference was achieved. The method was applicable to the determination of CS in different water samples at a sub-micromolar level. The interference of AS was diminished considerably by organic solvent washing with water, DSB and barium acetate solution. Repeatability, limit of detection, and analyte recovery were adequate for environmental studies of CS and the consumption of the organic solvent and sample compared to the standard procedure was highly reduced.

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6.7.1. Supplementary information – paper 2

Figure S1. Control circuit for the DC motor used for in-syringe stirring. By using two different auxiliary analog outputs on the multisyringe device, stirring enabling and selection of two different stirring velocities were achieved.

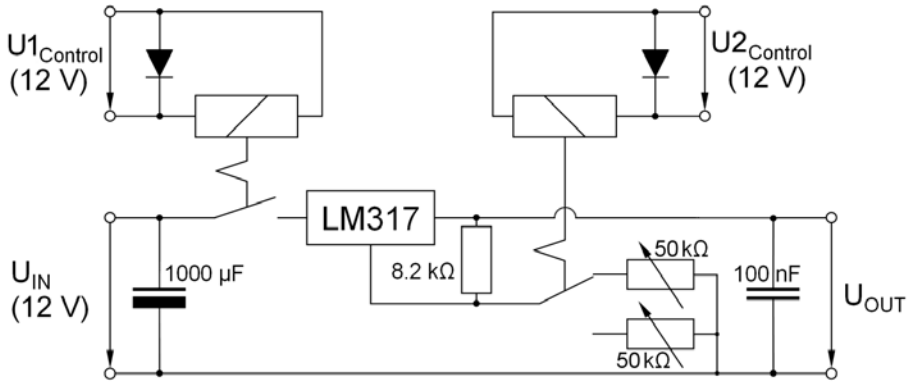


Table 2. Procedure 1 for automated in-syringe stirring-assisted DLLME of cationic surfactants without organic phase washing (simple extraction).

No	Instrument	Instruction	Comment
1a	SV	Move to position 4	
1b	MS	Pickup 0.60 ml at 7.5 mL/min [V in ON, U1 ON, U2 OFF], Wait 2 s	Clean syringe with sample with stirring at high speed, 3x repetition
1c	MS	Empty at 15 mL/min [V in OFF, U1 OFF, U2 OFF]	
2a	SV	Move to position 5	
2b	MS	Pickup 0.25 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 2 s	Aspiration of buffer
3a	SV	Move to position 6	
3b	MS	Pickup 0.15 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 2 s	Aspiration of dye
4a	SV	Move to position 4	
4b	MS	Pickup 4.00 ml at 7.5 mL/min [V in ON, U1 ON, U2 ON], Wait 2 s	Aspiration of sample with stirring at low speed
5	MS	No flow step [V in OFF, U1 OFF, U2 OFF]	Stop stirring
6a	SV	Move to position 7	
6b	MS	Pickup 0.22 ml at 2.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1 s	Aspiration of organic phase
7a	SV	Move to position 2	
7b	MS	Pickup 0.22 ml at 2.5 mL/min [V in ON, U1 ON, U2 ON]	Aspiration of air with stirring at low speed
8a	MS	No flow step [V in OFF, U1 ON, U2 OFF]	Stirring at high speed for DLLME
8b	Wait	Wait 35 s	
9a	MS	No flow step [V in OFF, U1 ON, U2 ON]	Decreasing to stirring at low speed
9b	Wait	Wait 5 seconds	
10a	MS	No flow step [V in OFF, U1 OFF, U2 OFF]	Stop stirring and phase separation
10b	Wait	Wait 35 seconds	
11a	D	Measurement at 638 nm against 550 nm	
11b	MS	Dispense 0.75 ml at 1.5 mL/min [V in OFF, U1 OFF, U2 OFF]	Dispense of organic phase to detection cell and measurement
11c	D	Stop measure	
12	MS	Priming in dispense at 15 mL/min [V in OFF, U1 OFF, U2 OFF]	Empty syringe to waste at high flow rate

Table S3. Procedures 2 for automated in-syringe stirring-assisted DLLME of cationic surfactants with double organic phase washing.

No	Instrument	Instruction	Comment
1a	SV	Move to position 4	
1b	MS	Pickup 0.60 ml at 7.5 mL/min [V in ON, U1 ON, U2 OFF], Wait 2 s	Clean syringe with sample with stirring at high speed, 3x repetition
1c	MS	Empty at 15 mL/min [V in OFF, U1 OFF, U2 OFF]	
2a	SV	Move to position 5	
2b	MS	Pickup 0.25 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 2 s	Aspiration of buffer
3a	SV	Move to position 6	
3b	MS	Pickup 0.15 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 2 s	Aspiration of dye
4a	SV	Move to position 4	
4b	MS	Pickup 4.00 ml at 7.5 mL/min [V in ON, U1 ON, U2 ON], Wait 2 s	Aspiration of sample with stirring at low speed
5	MS	No flow step [V in OFF, U1 OFF, U2 OFF]	Stop stirring
6a	SV	Move to position 7	
6b	MS	Pickup 0.26 ml at 2.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1 s	Aspiration of organic phase
7a	SV	Move to position 2	
7b	MS	Pickup 0.22 ml at 2.5 mL/min [V in ON, U1 ON, U2 ON]	Aspiration of air with stirring at low speed
8a	MS	No flow step [V in OFF, U1 ON, U2 OFF]	Stirring at high speed for DLLME
8b	Wait	Wait 35 s	
9a	MS	No flow step [V in OFF, U1 ON, U2 ON]	Decreasing to stirring at low speed
9b	Wait	Wait 5 seconds	
10a	MS	No flow step [V in OFF, U1 OFF, U2 OFF]	Stop stirring and phase separation
10b	Wait	Wait 35 seconds	
11a	SV	Move to position 2.	
11b	MS	Dispense 0.28 ml at 2.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1 s	Dispense organic phase into holding coil
12	MS	Priming in dispense at 15 mL/min [V in OFF, U1 OFF, U2 OFF], Wait 1 s	Dispense rest content of syringe to waste
13a	SV	Move to position 3.	
13b	MS	Pickup 2.00 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1 s	Aspiration of water
14a	SV	Move to position 2	
14b	MS	Pickup 0.22 ml at 7.5 mL/min [V in ON, U1 ON, U2 ON]	Aspiration of air with stirring at low velocity
15	Wait	Wait 20 s	Stirring at low speed for extract washing with water
16a	MS	No flow step [V in OFF, U1 OFF, U2 OFF]	Stop stirring and phase separation
16b	Wait	Wait 20 seconds	
17a	SV	Move to position 2.	
17b	MS	Dispense 0.28 ml at 2.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1 s	Dispense solvent into HC
18a	MS	Priming in dispense at 15 mL/min [V in OFF, U1 OFF, U2 OFF], Wait 1 s	Dispense rest content of syringe to waste
18b	SV	Move to position 3.	
18c	MS	Pickup 2.00 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1 s	Aspiration of water
19a	SV	Move to position 5.	
19b	MS	Pickup 0.15 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1	Aspiration of barium acetate

20a	SV	Move to position 6.	
20b	MS	Pickup 0.20 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF], Wait 1 s	Aspiration of dye
21a	SV	Move to position 2	
21b	MS	Pickup 0.22 ml at 7.5 mL/min [V in ON, U1 OFF, U2 OFF]	Aspiration of air with stirring at low speed
22a	MS	No flow step [V in OFF, U1 ON, U2 OFF]	Stirring at high speed for DLLME
22b	Wait	Wait 35 s	
22c	MS	No flow step [V in OFF, U1 ON, U2 ON]	Decreasing to stirring at low speed
23a	MS	No flow step [V in OFF, U1 OFF, U2 OFF]	Stop stirring and phase separation
23b	Wait	Wait 35 seconds	
24a	D	Measurement at 638 nm against 550 nm	
24b	MS	Dispense 0.75 ml at 1.5 mL/min [V in OFF, U1 OFF, U2 OFF]	Dispense of organic phase to detection cell and measurement
24c	D	Stop measure	
25	MS	Priming in dispense at 15 mL/min [V in OFF, U1 OFF, U2 OFF]	Empty syringe to waste at high flow rate

Figure S4. Example of peak signals of calibration with water standards of blank, 0.25 µmol L^{-1}, 0.50 µmol L^{-1}, and 0.75 µmol L^{-1} using 3 mL of standard and 1 mL of water. Conditions as in figure 4 C.

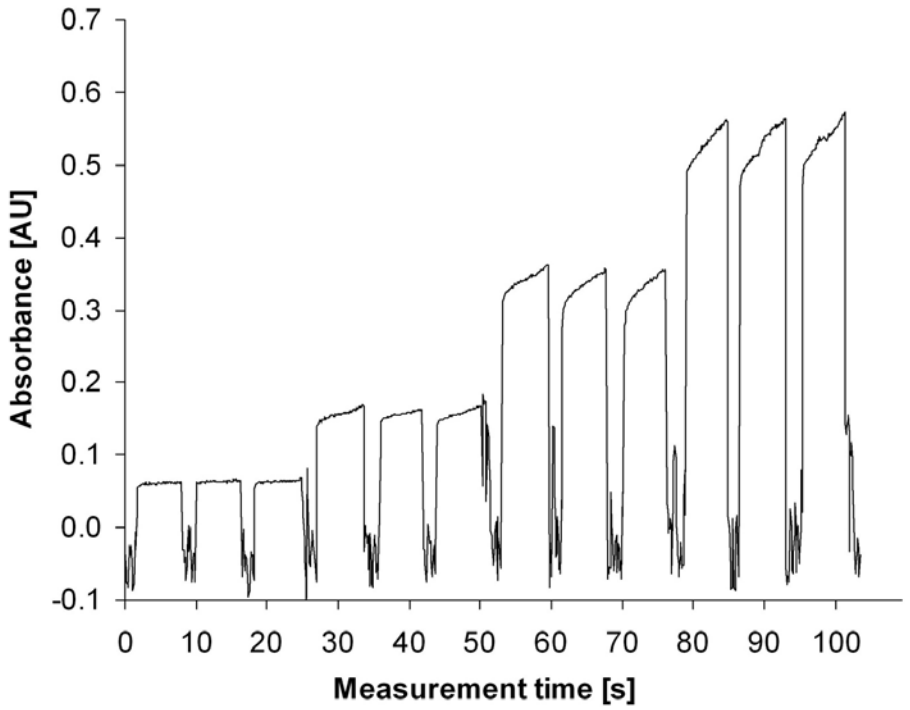


Figure S5. Box-Behnken experimental design for the optimization of the volumes of buffer and DSB stock solutions and extraction time. Conditions: blank and 4.1 mL 1 µmol L^{-1} CTAB solution with the addition of 0.2 µmol L^{-1} SDS, phase separation time 35 s.

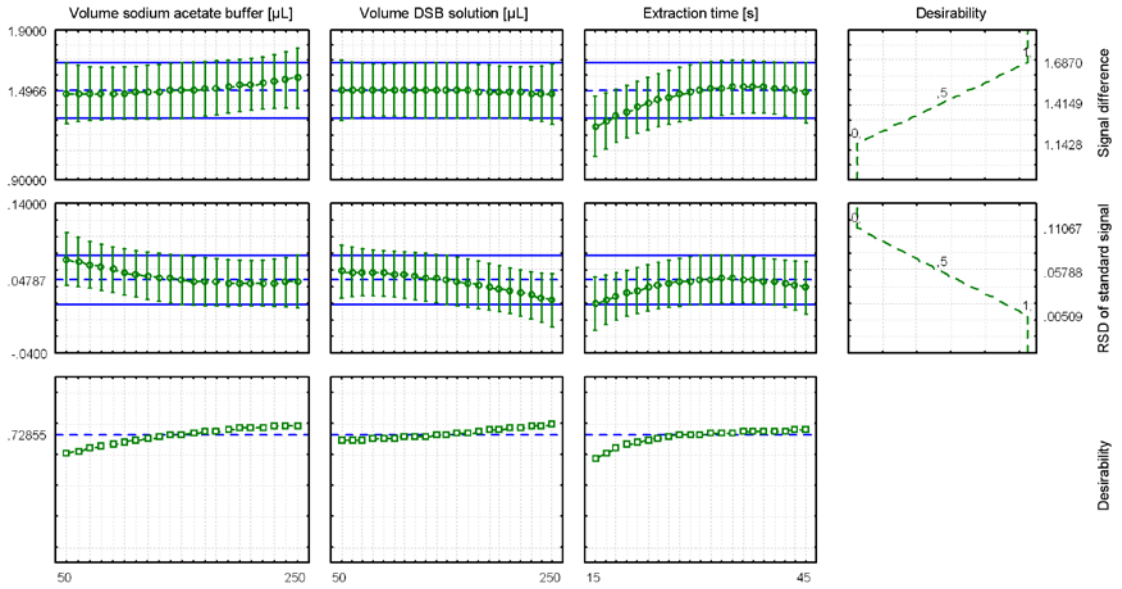
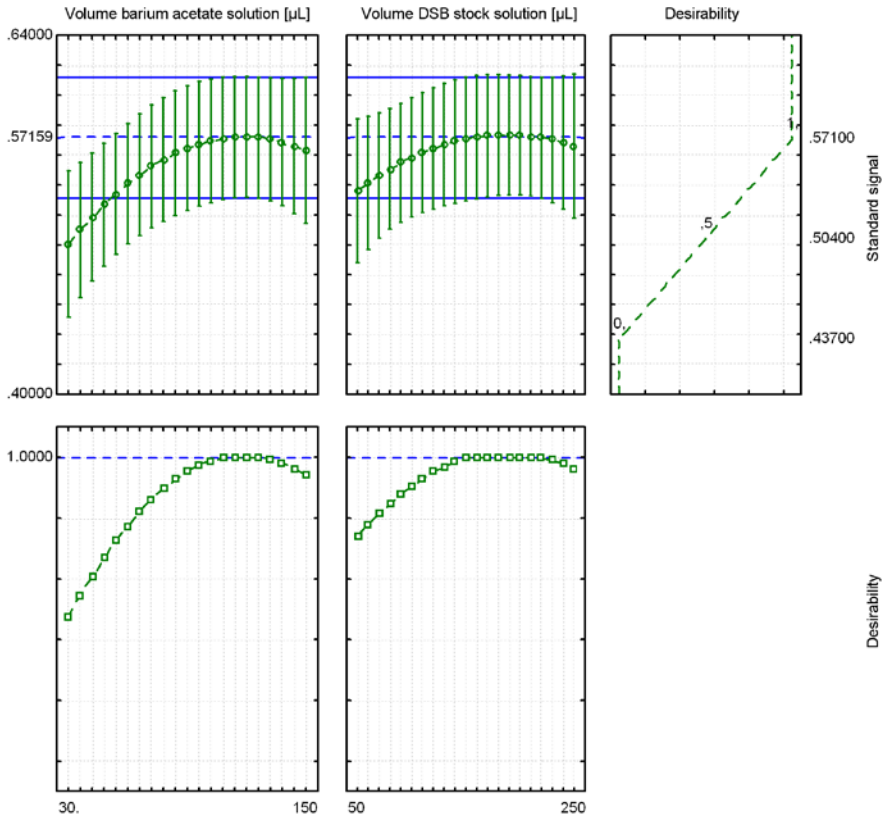


Figure S6. Box-Behnken experimental design for the optimization of the volumes of barium acetate and DSB stock solutions for extraction solvent washing. Conditions as given in figure 4 C but using 150 µL of 1 mmol L-1 DSB for the first extraction and 35 s for the organic phase washing.



CHAPTER 7

ON-LINE UV FILTERS DETERMINATION BY IN-SYRINGE MSA-DLLME COUPLED TO HPLC

7. On-line UV filters determination by in-syringe MSA-DLLME coupled to HPLC

7.1. General remarks about UV filters

It is well-known that approximately, the visible (VIS) radiation (400-760 nm) represents 44.3 % of the solar radiation reaching the Earth's surface, while 49.5% is made up of infrared (IR) radiation (>760 nm) and only 6.2 % is ultraviolet (UV) radiation (100-400 nm) because of the mitigation enabled by the stratospheric ozone layer. The UV spectrum is subdivided into three regions, the 98 % of the UV radiation is due to ultraviolet A (UVA) (320-400 nm), and the other 2% corresponds to ultraviolet B (UVB) (290-320 nm) meanwhile the ultraviolet C (UVC) (100-290 nm) which has the highest energy values, fortunately does not reach the Earth's surface.

Exposure to UV radiation in small amounts has a therapeutic effect since it improves the endogenous vitamin D production by the human body [1]. This increases calcium absorption and thus prevents osteoporosis and rickets, and also has beneficial effects on human health such as blood-pressure regulation [2]. However, in the last decades there has been a progressive increase in the UV radiation reaching the Earth's surface, due to the damage in the stratospheric ozone layer. This has considerably increased the concerns about the health risks associated with solar UV radiation exposure.

Consequently, UV filters are commonly used in many cosmetic products [3] in order to mitigate the deleterious effects of sunlight which promotes skin ageing as well as other harmful effects on human health, such as skin tumours, cutaneous photo-aging and damage to the skin's immunological system [4]. Moreover, UV filters are not only present in cosmetics products but also in textiles and plastics. This excessive use of them has lead to increase their presence in the environment, especially in the aquatic media by direct sources (e.g., sunbathing or swimming) [5] and/or indirect sources through wastewater treatment plants (WWTP) as Figure 7.1 illustrates. Due to their incomplete removal in WWTPs and continuous release, UV filters have been widely detected in surface waters [6], seawater [7], wastewaters [8], and even tap water [9].

The concern about UV filters relies on their lipophilic and stable properties that allow them to bioaccumulate in solid environmental matrices such as sediment [10], sewage sludge [11] and even in biota acting as endocrine disruptors [12]. Furthermore, once

discharged into the environment UV filters may suffer alteration resulting in highly toxic transformation products [5, 13].

For all this, UV filters have been recently catalogued as emergent contaminants. Accordingly, their monitoring in the aquatic environment has gained special attention over the last decade, and hence there is a growing need to develop sensitive and selective analytical methods for their determination at trace levels in the aquatic environment.

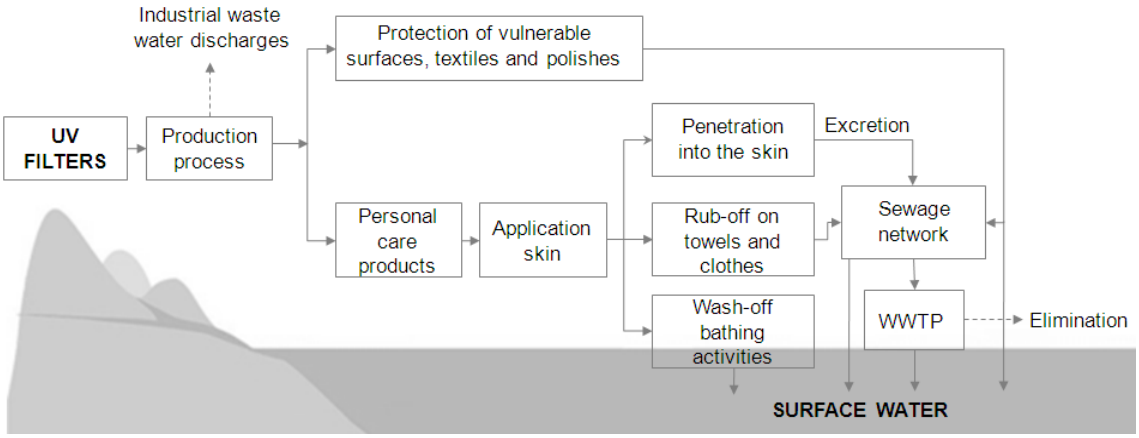


Figure 7.1 Entry pathways of UV filters into environmental water

UV filters can be classified into two groups according to their nature, i.e. inorganic and organic UV filters. The inorganic UV filters also called physical filters, include titanium dioxide (TiO_2) and zinc oxide (ZnO). These are micropigments that reflect and scatter UV radiation. These inorganic components are commonly used in the formulation of sunscreens in the form of nanoparticles [14]. Organic UV filters also called chemical filters are designed to absorb the UV radiation, and are classified into different families, e.g. benzophenone derivatives, salicylates, cinnamates, camphor derivatives, p-aminobenzoic acid and its derivatives [15]. In this study, we focussed in the determination of organic UV filters in water sample matrices.

The structures and relevant physico-chemical properties of the UV filters studied are given in Table 7.1. The most relevant properties are summarized below:

- Solubility in water: their solubility varies depending on each compound, but generally these are in the range of mg/L.

- Dissociation (pKa): the equilibrium constant defines the grade of dissociation of a compound at a certain pH. As a rule, these compounds are unlikely to dissociate at environmental pH (6–7), resulting in increased aqueous mobility, since their functional groups are not dissociated.

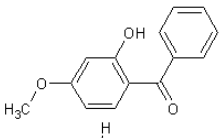
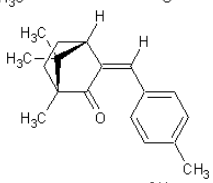
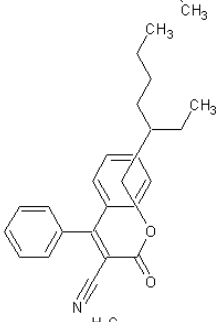
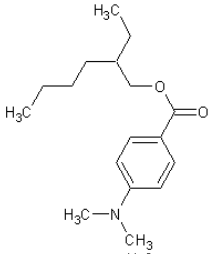
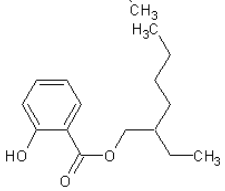
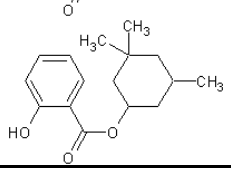
- Octanol-water partition coefficient (K_{ow})*:* this coefficient expresses the ratio of the concentration of a chemical in octanol and water at equilibrium at a specific temperature. Compounds with log K_{ow}<1 are hydrophilic and distribute into water, on the other hand, high log K_{ow} compounds bind to organic matter, sediments or biota.

- Volatility: UV filters have relatively low volatility values, from 10^{-6} to 10^{-8}atm m^3 mol^{-1}. This hinders the volatilization of the compounds from moist, dry soil or water surfaces. Thus, generally UV filters are considered non-volatile compounds.

- Absorption maximum: UV filters absorb different ranges of sun's radiation depending on their molecular structure. Commonly, UV filters absorb light between 240-326 nm.

Thus, the concern over the possible negative effects of UV filters has led to the provision of guidelines in order to minimize their negative impacts by reducing their concentration in the environment, as well as focusing efforts in designing improved substances with lower toxicity. Therefore, in order to guarantee consumers' health, the European Union (EU) 1223/2009 regulation permits the use of 26 UV filters in cosmetics and the maximum content allowed is regulated by actual legislations, between 0.1 % and 10 % (w/w) [16].

Table 7.1 Structure and physico-chemical properties of target compounds. [a]K_{ow}, octanol-water partition coefficient.

Analyte	Structure	Formula	CAS no	log K_{ow} [a]	pK_a
Benzophenone-3 (BZ3)		$C_{14}H_{12}O_3$	131-57-7	3.64	7.56
4-Methylbenzylidene camphor (MBC)		$C_{18}H_{22}O$	36861-47-9	4.95	7.56
2-Ethylhexyl 2-cyano -3,3-diphenylacrylate (OCR)		$C_{24}H_{27}NO_2$	6197-30-4	7.53	8.00
Ethylhexyl dimethyl p-aminobenzoate (EDB)		$C_{17}H_{27}NO_2$	21245-02-3	6.15	8.00
Ethylhexyl salicylate (EHS)		$C_{13}H_{18}O_3$	118-60-5	5.97	8.13
Homosalate (HMS)		$C_{16}H_{22}O_3$	118-56-9	6.16	8.09

7.2. Detection techniques for UV filters

Despite the restrictions of use established for UV filters, there isn't any official analytical method for the determination of these compounds neither in cosmetics nor in any other matrix. Therefore, since these compounds are found in the environment at low levels (µg/L or ng/L), it is necessary to develop efficient and sensitive analytical methods to control the UV filters contents and to supervise not exceeding the limits established by the current legislation. In this sense, the most used analytical technique to determine UV filters is HPLC coupled to diode array or MS detection [17, 18] since UV filters are generally non-volatile compounds. Nevertheless, other chromatographic techniques such

as GC-MS, have also been applied for this purpose [19, 20].

7.3. UV filters extraction and preconcentration techniques

UV filters are at trace levels in environmental samples. Therefore, enrichment techniques are usually employed to improve the sensitivity and LOD prior chromatographic analysis. Furthermore, sample clean-up enhances the performance of the instruments and enlarges their lifetime. Most frequently used extraction techniques for UV determination are LLE and SPE. Both, HPLC and GC have been widely combined with SPE for analyte enrichment and sample clean-up [21]. Research trends in separation science have been oriented toward minimization of the sample pretreatment steps and process downscaling. Thus, various pretreatment techniques have been successfully applied to extract UV filters from aqueous samples, e.g. solid phase microextraction (SPME) [22], SDME [6], membrane-assisted liquid–liquid extraction (MALLE) [23], HF-LPME [24] and DLLME [7].

On the other side, the use of classic organic solvents used as extractants in LPME techniques is being replaced by more green solvents, such as ionic liquids (ILs). ILs are ionic, non-molecular solvents with melting points below 100 °C. Their structure is formed exclusively by ions, in this sense ILs are completely different from classical solvents. Generally, ILs are formed by cations and anions. Cations employed are mostly organic, whereas anions may be organic or inorganic and consequently its combination results in a tremendous number of ILs and makes custom-synthesis feasible. It is estimated that there could be up to 1000 ILs available [25]

Most common cations used are: idazolium, pyridinium, pyrrolidinium, ammonium or phosphonium cations. The anions are either organic or inorganic, including: hexafluorophosphate [PF_6]; tetrafluoroborate [BF_4]; trifluoromethylsulfonate [CF_3SO_3]; bis[(trifluoromethyl)sulfonyl]amide [$(CF_3SO_2)_2N$]; trifluoroethanoate [CF_3CO_2]; acetate; nitrate and halide [26].
The physicochemical properties of ILs depend on nature and ion size of both their cation and anion constituents; including their negligible vapor pressure at room temperature, high thermal stability, and variable viscosity. Their miscibility in water depends mainly on the anions, while other characteristics such as viscosity or density depend on the cations.

Table 7.2 illustrates some physicochemical properties of some ILs commonly used in analytical chemistry [26].

Table 7.2 Some physicochemical properties of the commonly used ILs in analytical chemistry

Ionic liquid	Melting point (ºC)	Density (g/mL) 25 ºC	Viscosity (mPa s) 25 ºC	Miscibility in H_2O (g in100 mL)
[C_4MIM][PF6]	10.8	1.36-1.37	148-450	1.88
[C_6MIM][PF6]	-61	1.29-1.31	560-586	0.75
[C_8MIM][PF6]		1.20-1.23	682-710	0.20

[C_4MIM][PF6]:1-butyl-3-methylimidazolium hexafluorophosphate; [C_6MIM][PF6]: 1-hexyl-3-methylimidazolium hexafluorophosphate; [C_8MIM][PF6]: 1-octyl-3-methylimidazolium hexafluorophosphate

Thus, ILs are receiving much attention owing to their advantages over traditional organic solvents, such as negligible vapour pressure, high stability, high viscosity, moderate dissolvability of organic compounds, adjustable miscibility and polarity, good extractability for different organic and inorganic compounds, making them attractive alternatives to environmental unfriendly solvents. As a result of these properties, ILs have been considered as green solvents and have been employed in a number of analytical applications [27-30]. These have also been previously employed for the determination of UV filters due to their excellent characteristics [6, 24, 31].

7.4. Automation of a green method for UV filters determination exploiting in-syringe MSA-DLLME coupled to HPLC

As it has been mentioned throughout this thesis, flow analysis techniques play a crucial role in monitoring. In this sense, flow analysis techniques have been coupled on-line to chromatographic systems providing shorter analysis time, higher analyst safety, lower reagent consumption and low waste generation [32]. In this scenario, to the best of our knowledge there is only a previous work for automating UV filters determination [22] in which an on-line BI-LOV system was coupled to HPLC to determinate four UV filters in environmental samples.

Thus, the aim of this research was to develop a fully automated method, based on on-line in-syringe MSA-DLLME of a group of UV filters coupled to HPLC with ultraviolet detection. Thus, several green solvents, i.e. ILs, were evaluated. The extraction was enabled within the syringe containing a magnetic stirrer for homogenization of the sample and the required reagents. Afterwards, the enriched droplets of the IL accumulated at the bottom of the syringe were transferred to a loop coupled to HPLC.

The best chromatographic resolution was attained under the following conditions: C18 column (3.0 x 250 mm, Symmetry ®) and 85:15 (v/v) ACN/H_2O containing 0.5 % acetic acid as mobile phase in isocratic mode pumped at 0.8 mL/min and detected at 307 nm. Various parameters that affected the extraction efficiency were studied using multivariate optimization approach, including the type and volume of extraction and dispersive solvents, extraction and sedimentation time, ionic strength and pH. Under optimized conditions, LODs were within the range of 0.08 - 12 µg/L, for 3.5 mL sample volume. Intra- and inter-assay precision were 6 and 8%, respectively. The proposed method was successfully applied to the determination of UV filters in surface seawater and swimming pool water samples attaining recoveries in the range of 89-114 and 86-107 %, respectively.

In addition, the method permits simultaneous trace analysis of UV filters in less than 12 min for the whole process by the synchronism of the sample pretreatment and the chromatographic analysis. This is a significant improvement in analysis throughput (5 samples/h) in comparison to previously reported methods, proving its suitability for routine analysis. Furthermore, the use of an IL as extractant makes of the present automatic method an efficient and environmental friendly tool for UV-filters determination in bath waters.

More detailed information is given below in an original research paper which has been submitted for publication to an international research journal.

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7.6. Original paper

Title: On-line in-syringe magnetic stirring assisted dispersive liquid-liquid microextraction HPLC – UV method for UV filters determination using 1-hexyl-3-methylimidazolium hexafluorophosphate as extractant

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On-line in-syringe magnetic stirring assisted dispersive liquid-liquid microextraction HPLC – UV method for UV filters determination using 1-hexyl-3-methylimidazolium hexafluorophosphate as extractant

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Abstract

An environmental friendly and fully automated method using in-syringe magnetic stirring assisted dispersive liquid-liquid microextraction coupled with high-performance liquid chromatography has been developed for the determination of UV filters in environmental water samples. The main “green” features on this method are the use of an ionic liquid as extracting solvent, avoiding the use of chlorinated solvents, and the on-line microextraction, preconcentration, separation and detection minimizing the use of reagents and so the waste generation. After sample treatment, 20 µL of the organic droplet was injected onto the HPLC - UV system. Various parameters affecting the extraction efficiency were studied using multivariate optimization approach, including the quantity of extraction and dispersive solvents, extraction and sedimentation time, ionic strength and pH. Under optimized conditions, limits of detection were within the range of 0.08–12 µg/L, for 3.5 mL sample volume. Linearity ranges were up to 500 µg/L for the UV-filters studied. Furthermore, enrichment factors ranging from 11 to 23 folds were obtained. Intra- and inter-assay precisions were 6 and 8 %, respectively. Finally, the proposed method was successfully applied to determine UV filters in surface seawater and swimming pool samples attaining satisfactory recoveries over the range of 89-114 and 86-107 %, respectively.

Keywords:

Ionic liquids; in-syringe magnetic stirring assisted dispersive liquid-liquid microextraction; HPLC-UV; on-line coupling; UV filters; environmental water analysis.

1. Introduction

UV radiation has progressively increased due to the ozone layer reduction in the last decades, being an issue of great concern. This promotes harmful effects on human health such as skin cancer [1]. In order to protect human skin from direct exposure to sunlight, the use of personal care products containing UV filters has increased [2]. In addition, UV filters are not only present in cosmetics products but also in textiles and plastics. This excessive use of them has lead to increase their presence in the environment, especially in the aquatic media (rivers, lakes and coastal sea water) highlighting their potential toxicity [3, 4]. In fact, despite these are usually found in environmental waters at trace levels, they are not far below the dose that causes toxic effects in animals [5]. Therefore, UV filters have recently been classified as emerging pollutants. In order to guarantee consumers' health, the European Union (EU) 1223/2009 regulation permits the use of 26 UV filters in cosmetics and the maximum content allowed is regulated by actual legislations, between 0.1 % and 10 % (w/w) [6].

UV filters are commonly determined by chromatographic techniques [7]. Despite of the fact that gas chromatography (GC) presents higher resolution, offers good separation and lower limits of detection (LOD) for the environmental analysis of UV filters [8, 9], high performance liquid chromatography (HPLC) [10, 11] is a good option to determine UV filters since it is a more affordable technique and good performance is accomplished. Furthermore, due to the low volatility character of UV filters, HPLC is preferred because analyte derivatisation is avoided.

Coastal waters present a challenging matrix owing to the expected variety of parameters. These waters are often highly stressed due to recreational activities, being important to evaluate marine contamination in order not only to assess global effects but also to ensure their quality. Thus, sample pretreatment prior chromatographic analysis is mandatory. HPLC has been widely combined with solid phase extraction (SPE) for analyte enrichment and sample clean-up [12]. However, traditional preparation techniques are time, sample, solvent and labour-consuming. Research trends in separation science have been oriented toward minimization of the sample pretreatment steps and process downscaling. Thus, various pretreatment techniques have been successfully applied to extract UV filters from aqueous samples, e.g. solid phase microextraction (SPME) [13], single drop microextraction (SDME) [14], membrane-assisted liquid–liquid extraction (MALLE) [15], and hollow fiber liquid phase microextraction (HF-LPME) [16]. A promising method to reduce solvent, time and labour effort is dispersive liquid–liquid microextraction (DLLME), which was introduced by Rezaee et al. in 2006 [17]. DLLME is based on a ternary solvent system in which a small volume of extracting solvent is dispersed by the action of a second solvent. The

dispersive solvents, which are miscible in both aqueous and organic phases, are involved to improve and accelerate the extraction by enhancing the contact between aqueous sample solution and the extractant. Thus, fast extraction rates and high enrichment factors (EF), as well as simplicity of operation, low cost of implementation and minimal waste generation are obtained [18]. However, in some cases kinetic energy had to be implemented in order to achieve better extraction efficiencies leading to vortex-assisted [19], ultrasound-assisted [20] and magnetic-stirring-assisted (MSA) dispersion [21]. More recently, the concepts of DLLME and flow techniques (FT) were combined [22, 23]. Here, in-syringe DLLME has demonstrated to be a promising technique for the automation of DLLME [24] due to its simplicity and versatility, permitting the implementation of magnetic stirring assisted DLLME systems to improve mixing efficiency and speed up extractions [25].

Furthermore, flow techniques (FT) can be coupled on-line to HPLC by the use of an appropriate interface [26]. This usually consists of a high-pressure injection valve with a loop, whose size can be modified according to the requirements of each experiment. Reproducible injection into the chromatograph relies on accurate synchronized operation of the whole system. Most FT-HPLC coupled methods provide shorter analysis time, higher analyst safety, lower reagent consumption and low waste generation [13, 27].

Classic organic solvents used as extractants in liquid phase microextraction techniques are being replaced by ionic liquids (ILs), which are receiving much attention owing to their excellent properties [28]. ILs have various advantages over traditional organic solvents, such as negligible vapour pressure, high stability, high viscosity, moderate dissolvability of organic compounds, adjustable miscibility and polarity, good extractability for different organic and inorganic compounds, making them attractive alternatives to environmental unfriendly solvents. As a result of these properties, ILs have been considered as green solvents and have been employed in a number of analytical applications [29-32]. The low volatility of the ILs also makes of liquid chromatography a preferred option in front of GC.

The aim of this work was to develop a fast, simple, fully automated, cost-effective and environmental friendly method based on in-syringe MSA-DLLME coupled to HPLC allowing the on-line extraction, preconcentration, separation and detection of six UV filters in water samples. The main advantage of the method relies in the use an on-line sample treatment through the in-syringe MSA-DLLME system, exploiting an IL as extractive solvent instead of chlorinated solvents, becoming a greener alternative to existing methods.

2. Materials and methods

2.1 Reagents and samples

2-Hydroxy-4-methoxybenzophenone (benzophenone-3) (BZ3) 98%, 3-(4 methylbenzylidene) camphor (MBC) 99.7%, 2-ethylhexyl 2- cyano-3,3-diphenylacrylate (octocrylene) (OCR) >98%, 2-ethylhexyl 4-dimethylaminobenzoate (EDB) 99.8%, ethylhexyl salicylate (octisalate) (EHS) 99% and homosalate (HMS) obtained from Sigma Aldrich (Steinheim, Germany), were used as standards.

Standard stock solutions of each UV filter (1000 mg/L) were prepared in acetonitrile (ACN). Multicomponent working standard solutions were freshly prepared daily by proper dilution of the standard stock solutions with bi-distilled water. Bi-distilled quality water (resistivity >18 MΩ cm) was obtained using a Direct-8 purification system (Millipore, Millipore Iberica S.A.U., Spain). ACN, methanol (MeOH), acetone and acetic acid were obtained from Sigma Aldrich (Steinheim, Germany) of HPLC grade. ILs, 1-butyl-3-methylimidazolium hexafluorophosphate [C_4MIM][PF_6], 1-hexyl-3-methylimidazolium hexafluorophosphate [C_6MIM][PF_6] and 3-methyl-1-octylimidazolium hexafluorophosphate [C_8MIM][PF_6] were purchased from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid, sodium hydroxide and sodium chloride all from Scharlab SA (Barcelona, Spain) were used to adjust the pH and the ionic strength, respectively.

Surface seawater samples were collected in summer 2014 from six different beaches from Mallorca (Spain). Water samples from two private swimming pools were also analysed. Samples were collected in 500 mL Pyrex borosilicate amber glass containers with caps. They were stored in the dark at 4 ºC and filtered through 0.22 µm membrane filters just before being analysed. To avoid contamination, all glassware was soaked in acetone for at least 30 min and dried at 400°C for at least 4 h. All material was stored in aluminium foil to avoid adsorption of organic compounds.

2.2 In-syringe MSA-DLLME–HPLC system

The proposed in-syringe MSA-DLLME–HPLC system used for the preconcentration, separation and determination of UV filters is depicted in Fig. 1 following prior designs [25]. PTFE tubing of 0.8 mm inner diameter (id) was used for the entire manifold. The setup consists of a 5000-step multisyringe pump (MSP), a rotary 8-port multiposition valve (MPV) both from Crison SL (Alella, Barcelona) and a rotary 6-port high pressure injection valve (IV) (loop volume 20 µL) from Sciware System SL (Bunyola, Spain) which was employed as interface between the flow system and the HPLC equipment. The MSP used as propulsion unit was equipped with two syringes (Hamilton, Bonaduz, Switzerland) of 5 mL (S1 and S2). Each syringe has a three-way solenoid valve (N-Research, Caldwell, NJ) at the head enabling multicommutation operations, i.e. S1 is

connected to the central port of the MPV when activated (position ON) and to the IV when deactivated (position OFF) and S2 to the IV (position OFF) and to its reservoir (position ON). In-syringe MSA-DLLME was carried out in S1, while S2 was used for propelling the extract through the interface line to fill the HC of the IV. In this work, since the extraction solvent had a density higher than water, the syringe module was placed upside-down, accumulating the organic droplet at the bottom. The positions (OFF) from each syringe are joined via a T-piece leading to holding coil 2 (HC2) of 500 µL. Peripheral ports of the MPV were connected to reservoirs of waste (1), ACN (2), IL/ACN (3), sample (4) and air (5). The connection between the common port of the MPV and the S1 head-valve was through HC1 of 125 µL. To enhance the extraction efficiency, a MSA system (Sciware Systems) was placed around S1 as it was described elsewhere [25].

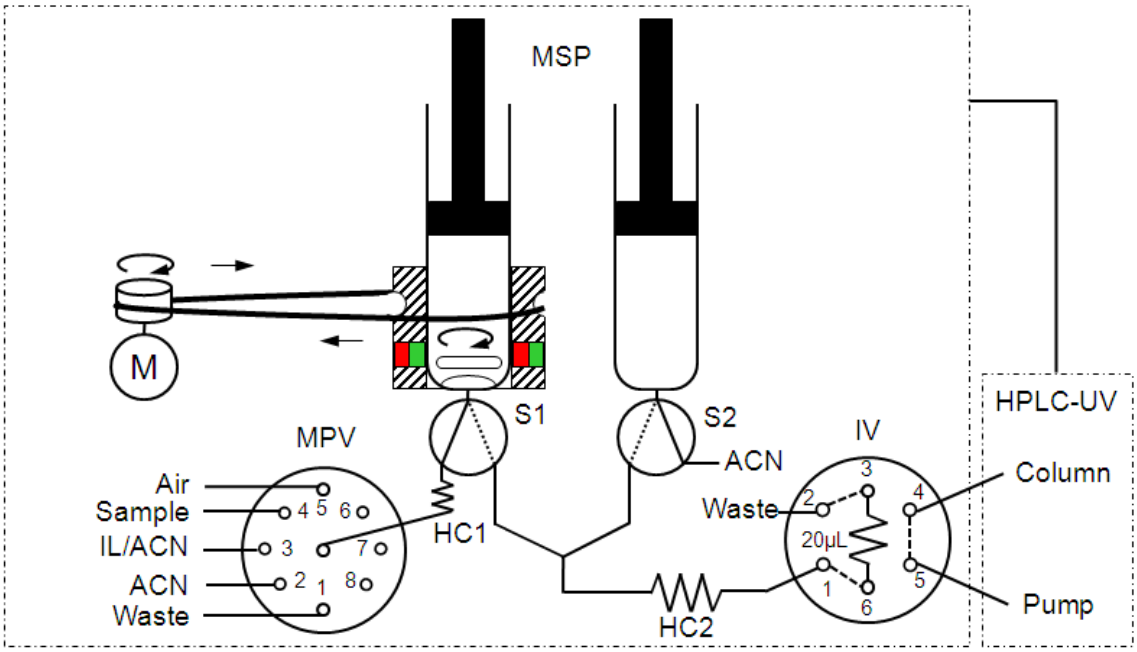

Figure 1 Schematic manifold of the in-syringe magnetic stirring-assisted dispersive liquid-liquid microextraction system coupled to HPLC (in-syringe MSA-DLLME-HPLC) for UV filters determination. The manifold was composed of a multisyringe pump (MSP) with two syringes (S1 and S2) with a magnetic stirring system on S1, a multiposition valve (MPV), a high injection valve (IV), a DC motor (M) and a HPLC system.

The chromatographic separation was carried out on a Waters LC system (Milford, MA, USA) equipped with a quaternary Pump (600), UV/vis Detector (2996) and a column oven. Injections were made with an external injection valve equipped with a 20 µL loop (see Fig.1). Separations were carried out using a Symmetry® C18 analytical column (250mm x 3mm id 5 µm) preceded by a C18 guard column (5mm x 4.6mm id), both from Waters (Torrance, CA, USA). Separation was conducted by isocratic mode using

85/15, (v/v) ACN/H_2O containing 0.5% acetic acid, at a flow rate of 0.8 mL/min, and analytes were monitored at 307 nm. HPLC system and data management were controlled by Empower software (Waters), while the extraction system was controlled by the AutoAnalysis 5.0 package (Sciware Systems) achieving complete automation of the analytical protocol (see section 2.3) [33] . The synchronism of the HPLC with the in-syringe MSA-DLLME-HPLC system was performed through a net command between AutoAnalysis and Empower used for activation of HPLC by AutoAnalysis when the eluate was injected into the HPLC interface (IV loop). All the chromatographic experiments were carried out at room temperature (22±2 ºC). Before use, mobile phases and samples were vacuum filtered through a 0.22 µm PVDF and Fluoropore FGLP membranes (Millipore).

2.3 Analytical protocol

The method for in-syringe MSA-DLLME-HPLC is detailed in supplementary material S-1 and briefly described below. First of all, the syringes were cleaned by aspiration of 1 mL of ACN (stirring activated) and discharge to waste in order to avoid possible sample carry-over and to achieve better reproducibility. Then, the following solutions were subsequently aspirated into S1: mix of (200 µL of ACN and 190 µg of IL) and 3.5 mL of sample, under low-speed stirring, i.e. at 1000 rpm, to have an efficient mixing. Then, 250 µL of air was aspirated to drive all the sample volume into the syringe. During air-aspiration plus 150 s, rapid-speed stirring, i.e. 2000 rpm, was activated. Thus, the IL was dispersed into small droplets enabling DLLME. During the last five seconds, the stirring speed was decreased, which favoured the coalescence of the fine ionic liquid droplets. Afterwards, during a sedimentation time of 40 s, the enriched droplets (~140 µL) were accumulated at the head of the syringe. The IL is too viscous to be injected directly into the HPLC system, for this reason the organic droplet was diluted 1:1 with ACN (contained in S2) in the HC2 with the IV in load position. ACN was chosen to dilute the extract since it is the major component of the mobile phase used. Afterwards, the IV was switched to inject position, the HPLC was activated by a net command and 20 µL of the diluted droplet was injected into the HPLC. At this point, the liquid contained in S1 was discharged to waste. The HPLC separation was synchronized with the in-syringe MSA-DLLME procedure, i.e. a chromatographic separation was performed, while the ensuing sample was being processed in the flow system. Total sample treatment from extraction to injection of the eluate into HPLC takes ca. 5.5 min and chromatographic separation was performed in 12 min. This allows the overlapping of the in-syringe MSA-DLLME with the chromatographic run,

increasing the overall sample throughput. All experiments were performed in quadruplicate.

3. Results and discussion

3.1 Preliminary remarks

In DLLME several variables, such as type and volume of dispersive solvents, type and mass of extractant, ionic strength, sample pH, extraction time and sedimentation time, can affect the extraction yield and in most cases they are correlated. Therefore, it is advisable to perform a multivariate optimization [34] because it provides relevant knowledge of the effect of the variables within the entire experimental domain selected as individuals and also of their interactions, and of the variance of the estimate response at every point of the domain. Thus, firstly a preliminary study was performed to find out which extractant and dispersive solvent provided the best extraction efficiency. Then, in order to obtain the optimum experimental conditions for extraction of UV filters from the water samples a screening studying the effect of the following variables: dispersive solvent volume, extractant mass, ionic strength, pH and extraction time, and a central composite design (CCD), with the variables: ionic strength IL amount and dispersive solvent volume), were applied. For this purpose STATISTICA 7.0 statistical package (Statsoft Inc., Tulsa, USA) was used to generate the experimental matrix and to evaluate the results.

Given the fact that the number of variables involved in the study was too high, some variables were not included in the multivariate approach. Sedimentation time was fixed at 240 s in order to assure the complete drop formation and later optimized using a univariate approach. All experiments were made using 3.5 mL of distilled water spiked with UV filters at the following levels of concentration: 25 µg/L BZ3, 10 µg/L MBC, 25 µg/L OCR, 10 µg/L EDB, 60 µg/L EHS and 60 µg/L HMS. The peak area was used as the analytical response to optimize the system, except for the multivariate experimental design that was accomplished by using normalized peak area. Normalization is necessary to eliminate possible effects that could influence the analytical response. In this case the normalized peak area corresponded to the ratio of UV filters peak area by the drop volume to eliminate possible dilution effects.

3.2 Selection of the extractant

The selection of an appropriate extraction solvent is a critical point for efficient DLLME. An ideal extraction solvent in DLLME should possess some characteristics such as low solubility in water, good extraction capability of the interested compounds, low volatility and good chromatographic behaviour. Based on these facts, three ILs, $[C_4MIM][PF_6]$,

$[C_6MIM][PF_6]$ and $[C_8MIM][PF_6]$ were studied as extractant solvent. $[C_4MIM][PF_6]$ was miscible in aqueous phase and no or little organic phase appeared at the bottom of the syringe after stirring, given its relatively high solubility in water. $[C_8MIM][PF_6]$ is more viscous than $[C_6MIM][PF_6]$ which makes difficult its dispersion in water. Although the values of extraction efficiency obtained were similar, better reproducibility was obtained with $[C_6MIM][PF_6]$ (3% expressed as RSD) than with $[C_8MIM][PF_6]$ (6%). Moreover, $[C_8MIM][PF_6]$ provides a smaller volume of sediment what represents a limitation in the hyphenation between FT and HPLC system. Therefore, $[C_6MIM][PF_6]$ was chosen as the extraction solvent for further experiments.

3.3 Selection of the dispersive solvent

A dispersive solvent must be miscible in both the organic and the aqueous phase. For this purpose, ACN, MeOH and acetone were tested. According to our results, MeOH and acetone were discarded since these showed an RSD higher than 10 % (n= 4). On the other hand, ACN showed good dispersive ability, leading to the formation of very fine droplets and increasing the contact surface area of the selected extraction solvent with good recoveries and better reproducibility. Thus, ACN was chosen as the dispersive solvent for further experiments.

3.4 Multivariate optimization of experimental conditions

An experimental two level full factorial screening design in two blocks (2^5) composed by 38 experiments was carried out to estimate the main variables affecting the extraction efficiency. Three centre points were included in each block to identify any curvature and to estimate the error. The five variables studied were: dispersive solvent volume (500-1000 µL), extractant mass (80-240 µg), ionic strength (0-59 g/L), pH (3-8) and extraction time (60-300 s). An ANOVA test was used to statistically evaluate the data (S-2) and significant effects were determined using a *t-test* with a 95 % confidence level and graphically summarized using main effects Pareto chart (S-3). Results showed that the curvature and all the variables were significant in the studied experimental domain, except for the extraction time which was fixed at 300 s for further univariate optimization. Extractant mass was the most significant factor for all target analytes showing a positive effect. The pH showed a negative significant effect, however pH 3 was fixed because a lower pH would affect the chromatographic column lifetime. The remaining variables were considered in the following optimization step, using a face centered central composite design (CCD) in two blocks including three central points with 20 experimental runs. Taking into account the screening results, the ranges of these three variables were modified as follows, ionic strength (0-59 g/L),

IL amount (160-320 µg) and dispersive solvent volume (200-600 µL). The data obtained were evaluated by an ANOVA test (S-4). Critical values were obtained using the desirability function (S-5). All experimental responses were statistically analysed, showing satisfactory results. The highest response for ionic strength was obtained with 29 mg/L. Nevertheless the range of the salinity in seawater is higher than the obtained value (35-39 mg/L) [35], so the ionic strength was fixed at 39 mg/L as a compromise between salinity found in the samples and the analytical response, in order to reduce matrix effect when analysing seawater samples. Regarding the amount of IL, the extraction of all UV filters was constant and higher in the range of 160–190 µg, so 190 µg IL were chosen due to better sedimentation reproducibility was attained. Overall, results obtained from the optimization lead to the following experimental conditions: 200 µL ACN, 190 µg IL and 39 mg/L of ionic strength which were used in further assays.

3.5 Extraction and sedimentation time

Extraction and sedimentation time were studied under the optimized conditions. Extraction time, which refers to the stirring time of the mixture of reagents and sample, was studied in the range from 20 to 300 s. It was observed that the peak areas increased nearly linearly with the extraction time up to 150 s, reaching a stable level and good RSD % values (2 %). Thus, an extraction time of 150 s was selected for further experiments. In order to ensure a reproducible formation of the extract, it is also important to study the sedimentation time which refers to the time in which both phases are separated. Sedimentation time was studied in the range from 5 to 150 s. The area increased rapidly reaching a maximum at 40 s with constant values for longer times. Therefore, 40 s were set for sedimentation time in further analysis in order to improve the analysis throughput.

Finally, the robustness of the critical values obtained through the experimental design was evaluated by using a two-level screening design, after varying system operating parameters, i.e. pH (3.00 ± 0.02), amount of IL (190 ± 1 µg) and ACN volume (200 ± 2 µL). No lack of fit was observed and the Pareto chart obtained demonstrates clearly that the pH, IL and ACN volume variables are robust in these intervals.

Fig. 2 shows typical chromatograms of raw seawater and spiked seawater analysed with the proposed system. As can be seen under optimized chromatographic conditions, BZ3, MBC, OCR, EDB, EHS and HMS peaks were well resolved and endogenous environmental water compounds did not give any interfering peaks.

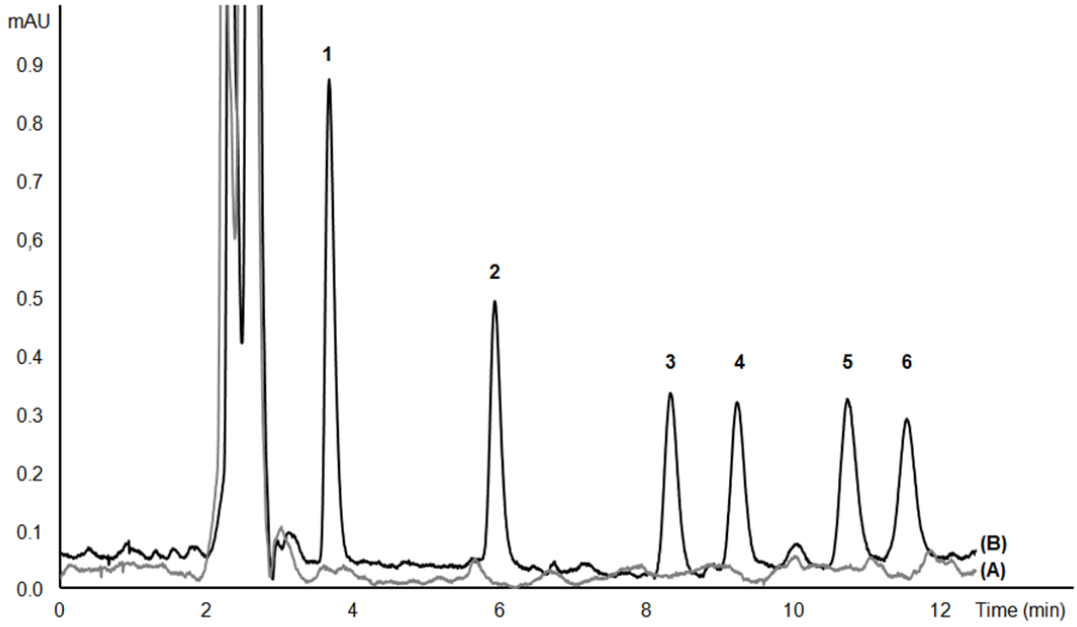

Figure 2 Chromatogram of UV filters analyzed with the proposed in-syringe MSA-DLLME-HPLC system: (A) surface seawater and (B) spiked surface seawater with 25 µg/L BZ3 (1), 10 µg/L MBC (2), 25 µg/L OCR (3), 10 µg/L EDB (4), 60 µg/L EHS (5), 60 µg/L HSM (6).

3.6 Analytical performance of the in-syringe MSA-DLLME-HPLC system

To evaluate the performance of the proposed in-syringe MSA-DLLME-HPLC method, its figures of merit, i.e. linearity, intra- and inter-day precision, the LODs and limits of quantification (LOQs) and EFs, were studied under optimized conditions and they are summarized in Table 1.

Table 1. Figures of merit of the proposed in-syringe MSA-DLLME-HPLC method

UV filter	Linearity (µg/L)	Slope ± SD (µg/L)	Intercept ± SD	r^2	LOD (µg/L)	LOQ (µg/L)	%RSD[a]	%RSD[b]	EF	ER %
BZ3	0.6-500	609.8 ± 19.5	0.90 ± 0.07	0.999	0.18	0.62	6	8	23	92
MBC	0.25-500	853.5 ± 38.3	0.45 ± 0.05	0.999	0.08	0.25	5	7	13	52
OCR	8.5-500	283.3 ± 0.7	-3.2 ± 0.3	0.993	2.50	8.34	4	6	12	48
EDB	3-500	658.5 ± 5.5	-2.4 ± 0.2	0.991	0.89	2.98	5	8	12	49
HMS	34-500	159.7 ± 0.9	-12.2 ± 1.5	0,997	10.24	34.15	6	8	13	51
EHS	40-500	138.0 ± 0.8	-11.9 ± 0.8	0.998	11.82	39.39	4	4	11	46

a: intra-day precision, b: inter-day precision. EF: enrichment factor, ER: enrichment recovery.

Calibration curves over the studied range were constructed for aqueous standards and for seawater samples spiked with the UV filters. The slopes of the calibration curves obtained with standards and seawater were statistically compared with a *t-test* and no significant differences were observed since all experimental *t* values obtained were lower than the critical *t* value at the 95 % confidence level. Thus, no difference is observed and samples can be quantified using calibration curves.

The calibration graph for each analyte was constructed with 6 standard solutions in quadruplicate containing all the analytes up to 500 µg/L with correlation coefficients (r^2) ranging from 0.991 to 0.999. The LODs for the analytes, calculated at a signal-to-noise ($S\ N^{-1}$) ratio of 3, ranged from 0.08 to 12 µg/L and the LOQs, calculated at $S\ N^{-1} = 10$, were from 0.25 to 40 µg/L, when a sample volume of 20 µL was injected. However, it is possible to enhance the sensitivity by for instance injecting larger volumes on the HPLC.

Intra- and inter-day precision of the method, expressed as RSDs % were evaluated for eight replicate experiments in one day and over a period of five consecutive days with a spiked surface seawater sample, respectively. The RSDs were below 6 % and 8 %, respectively, illustrating satisfactory repeatability and reproducibility. The EF, defined as the ratio of the concentration of a compound in the sedimented phase to the initial concentration of the same compound in the aqueous phase before the extraction, ranged between 11 and 23 as can be seen in Table 1.

The entire procedure allows a determination frequency of 5 samples per hour thanks to the synchronism between in-syringe MSA-DLLME and HPLC. Results demonstrate that this method is a reliable technique for analysing UV filters in environmental water samples. In Table 2 analytical performance of the developed in-syringe MSA-DLLME-HPLC has been compared with the most commonly used HPLC hyphenated techniques. As can be seen, some of the previously reported methods provide similar LODs and repeatability as the proposed method. Although some of these methods report higher EFs for some of the UV filters studied, the analysis time was longer than those reported in the present method, limiting their use in routine analysis. Furthermore, in most of them liquid-liquid microextraction was used as off-line sample treatment not achieving a fully automated analyser [14, 20, 21]. There is a fully automated method reported in bibliography exploiting SPE in a LOV system for UV filters [13] with better LODs. This is due to the capability of SPE to preconcentrate higher sample volumes what permits an injection volume 15 times higher than in our method, this being the main limitation of in-syringe DLLME systems. However, a higher frequency of analysis is achieved with the present work in comparison with the listed works. Overall, in-syringe MSA-DLLME-HPLC method presents good LODs and

repeatability, low extraction time, and the most important the coupling to HPLC that allows the performance of the entire procedure in about 12 min within a single instrumental assembly.

Table 2 Comparison of the analytical performance of the developed in-syringe MSA-DLLME-HPLC method with commonly used HPLC hyphenated techniques for UV filters determination.

Technique	*LOD (µg/L)*	*Extraction time (min)*	*RSD%*	*Sample (mL)*	*Analysis frequency(1/h)*	*Ref.*
IL-SDME	0.06 - 3	37	2.8 - 8.8	10	~ 2	[14]
LOV-BI	0.45.3.2	-	2.1 - 13	12	~ 3	[13]
MSA-DLLME	0.2-0.8	25	1.4 – 4.8	20	~ 3	[21]
IL-USA-DLLME	0.2-5	3	4-6.3	10	~ 3	[20]
In-syringe MSA-DLLME-HPLC	0.08-12	2.5	4-6	3.5	5	This method

IL-SDME: ionic liquid single drop microextraction; LOV-BI: lab-on-valve bead injection; MSA-DLLME: magnetic stirring assisted dispersive liquid-liquid microextraction; IL-USA DLLME: ionic liquid ultrasound-assisted dispersive liquid-liquid microextraction.

3.7 Method validation and application to bath water samples

The applicability of the proposed in-syringe MSA-DLLME-HPLC method was tested by the determination of six UV filters in 2 different matrix water samples (surface seawater and swimming pool water) under optimum conditions. Results are summarized in Table 3, (S1-S6 refer to surface seawater samples and S7-S8 to swimming pool water samples). As mentioned before samples were adjusted to pH 3. Moreover, the ionic strength of S8 had to be adjusted prior to DLLME. The statistical t-test confirmed the nonexistence of significant differences between the mean recovery ratios for spiked concentration values and the expected concentration values at the 95 % significance level. Therefore, results proved that there is no matrix effect on the developed in-syringe MSA-DLLME method for the two different matrix water samples analysed. One of the target analytes was detected in one of the samples (HMS in S7), indicating the rest were not present or below the LOQs in the analysed samples. Relative recoveries for the six UV filters ranged from 89 to 114% for surface seawater samples and 86 to 107% for swimming pool water samples.

Table 3. Application of in-syringe MSA-DLLME-HPLC method to bath water samples and addition-recovery test at three spiking levels.

Analytes																		
Sample	BZ3			MBC			OCR			EDB			HSM			EHS		
	Ca (µg/L)	Cf ± SD (µg/L)	RR (%)	Ca (µg/L)	Cf ± SD (µg/L)	RR (%)	Ca (µg/L)	Cf ± SD (µg/L)	RR (%)	Ca (µg/L)	Cf ± SD (µg/L)	RR (%)	Ca (µg/L)	Cf ± SD (µg/L)	RR (%)	Ca (µg/L)	Cf ± SD (µg/L)	RR (%)
S1	0.0	<LOQ	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	<LOQ	-	0.0	<LOQ	-
	2.4	2.2 ± 0.2	106	1.0	1.0 ± 0.1	99	26.9	26.7 ± 1.5	99	9.8	10.4 ± 0.6	106	239.0	242 ± 10	101	242.1	242 ± 10	101
	6.0	6.34 ± 0.11	105	2.4	2.28 ± 0.05	102	67.3	69 ± 2	102	24.5	23.9 ± 0.8	97	597.6	597 ± 24	100	605.3	597 ± 21	100
	12.0	11.9 ± 0.3	99	4.8	4.8 ± 0.2	99	134.7	134 ± 4	99	49.0	49.2 ± 0.8	100	1195.2	1192 ± 34	100	1210.6	1192 ± 38	100
S2	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-
	2.4	2.51 ± 0.10	101	1.0	0.97 ± 0.06	94	26.9	25.3 ± 1.2	94	9.8	9.7 ± 0.6	99	239.0	216 ± 7	90	242.1	216 ± 8	101
	6.0	5.9 ± 0.3	97	2.4	2.41 ± 0.08	102	67.3	69 ± 5	102	24.5	24.6 ± 1.0	101	597.6	623 ± 69	104	605.3	623 ± 8	99
	12.0	12.1 ± 0.3	101	4.8	4.8 ± 0.2	99	134.7	133 ± 7	99	49.0	49.0 ± 1.2	100	1195.2	1180 ± 138	98	1210.6	1180 ± 85	98
S3	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-
	2.4	2.6 ± 0.2	95	1.0	0.95 ± 0.05	101	26.9	27.3 ± 1.4	101	9.8	10.1 ± 0.3	103	239.0	249 ± 10	104	242.1	249 ± 8	105
	6.0	5.6 ± 0.4	94	2.4	2.46 ± 0.09	99	67.3	66 ± 3	99	24.5	23.8 ± 1.0	96	597.6	576 ± 37	96	605.3	576 ± 40	96
	12.0	12.17 ±0.15	101	4.8	4.8 ± 0.4	100	134.7	134.8 ± 1.2	100	49.0	49.3 ± 0.6	101	1195.2	1198 ± 23	100	1210.6	1198 ± 36	100
S4	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-
	2.4	2.4 ± 0.2	100	1.0	1.00 ± 0.08	112	26.9	30.2 ± 0.7	112	9.8	11.0 ± 0.4	113	239.0	261 ± 4	109	242.1	261 ± 38	110
	6.0	6.0 ± 0.3	99	2.4	2.4 ± 0.2	92	67.3	62.2 ± 1.9	92	24.5	22.6 ± 0.6	92	597.6	553 ± 7	93	605.3	553 ± 19	91
	12.0	12.1 ± 0.2	101	4.8	4.8 ± 0.2	105	134.7	141 ± 8	105	49.0	51 ± 3	104	1195.2	1212 ± 51	99	1210.6	1212 ± 562	105
S5	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	<LOQ	-	0.0	<LOQ	-
	2.4	2.2 ± 0.2	103	1.0	1.04 ± 0.05	112	26.9	30.0 ± 0.6	112	9.8	11.0 ± 0.4	112	239.0	278 ± 9	116	242.1	278 ± 8	114
	6.0	6.5 ± 0.6	108	2.4	2.3 ± 0.2	93	67.3	62 ± 2	93	24.5	22.7 ± 0.6	93	597.6	532 ± 18	89	605.3	532 ± 20	91
	12.0	11.8 ± 0.6	98	4.8	4.8 ± 0.3	102	134.7	137 ± 4	102	49.0	50.0 ± 1.2	102	1195.2	1226 ± 38	103	1210.6	1226 ± 36	102
S6	0.0	<LOQ	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	<LOQ	-	0.0	<LOQ	-
	2.4	2.4 ± 0.2	108	1.0	1.00 ± 0.09	107	26.9	28.9 ± 0.5	107	9.8	9.6 ± 0.4	98	239.0	259 ± 12	107	242.1	259 ± 10	104
	6.0	6.2 ± 0.4	102	2.4	2.4 ± 0.2	93	67.3	63.0 ± 2.5	93	24.5	25.0 ± 1.1	102	597.6	569 ± 10	95	605.3	569 ± 15	98
	12.0	12.1 ± 0.9	100	4.8	4.8 ± 0.3	101	134.7	136.5 ± 3.5	101	49.0	48.8 ± 1.1	100	1195.2	1211 ± 45	101	1210.6	1211 ± 19	101
S7	0.0	<LOQ	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	34.6 ± 0.5	-	0.0	<LOQ	-
	2.4	2.2 ±0.2	103	1.0	1.00 ± 0.09	103	26.9	27.7 ± 1.9	103	9.8	10.5 ± 0.3	107	239.0	241 ± 5	86	242.1	241 ± 5	103
	6.0	6.4 ± 0.3	106	2.4	2.4 ± 0.2	98	67.3	65.9 ± 1.7	98	24.5	23.5 ± 0.3	96	597.6	595 ± 7	94	605.3	595 ± 6	98
	12.0	11.8 ± 0.7	98	4.8	4.8 ± 0.2	100	134.7	134.8 ± 2.7	100	49.0	49.4 ± 0.7	101	1195.2	1196 ± 8	97	1210.6	1196 ± 18	100
S8	0.0	<LOQ	-	0.0	nd	-	0.0	nd	-	0.0	nd	-	0.0	<LOQ	-	0.0	<LOQ	-
	2.4	2.2 ± 0.2	103	1.0	0.99 ± 0.09	98	26.9	26.3 ± 0.8	98	9.8	8.9 ± 0.6	91	239.0	231 ± 7	97	242.1	231 ± 6	96
	6.0	6.3 ± 0.4	105	2.4	2.36 ± 0.14	100	67.3	67.7 ± 2.8	100	24.5	26 ± 2	104	597.6	610 ± 27	102	605.3	610 ± 20	102
	12.0	11.9 ± 0.6	99	4.8	5 ± 2	100	134.7	134.2 ± 6.1	100	49.0	48 ± 4	98	1195.2	1191 ± 55	100	1210.6	1191 ± 29	99

Ca: added concentration, Cf: found concentration, RR: relative recovery and nd: not detected.

4. Conclusions

The proposed in-syringe MSA-DLLME-HPLC method allows efficient extraction and preconcentration of most commonly used UV filters: BZ3, MBC, OCR, EDB, EHS and HMS from sea and pool water samples. The method permits simultaneous trace analysis of UV filters, in less than 12 min for the whole process by the synchronism of the sample pretreatment and the chromatographic analysis. This is a significant improvement in analysis throughput (5 samples/h) in comparison to previously reported methods, proving its suitability for routine analysis. The use of in-syringe MSA-DLLME allows efficient and rapid clean-up and extraction of UV filters from bath water samples, minimizing matrix-induced effects. Furthermore, the use of an IL as extractant makes of the present automatic method an efficient and environmental friendly tool for UV-filter determination in bath waters.

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7.6.1. Supplementary information

Table S-1. Detailed procedure for the in-syringe stirring assisted dispersive liquid-liquid microextraction coupled to HPLC to determine UV filters.

No	Instrument	Operation	Comment
1	IV	Load	
2	MPV	Valve A move to position 2	Clean syringe with ACN
3	MSP	Pickup 1 mL at 5 mL/min V [On] after 0.400 mL activate agitation	
4	MSP	Dispense 1 mL at 5 mL/min V [Off] without agitation	
5	MPV	Valve A move to position 3	Aspiration of mix (IL/ACN)
6	MSP	Pickup 1 mL at 3 mL/min V [On]	
7	Wait	Wait 2 seconds	
8	MPV	Valve A move to position 4	Aspiration of sample
9	MSP	Pickup 3.5 mL at 5 mL/min V [On] and activate and deactivate agitation each 0.200 mL	
10	Wait	Wait 2 seconds	
11	MSP	Deactivate agitation	
12	MPV	Valve A move to position 5	Aspiration of air
13	MSP	Pickup 0.250 mL at 2.5 mL/min V [On] and activate agitation	
14	Wait	Wait 150 seconds	MSA-DLLME
15	MSP	Deactivate agitation	Phase separation and droplet aggregation
16	Wait	Wait 40 seconds	
17	MSP	Dispense 0.730 mL at 1 mL/min V [Off]	Discharge through IV
18	Wait	Wait 15 seconds	
19	IV	INJECT	Both instruction are done at the same time
20	HPLC	ACTIVATION	
21	MPV	Valve A move to position 1	Empty syringe rapidly to waste
22	SP	Empty at 5 mL/min V [Off]	
23	HPLC	Chromatographic analysis	
24	HPLC	Stop	

IV: injection valve, D: detector, MSP: multiposition valve, MPV: multisyringe pump, IL: ionic liquid, ACN: acetonitrile, MSA-DLLME: magnetic-stirring-assisted dispersive liquid-liquid microextraction.

Table S-2. Analysis of variance (ANOVA) for the screening design. Variables correspond to (1) pH, (2) ionic strength, (3) extraction time, (4) dispersant volume and (5) extractant mass.

Factor	Sum of Squares	Df[a]	Mean Square	F-Ratio[b]	P-Value
Blocks	2.854	1	2.854	0.9009	0.396296
Curvature	739.068	1	739.068	233.2659	0.000107
(1)pH	32.005	1	32.005	10.1013	0.033591
(2)NaCl	165.778	1	165.778	52.3232	0.001938
(3)text	0.191	1	0.191	0.0603	0.818055
(4)Vdis	58.539	1	58.539	18.4761	0.012662
(5)Mext	2278.592	1	2278.592	719.1731	0.000011
1 by 2	229.04	1	229.04	72.29	0.001049
1 by 3	53.429	1	53.429	16.8633	0.014774
1 by 4	2.088	1	2.088	0.6592	0.462403
1 by 5	23.068	1	23.068	7.2809	0.054189
2 by 3	11.825	1	11.825	3.7321	0.125545
2 by 4	67.561	1	67.561	21.3237	0.009898
2 by 5	122.097	1	122.097	38.5365	0.003426
3 by 4	1.583	1	1.583	0.4998	0.518611
3 by 5	0.195	1	0.195	0.0616	0.816235
4 by 5	89.81	1	89.81	28.3458	0.005989
1*2*3	31.287	1	31.287	9.8748	0.034767
1*2*4	0.008	1	0.008	0.0024	0.963456
1*2*5	203.911	1	203.911	64.3589	0.00131
1*3*4	97.585	1	97.585	30.7998	0.005158
1*3*5	40.228	1	40.228	12.6969	0.023517
1*4*5	0.349	1	0.349	0.1102	0.756599
2*3*4	87.191	1	87.191	27.5195	0.006315
2*3*5	11.856	1	11.856	3.7419	0.125182
2*4*5	40.881	1	40.881	12.9029	0.02292
3*4*5	1.595	1	1.595	0.5033	0.517204
Lack of Fit	271.32	6	45.22	14.2724	0.01125
Pure Error	12.673	4	3.168		
Total SS	4676.607	37			

[a]Df. Degrees of freedom, [b] Test for comparing model variance with residual (error) variance
R^2 = 0.939, R^2 adjusted= 0.775

Figure S3. Standardized main Pareto chart from the screening design. Vertical line in the chart defines 95% of confidence level.Variables (1) pH, (2) ionic strength, (3) extraction time, (4) dispersant volume and (5) extractant mass.

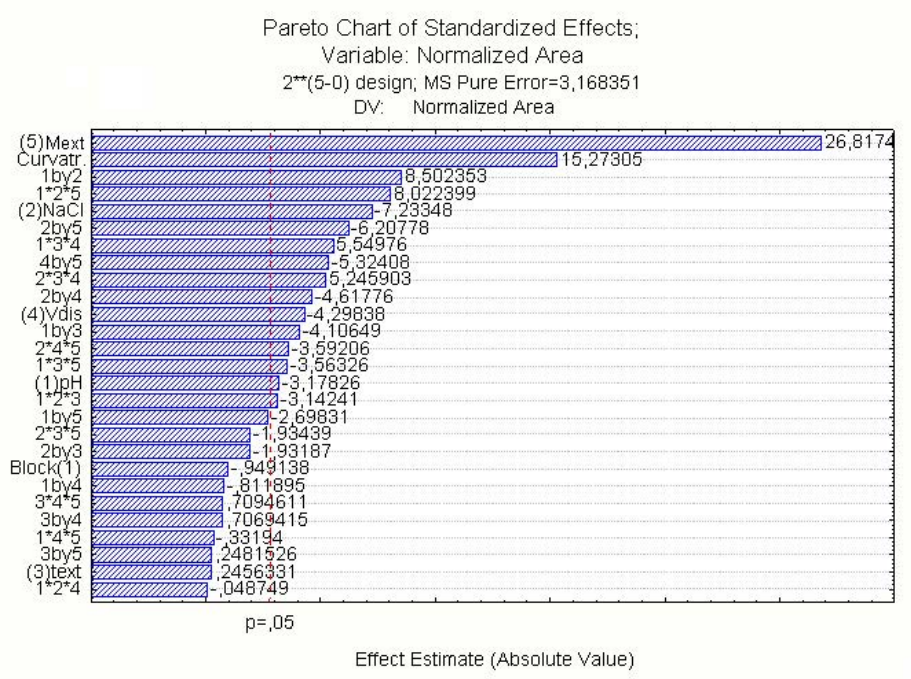
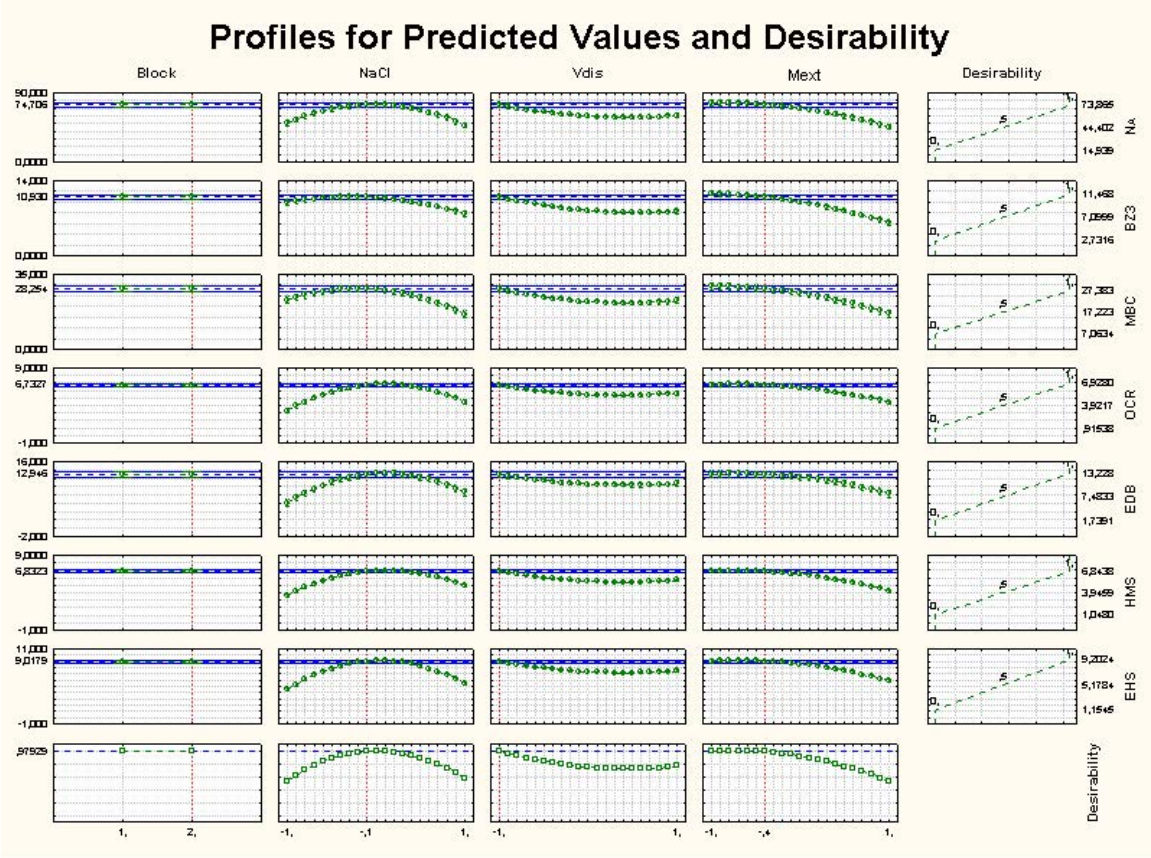


Table S4. Analysis of variance (ANOVA) for the CCD design. Variables correspond to (1) ionic strength, (2) dispersant volume and (3) extractant mass.

Factor	Sum of Squares	Df[a]	Mean Square	F-Ratio[b]	P-Value
Blocks	85.160	1.000	85.160	49.938	0.002
(1)NaCl (L)	7.213	1.000	7.213	4.230	0.109
NaCl (Q)	1736.207	1.000	1736.207	1018.106	0.000
(2)Vdis (L)	447.284	1.000	447.284	262.286	0.000
Vdis (Q)	176.740	1.000	176.740	103.640	0.001
(3)Mext (L)	1980.766	1.000	1980.766	1161.515	0.000
Mext (Q)	190.608	1.000	190.608	111.772	0.000
1L by 2L	2.650	1.000	2.650	1.554	0.281
1L by 3L	66.258	1.000	66.258	38.853	0.003
2L by 3L	11.328	1.000	11.328	6.642	0.062
Lack of Fit	166.448	5.000	33.290	19.521	0.007
Pure Error	6.821	4.000	1.705		
Total SS	5775.706	19.000			

[a]Df. Degrees of freedom, [b] Test for comparing model variance with residual (error) variance
R^2 = 0.970, R^2 adjusted= 0.936

Figure S-5. Desirability chart used to obtain the critical values of ionic strength (NaCl), dispersant volume (Vdis) and extractant mass (Mext). N_A: normalized area.



CHAPTER 8
CONCLUSIONS

8. Conclusions

In the present thesis new automated approaches for monitoring of parameters of environmental interest exploiting microextraction flow analysis techniques and separative techniques have been developed especially suited for analysis of environmental samples. The versatility of flow analysis techniques to accommodate a plethora of pretreatment and detection techniques has been demonstrated.

Furthermore the use of flow analysis techniques to automate the present analytical methods achieving maximal robustness, simplicity and stability has resulted in precise and accurate results, together with time and reagents saving, and therefore higher sample frequency, minimal handling of sample and reagents, and a lower environmental impact per analysis due to reduced waste production.

3-hydroxy-4-pyridinone (3,4-HPO) has proved to be an efficient colorimetric reagent for iron determination and speciation. Furthermore, the use of solid phase spectrometry (SPS) exploiting this reagent and nitrilotriacetic acid (NTA) Superflow resin in a μSI-LOV-SPS system resulted in enhanced environmental and cost effective performance. Furthermore by the implementation of SPS the method could be applied to samples with high salt content, i.e. seawater samples.

In-syringe dispersive liquid-liquid microextraction (DLLME) has proved to be a useful tool for analyte extraction and sample clean-up resulting in high recoveries, and providing several advantages such as reduction of the solvents volume, safer handling of volatile organic solvents, increased analysis throughputs, minimization of sample loss and sample contamination, reproducible extraction and potential use for routine analysis. In addition, in-syringe DLLME allowed the use of solvents denser than water as well as alternative extractants, such as ionic liquids and coupling with separation techniques. Moreover, the implementation of a magnetic stirring assisted (MSA) device into in-syringe DLLME systems helped improve the mixing efficiency and thus to speed up the analytical procedure. Furthermore, the use of in-syringe MSA-DLLME allowed efficient and rapid clean-up of the syringe, minimizing matrix-induced effects.

In-syringe DLLME capabilities were demonstrated in the development of a fully automated method for aluminium determination with fluorescence detection. Thus, the complete analytical procedure including sampling, buffering, reaction with the fluorescence reagent, extraction, phase separation, and quantification was completely

automated and carried out within 4 min. Furthermore, by implementing a MSA system rapid and homogeneous mixing of the sample with the required reagents was accomplished not requiring the use of a dilution chamber or the disperser solvent, allowing the reduction of solvents and achieving a higher preconcentration factor, higher sensitivity and shorter analysis time.

Placing the syringe burette up-side down permitted the handling of organic solvents denser than water in in-syringe MSA-DLLME systems and also the simplification of the MSA system. This configuration permitted the development of two simplified and automated methods for anionic and cationic surfactants determination based on the standard protocols using chloroform as extractant. Thus, the developed in-syringe MSA-DLLME systems permitted a great reduction of the chloroform and the sample volume, and a huge increase of the analysis frequency since the entire procedure including sample mixing with reagents, extraction, phase separation, detection and system cleaning, involved just few minutes. In addition, a significant reduction of interferences in the method for cationic surfactants determination was achieved by washing of the organic solvent.

The hyphenation of flow analysis techniques and chromatographic techniques has proved to be a useful strategy to enhance chromatographic performance and method's selectivity, accuracy and sensitivity. Thus, the coupling of in-syringe MSA-DLLME using a green extraction solvent to HPLC permitted the development of an efficient fully automated system for UV filters determination in terms of cost, time and accuracy. The method's throughput was increased by the synchronism of the sample pretreatment and the chromatographic analysis. Furthermore, the use of an ionic liquid as extractant made of the developed automatic method an efficient and environmental friendly tool for UV-filters determination in bath waters.

In conclusion, the implementation of microextraction techniques in flow based systems and the coupling to chromatographic techniques improved methods' selectivity and sensitivity. Moreover, the automation of the sample pretreatment provides enhanced reproducibility, analysis throughput and reduces analysis costs, cross contamination and waste production. Thus, as a result more efficient analytical methods are obtained in terms of precision, environmental impact and cost.

8. Conclusiones

En esta tesis se han desarrollado nuevos sistemas automáticos para el control de parámetros de interés ambiental utilizando técnicas de microextracción en flujo y técnicas separativas para el análisis de muestras medioambientales. Se ha demostrado la gran versatilidad de las técnicas de análisis en flujo en su acoplamiento a una gran variedad de técnicas de detección y pretratamientos.

Además, el uso de técnicas de análisis en flujo para automatizar los métodos analíticos desarrollados ha aportado gran robustez, simplicidad y estabilidad lo cual se ha traducido en resultados precisos y exactos, junto con el ahorro de tiempo y reactivos, una mayor frecuencia de muestreo, una mínima manipulación de la muestra y reactivos por parte del analista, y un menor impacto ambiental por análisis debido a la reducción de los residuos generados.

3-hidroxi-4-piridinona (3,4-HPO) ha demostrado ser un reactivo colorimétrico eficiente para la determinación y especiación de hierro. Además, la combinación de este reactivo con espectrometría en fase sólida (SPS) utilizando una resina de ácido nitrilotriacético Superflow en un sistema µSI-LOV-SPS ha mejorado el funcionamiento del sistema en términos medioambientales y económicos. Cabe destacar que el uso de la SPS ha permitido expandir la aplicabilidad del método a muestras con alto contenido salino, es decir, a muestras de agua de mar.

La microextracción líquido-líquido dispersiva (DLLME) en jeringa ha demostrado ser una herramienta útil para la extracción de analitos y eliminación de la matriz de la muestra presentando altas eficiencias de extracción y proporcionando varias ventajas, tales como la reducción del volumen de los disolventes utilizados, así como una manipulación más segura de los disolventes orgánicos volátiles usualmente utilizados, el aumento de la frecuencia de análisis, la minimización de la pérdida y contaminación de la muestra y una mayor reproducibilidad. Todo esto fundamenta su uso para análisis de rutina. Además, la DLLME en jeringa permite el uso de disolventes más densos que el agua, así como extractantes alternativos, como líquidos iónicos, y su acoplamiento con técnicas de separación. Por otra parte, la implementación del sistema de agitación magnética asistida (MSA) en sistemas DLLME en jeringa ha ayudado a mejorar la eficiencia de mezcla y por tanto a acelerar el procedimiento analítico. Además, el uso de sistemas MSA-DLLME en jeringa ha permitido la limpieza

de la jeringa de forma eficiente y rápida, reduciendo al mínimo los efectos inducidos por la matriz.

Las capacidades de la DLLME en jeringa han quedado demostradas con el desarrollo de un método totalmente automático para la determinación de aluminio con detección por fluorescencia. Así, el procedimiento analítico completo incluyendo el cambio de muestra, ajuste de pH, reacción con el reactivo de fluorescencia, extracción, separación de fases, y la cuantificación, fue llevado a cabo de forma completamente automatizada en tan solo 4 min. Además, se consiguió una mezcla rápida y homogénea de la muestra con los reactivos necesarios mediante la implementación del sistema MSA, no requiriendo el uso de una cámara de dilución ni disolvente dispersante, lo cual ha permitido la reducción de los disolventes utilizados y un mayor factor de preconcentración, mayor sensibilidad y menor tiempo de análisis.

La colocación de la bureta hacia abajo permitió la manipulación de disolventes orgánicos más densos que el agua en sistemas MSA-DLLME en jeringa y también la simplificación del sistema de agitación. Esta nueva configuración permitió el desarrollo de dos métodos simplificados y automáticos para la determinación de tensioactivos aniónicos y catiónicos basados en protocolos estándar utilizando cloroformo como extractante. Así, los sistemas basados en MSA-DLLME en jeringa desarrollados permitieron una gran reducción del volumen de cloroformo, así como del volumen de la muestra y un gran aumento de la frecuencia de análisis, ya que todo el procedimiento, incluyendo la mezcla de la muestra con los reactivos, extracción, separación de fases, detección y limpieza del sistema, requiere apenas unos minutos. Además, se ha conseguido una reducción significativa de las interferencias en el método para la determinación de tensioactivos catiónicos mediante una etapa de lavado del disolvente orgánico.

El acoplamiento de técnicas en flujo y cromatográficas ha demostrado ser una estrategia útil para mejorar el rendimiento cromatográfico, así como la selectividad, precisión y sensibilidad de los métodos analíticos. Así, el acoplamiento entre un sistema MSA-DLLME en jeringa y HPLC utilizando un disolvente de extracción respetuoso con el medioambiente, ha permitido el desarrollo de un sistema totalmente automático, eficiente en términos de coste, tiempo y precisión para la determinación de filtros UV. La frecuencia de análisis del método ha aumentado gracias al sincronismo del pretratamiento de la muestra y el análisis cromatográfico. Además, el uso de un líquido iónico como extractante hace del método desarrollado, una herramienta

eficiente y respetuosa con el medio ambiente para la determinación de filtros UV en aguas de baño.

En conclusión, la aplicación de técnicas de microextracción en sistemas en flujo y el acoplamiento con técnicas cromatográficas han ayudado a mejorar la selectividad y sensibilidad de los métodos desarrollados. Por otra parte, la automatización del pretratamiento de la muestra proporciona reproducibilidad, mejora de la frecuencia de análisis y reducción de los costes de análisis, de la contaminación cruzada y de la producción de residuos. Como resultado se han obtenido métodos analíticos más eficientes en términos de precisión, impacto ambiental y rentabilidad.

ANNEX

Exploiting the use of 3,4-HPO ligands as nontoxic reagents for the determination of iron in natural waters with a sequential injection approach

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Iron speciation by microsequential injection solid phase spectrometry using 3-hydroxy-1 (H)-2-methyl-4-pyridinone as chromogenic reagent

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Author: Ruth Suárez, Burkhard Horstkotte, Carlos M. Duarte, et al

Publication: Analytical Chemistry

Publisher: American Chemical Society

Date: Nov 1, 2012

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In-syringe-stirring: a novel approach for magnetic stirring-assisted dispersive liquid-liquid microextraction

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In-syringe magnetic stirring assisted dispersive liquid-liquid micro-extraction with solvent washing for fully automated determination of cationic surfactants

B. Horstkotte, R. Suárez, P. Solich and V. Cerdà, Anal Methods, 2014, **6**, 9601

DOI: 10.1039/C4AY01695E

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