

U. PORTO



**FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO**

**Automatic methodologies for
environmentally friendly processing of
samples and coupling to liquid
chromatography**

Hugo Miguel Rodrigues Cunha Oliveira

**Tese do 3º Ciclo de Estudos Conducentes ao Grau de Doutor em
Ciências Farmacêuticas com especialização em Química Analítica**

Porto

Setembro de 2010

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Acknowledgements

To Faculdade de Farmácia da Universidade do Porto for receiving me as PhD student.

To Fundação para a Ciência e Tecnologia for the PhD grant SFRH/BD/22494/2005.

To Prof. Costa Lima for receiving me in his department, for his enthusiasm and wisdom.

To Dr. Marcela Segundo for her excellent supervision, friendship and inspiration to pursue more and better every single day.

To Prof. Victor Cerdà for receiving me in his lab at the University of Balearic Islands and also for his friendship along these years. A special thanks also to Prof. Manuel Miró for the fruitful discussions and contributions to my work. I'm also grateful to all colleagues and friends for the great time that I spent there. I'd like also to thanks to Ferran Hierro for the sorbent pictures.

To Chris Measures, Jarda Ruzicka and all the trace metals team (Mariko, Max and Bill) for the wonderful Hawaiian experience. In addition, a big aloha for all members of the Department of Oceanography of the University of Hawaii at Manoa for their friendship and kindness.

To all my colleagues and friends of the lab for the excellent atmosphere and all great moments that we spent together throughout these years.

To all visitors of our lab for sharing the beauties of the different cultures.

To my family for their unconditional support in all moments of my life.

To Marieta, for several reasons...

Abstract

In the present dissertation, different manifolds based on multisyringe flow injection analysis (MSFIA) were developed, aiming the introduction of new hyphenated methodologies coupling sample preparation procedures and liquid chromatography (LC), or for attaining relevant information about sorption mechanisms of a group of analytes or mesofluidic sorbents handling in order to implement new automatic solid-phase extraction (SPE) protocols.

Four polystyrene-divinylbenzene sorbents with different particle size and cross-linking degree, which were able to perform the sorption of the eleven priority phenolic pollutants by a reversed-phase mechanism, were compared under the same experimental conditions provided by a multisyringe flow injection system coupled on-line to LC. Sorbent capacity, breakthrough volume and enhancement factor (EF) were assessed. A positive correlation between the hydrophobicity of the phenolic compounds and EF was observed for the sorption in sorbents with low cross-linking degree (Amberlite-XAD 4) while a negative correlation was found between EF and molecular weight when the phenolic compounds were preconcentrated in sorbents with high cross-linking degree (Macronet-MN 200 and Lichrolut EN), indicating different predominant interactions between the target analytes and the sorbent. Lichrolut EN provided the best performance for all the parameters studied and it was selected for the development of a new automatic methodology.

A similar MSFIA manifold was used for the automatic screening of phenolic compounds in environmental samples. Variable volumes (up to 100 mL) of water from different sources or soil extract were preconcentrated and analyzed by the hyphenated methodology. High enhancement factors (average value of 176 for 50 mL of sample) and limits of detection below $1 \mu\text{g L}^{-1}$ (for 100 mL of sample) were attained. Due to the fast chromatographic analysis and sample preparation procedure, which were performed within the same time frame, up to 10 samples per hour could be analyzed by this method with high precision and accuracy.

The association between MSFIA, lab-on-valve (LOV) and LC was used for the design of a renewable SPE strategy aiming the extraction and determination of UV filters from bathing water samples. Hence, the MSFIA-LOV system performed the SPE of water samples in a fully automatic fashion, by applying the bead injection (BI) concept, which relies on the use of a new sorptive surface for each determination. By processing sample volumes up to 12.0 mL, the four UV filter molecules were detected at $\mu\text{g L}^{-1}$ levels without carryover effect between consecutive samples, previously reported procedures that used a

permanent sorbent column. The association between the automatic sample preparation and a fast reversed-phase chromatographic methodology, based on a monolithic column and an accelerated isocratic elution, made possible a determination frequency of 6 h⁻¹.

Bead injection concept in LOV format applied to irregularly-shaped and non-uniformly sized bead suspensions was investigated, since these materials have been a source of lump formation and clogging of the microchannels of the LOV, excluding them from SPE protocols under this format. In order to handle the suspensions in a repeatable fashion, four different sorbents, comprising polymeric and silica-based materials (Oasis HLB, SupelMIP β -receptors, Lichrolut EN and Discovery DSC-MCAX), were suspended in different solvents and submitted to different fluidic protocols. By using an appropriate protocol sequence and / or suspension milieu it was possible to pack into the LOV microchannel heterogeneously sized and shaped beads with satisfactory precision (RSD < 14%). Therefore, a universal approach for beads handling under LOV format was proposed, making possible the use of new families of sorbents, and consequently, enlarging the scope of this technique as front end to liquid chromatography.

Molecularly imprinted solid-phase extraction (MISPE) was implemented in a MSFIA-LOV manifold and coupled to liquid chromatography for the determination of riboflavin in foodstuff samples. Samples with high complexity such as infant milk formulas or energetic drinks were processed by the flow methodology, aiming the selective extraction of the target analyte. The automatic manifold controlled all the steps of the MISPE protocol, including the sorbent packing and discarding. This innovative strategy demonstrated a high precision (RSD < 5.5%), a short analysis time (9.6 min) and a low consumption of chemicals and solvents. By this way, it was introduced a new, fast and reliable methodology suitable to be used for the routine analysis of riboflavin in complex foodstuff samples.

Finally, accuracy was assessed by analyzing certified reference materials or spiked samples. All the details respecting to the development of the proposed systems, as well their advantages and shortcomings are critically discussed.

Keywords: automation, sample preparation, solid-phase extraction, flow injection analysis, liquid chromatography.

Resumo

Na presente dissertação, foram desenvolvidos diversos sistemas baseados na análise por injeção em multi-seringa (MSFIA, do inglês *multisyringe flow injection analysis*) que tiveram dois objectivos distintos: a introdução de novas metodologias analíticas hifenizadas, que englobaram a preparação das amostras através de um sistema de fluxo e o acoplamento a cromatografia líquida (LC), e a obtenção de informação relevante sobre os mecanismos de adsorção analitos ou formas de manipular fases sólidas sob formato mesofluídico de modo a implementar novos procedimentos automáticos para extracção em fase sólida (SPE, do inglês *solid-phase extraction*).

Quatro fases sólidas, com diferentes tamanhos de partícula e esqueleto polimérico de estireno-divinilbenzeno com diferentes graus de interligação, foram avaliadas perante as mesmas condições experimentais usando um sistema MSFIA hifenizado à cromatografia líquida de modo a obter informação relevante sobre a sua performance para retenção de compostos fenólicos através de um mecanismo de fase reversa. Os parâmetros em estudo foram a capacidade da fase sólida, o volume de ruptura e o aumento de sensibilidade (EF). Foi observada uma correlação positiva entre a hidrofobicidade dos compostos e os valores de EF para as fases sólidas com baixo grau de interligação do polímero (Amberlite-XAD 4), enquanto foi encontrada uma correlação negativa entre os valores de FE e o peso molecular dos compostos para as fases sólidas com elevado grau de interligação do polímero (Lichrolut EN e Macronet MN-200. A fase sólida Lichrolut EN apresentou o melhor desempenho em todos os parâmetros em avaliação e por isso foi escolhida para o desenvolvimento de uma nova metodologia analítica.

A mesma configuração MSFIA, que incluiu uma coluna empacotada com Lichrolut EN, foi utilizada para a despistagem de compostos fenólicos em amostras ambientais. Volumes variáveis (até 100 mL) de água, bem como extractos de solo, foram pré-concentrados e analisados através da metodologia proposta, que foi baseada na hifenização do procedimento extractivo com a análise por cromatografia líquida. Foi obtido um elevado aumento de sensibilidade (em média 176 vezes para 50 mL de amostra) e limites de detecção inferiores a $1 \mu\text{g L}^{-1}$ (para 100 mL de amostra). Uma vez que de preparação de amostra e a análise cromatográfica foram efectuadas de uma forma paralela, no mesmo intervalo de tempo, foi possível efectuar até dez determinações por hora com elevada precisão e exactidão.

Um sistema de fluxo baseado em multi-seringa associada a uma plataforma mesofluídica *lab-on-valve* (LOV) foi acoplado a cromatografia líquida, tendo sido utilizado no desenvolvimento de uma metodologia analítica que visou a determinação de filtros UV

em amostras de águas balneares. O sistema de fluxo processou volumes de amostra até 12.0 mL de uma forma totalmente automática para além de assegurar a renovação da fase sólida extractora em cada determinação. Assim, foi possível detectar as quatro moléculas alvo em baixos teores ($\mu\text{g L}^{-1}$) sem qualquer efeito de memória entre determinações consecutivas, efeito este mencionado anteriormente para configurações que aplicam uma coluna permanente de fase sólida. O método cromatográfico baseou-se na utilização de uma coluna monolítica bem como uma eluição isocrática acelerada, o que conduziu a uma redução acentuada do tempo de corrida cromatográfica, possibilitando uma frequência de determinação de 6 h^{-1} .

O processamento de suspensões de fases sólidas (*bead injection*) com forma e tamanho irregulares através de um sistema LOV foi objecto de estudo, uma vez que causam formação de aglomerados, ocasionando o bloqueamento dos seus canais. Assim, diferentes fases sólidas constituídas por esqueletos poliméricos ou de sílica (Oasis HLB, SupelMIP β -receptors, Lichrolut EN and Discovery DSC-MCAX), foram suspensas em diferentes solventes e processadas através de diferentes protocolos fluídicos. Através da utilização de um meio de suspensão e/ou um protocolo fluídico adequado foi possível empacotar cada uma das fases em estudo num dos canais do dispositivo LOV, com uma repetibilidade satisfatória ($\text{RSD} < 14\%$). Foi alcançada desta forma uma abordagem universal para o manuseamento destes materiais, aumentando a aplicabilidade dos sistemas LOV como técnica precedente à cromatografia líquida.

A extracção em fase sólida em modo automático através de polímeros de impressão molecular visando a determinação de riboflavina através de cromatografia líquida, foi implementada recorrendo a um MSFIA-LOV. Todas as fases do protocolo de extracção selectiva foram realizadas no sistema automático, incluindo o empacotamento e o descarte da fase sólida entre cada determinação, possibilitando o processamento de amostras de elevada complexidade, tais como leite infantil ou bebidas energéticas. Esta metodologia, associando o sistema de fluxo a um cromatógrafo, demonstrou uma elevada precisão ($\text{RSD} < 5.5\%$), um reduzido tempo de análise (9.6 min) e um reduzido consumo de reagentes e solventes, sendo adequada para análises de rotina de riboflavina em amostras alimentares de elevada complexidade.

A exactidão das metodologias propostas foi avaliada através da análise de materiais de referência ou de amostras fortificadas com os analitos alvo. Os detalhes respeitantes aos sistemas propostos bem como as suas vantagens e limitações são objecto de discussão crítica.

Palavras-chave: automatização, preparação de amostra, extracção em fase sólida, análise por injeção em fluxo, cromatografia líquida

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List of abbreviations

<i>Abbreviation</i>	<i>Definition</i>
246TCP	2,4,6-trichlorophenol
24DCP	2,4-dichlorophenol
24DMP	2,4-dimethylphenol
24DNP	2,4-dinitrophenol
2CP	2-chlorophenol
2NP	2-nitrophenol
46DNOC	2-methyl-4,6-dinitrophenol
4C3MP	4-chloro-3-methylphenol
4NP	4-nitrophenol
AAS	atomic absorption spectrometry
ACN	acetonitrile
APHA	American Public Health Association
AWWA	American Water Works Association
BI	bead injection
BMDBM	butylmethoxydibenzoylmethane
BP3	benzophenone-3
BZC	benzylcinnamate

List of abbreviations (continued)

<i>Abbreviation</i>	<i>Definition</i>
conc.	concentration
CPU	central processing unit
CTAC	cetyltrimethylammonium chloride
d. f.	degrees of freedom
DAD	diode array detector
DBM	dibenzoylmethane
DDC	diethyldithiocarbamate
DOSS	dioctyl sulfosuccinate
ED	electrochemical detection
EDTA	ethylenediamine tetraacetic acid
EF	enhancement factor
EHMC	ethylhexylmethoxycinnamate
ELS	evaporative light scattering
EPA	United States Environmental Protection Agency
FIA	flow injection analysis
FL	fluorimetric detection
GABA	γ -aminobutyric acid

List of abbreviations (continued)

<i>Abbreviation</i>	<i>Definition</i>
GC	gas chromatography
HMS	homosalate
HPLC	high performance liquid chromatography
i.d.	internal diameter
IC	ion-chromatography
ICP-AES	inductively coupled plasma atomic emission spectrometry
IL	ionic liquid
k'	capacity factor
k_{ow}	octanol-water partition coefficient
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LOV	lab-on-valve
MCFIA	multicommutation flow analysis
MeOH	methanol
MIP	molecularly imprinted polymer

List of abbreviations (continued)

<i>Abbreviation</i>	<i>Definition</i>
MISPE	molecularly imprinted solid-phase extraction
MPFS	multipumping flow systems
MPV	multiposition valve
MS	mass-spectrometry
MSC	multisyringe chromatography
MSFIA	multisyringe flow injection analysis
MW	molecular weight
Nitro-PAPS	2-(5-nitro-2-pyridylazo)-5-(N-propyl-3-sulfopropylamino)phenol
ODS	octadecylsilica
OPA	o-phthaldialdehyde
P	phenol
PAH	polycyclic aromatic hydrocarbon
PAR	4-(2-pyridylazo)-resorcinol
PCP	pentachlorophenol
PDC	Cu-pyrrolidine dithiocarbamate
PEEK	polyetheretherketone

List of abbreviations (continued)

<i>Abbreviation</i>	<i>Definition</i>
PEI	polyetherimide
PMMA	polymethylmethacrylate
PS-DVB	polystyrene-divinylbenzene
PSE	pressurized solvent extraction
PTFE	polytetrafluorethylene
RP	reversed-phase
RSD	relative standard deviation
SBSE	stir-bar sortive extraction
SD	standard deviation
SDS	sodium dodecyl sulfate
SFC	supercritical fluid chromatography
SFE	supercritical fluid extraction
SIA	sequential injection analysis
SIC	sequential injection chromatography
SLME	supported liquid membrane extraction
SPE	solid-phase extraction

List of abbreviations (continued)

<i>Abbreviation</i>	<i>Definition</i>
SPME	solid-phase microextraction
SPW	swimming pool water
SW	seawater
t_{calc}	<i>t</i> -test parameter (calculated)
TFA	trifluoroacetic acid
t_{tab}	<i>t</i> -test parameter (tabulated)
UV	ultra violet radiation
Vis	visible radiation
vol	volume
α	probability of confidence limits
λ	wavelength

1

Introduction

1.1 Analytical chemistry: a sustainable source of information

Analytical Chemistry is a decision maker. The results provided by the analytical methods are an essential source of information for relevant decisions with socio-economical impact. Furthermore, the growing concerns about environmental risk, food safety and human health transformed analytical chemistry in a transversal data source for all scientific fields. This increment in the demand for analytical results has driven the recent evolution in this field, comprising the introduction of new analytical instruments and protocols that have to accomplish the current regulations and customer requirements.

In the recent years, beyond the quality of its results, analytical chemistry also looks to the sustainability of the procedures. This is a result of the potential impact of the analytical methods in the environment. Although analytical chemistry could be considered a small-scale activity, the existence of control laboratories that can process a high number of samples per day, transform the analysis in an important source of pollutants. For these reasons, the objectives of the development of new analytical methods include not only a high analytical performance (selectivity, precision, accuracy, limit of detection) but also a high environmental performance, by reducing materials, human efforts and risks [1]. Consequently, new environmentally friendly methodologies are currently aimed, transforming analytical chemistry in a sustainable source of analytical information. Moreover, the environmental benefit is directly related with an economic benefit, and a positive economic impact is also achieved.

1.2 Green Analytical Chemistry

Although the environmentally friendly perspective seems to be in the genetic code of analytical chemistry, it has been only a topic of growing interest in the recent years [2]. The side effects of the analytical methods caused by the use of toxic reagents and solvents and waste generation are now a concern for the analytical chemist. By this way, current analytical chemistry tries to accomplish green chemistry principles (Table 1) [3].

Table 1.1. The twelve principles of Green Chemistry. The highlighted principles correspond to the most applicable to Analytical Chemistry. Adapted from [4]

1	<u>Prevention</u>
	It is better to prevent waste than to treat or clean up waste after it has been created.
2	Atom Economy
	Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3	Less Hazardous Chemical Syntheses
	Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
4	Designing Safer Chemicals
	Chemical products should be designed to effect their desired function while minimizing their toxicity.
5	<u>Safer Solvents and Auxiliaries</u>
	The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.
6	<u>Design for Energy Efficiency</u>
	Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7	Use of Renewable Feedstocks
	A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable
8	<u>Reduce Derivatives</u>
	Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
9	Catalysis
	Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10	Design for Degradation
	Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11	<u>Real-time Analysis for Pollution Prevention</u>
	Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12	<u>Inherently Safer Chemistry for Accident Prevention</u>
	Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Green chemistry is an approach to the synthesis, processing, and use of chemicals that reduce risks to human and the environment [3]. The risk could be defined as the the hazard of a particular substance multiplied by the exposure to that substance ($\text{Risk} = \text{Hazard} \times \text{Exposure}$) [3]. Since previous efforts in reducing risk were associated to the exposure side of the equation, green chemistry looks for eliminate or reducing the risk by the hazardous point of view. Hazard is a broad range definition, which includes the full range of consequences that could be realized from the use or generation of a substance [3]. By this way, green chemistry concept relies on the implementation of the twelve principles mentioned before (Table 1) aiming the hazard reduction / elimination, and consequently, pollution prevention.

Green analytical chemistry, recently updated by Armenta et al. [2], could be defined as the application of the green chemistry principles into the analytical field. For analytical methods, green chemistry means the design of new protocols that reduce or eliminate the hazardous substances [4]. By this way, collateral effects of the analytical methodologies could be minimized by three different perspectives [2]: (i) reduction of the amount of solvents required in sample pre-treatment, (ii) reduction in the toxicity and the amount of solvents and reagents employed in the measurement step, and (iii) development of alternative direct analytical methodologies not requiring solvents and reagents. Hence, green analytical chemistry purposes the application of its principles in all steps of the analytical procedure (Fig. 1). Nevertheless, most of the recent “green” efforts in the analytical field are focused in the sample preparation because it is commonly the most time consuming [5] and chemical demanding, labour intensive and pollutant step of the whole analytical procedure.

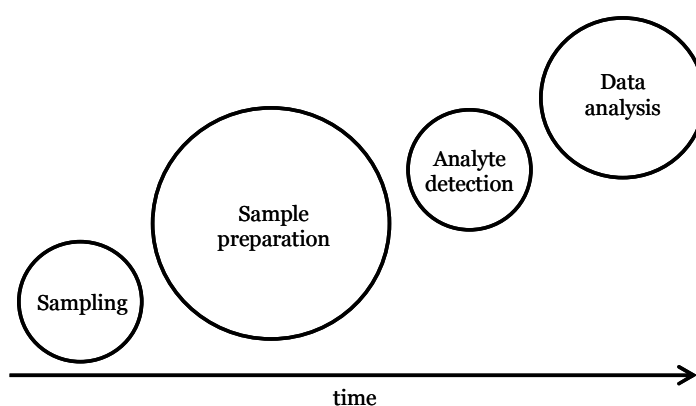


Fig. 1.1. The different steps of an analytical procedure in the respective time scale.

1.3 Sample preparation

Sample preparation is often necessary because most of the current instrumentation is not able to handle the samples without problems. Commonly, samples have to be introduced into the instruments in a liquid phase, which is not possible when solid or gas samples are present. Furthermore, sample matrix is a source of interferent species or detection artefacts. Hence, detection could be affected, resulting in under or overestimation of the analytical result. Therefore, sample preparation is usually mandatory and dependent of the nature of the analytes, the matrix and the levels at which the analysis needs to be carried out [6].

The complexity of the sample treatment is dependent on its nature and it looks for the separation between the analyte(s) of interest and the matrix [1]. An inefficient treatment of the sample is usually the major source of errors that could affect irreversibly the performance of the method. Although sample preparation is customized for each analytical method, a sample is commonly treated in two different complementary steps: preliminary sample treatment and extraction [1]. For example, in the first one the sample is manipulated in order to get a homogeneous and dry surface (solid samples) or to eliminate solid particles in suspension (liquid samples). Other operations such as pH adjustment or protein precipitation could also be performed. During the extraction step, the analyte(s) of interest are isolated and / or concentrated from the matrix by displacement to another phase (generally liquid or solid). The described two-parts procedure is often used for the analysis of organic compounds by liquid chromatography (LC), which was the subject of the present work. The analytical results are highly dependent of the sample treatment, especially when samples with complex matrices are handled. Furthermore, the use of organic solvents and hazardous chemicals, common in extraction procedures, combined with the growing demand for analytical results makes the sample preparation procedure in the major source of pollution of the analysis, sometimes more hazardous than the “pollutants” that are being assessed. For these reasons, it is not strange that sample preparation has been identified as one of the most important targets of green analytical chemistry [2].

Actually, some of the milestones in green analytical chemistry in the last thirty years [2] were related with new concepts in sample preparation procedures, particularly in the extraction procedures of organic compounds. The novel strategies were developed in order to replace the classical liquid-liquid extraction (LLE) and Soxhlet extraction that have been used during decades for extracting organic molecules from liquid and solid samples, respectively. New advances in sample preparation also include the replacement of organic solvents by new benign alternatives, such as ionic liquids and supercritical fluids.

For liquid samples, the common use of sorbent materials in solid-phase extraction (SPE) [7] and the introduction of non-exhaustive extraction procedures as solid-phase microextraction (SPME) [8] and stir-bar sorptive extraction (SBSE) [9] drastically reduced or even eliminated (when thermal desorption is used) the consumption of organic solvents. Another important strategy to minimize the use of organic solvents is the miniaturization of LLE. To this end, single drop microextraction [10], and liquid-liquid-liquid microextraction [11] were introduced and all employed a minimal amount of organic solvent when compared to the LLE in the classic separatory funnel format. Cloud point and phase separation phenomena of surfactant solutions, defined as cloud-point extraction (CPE) [12] was also used as a strategy to circumvent the use of organic solvents.

Concerning solid samples, novel strategies for replacing Soxhlet extraction included the use of microwave (microwave assisted extraction (MAE)) [13] or ultrasonic (ultrasound assisted extraction (UAE)) [14] radiation and also supercritical fluids (SFE) [15]. These are “green” effective approaches to extract solids because they avoid or use minimum amounts of organic solvents. The solid-liquid extraction performed at high temperatures and pressures, defined as pressurized solvent extraction (PSE) is another alternative to drastically reduce the organic solvent consumption [16]. Moreover, due to the possibility of using water as solvent [17], this technique may be free of organic solvents.

Regarding the replacement of organic solvents by “greener” alternatives, two families of solvents should be highlighted due to their current growing interest: supercritical fluids and ionic liquids. Supercritical fluids have gas-like and liquid-like properties [18] and have been essentially used for extracting organic compounds from solid samples. Carbon dioxide is the most used supercritical fluid since it is easy to obtain and provides high extraction efficiency. The use of relatively low pressures and temperatures combined with the flexibility of the technique are the most important advantages of SFE. On other hand, the difficult in the optimization of the extractions parameters and the complex instrumentation required are the major shortcomings [19]. Due to the complexity of the samples and nature of the analytes, SFE has been specially used for food and natural products analysis though applications for other matrices were reported in the literature [15]. Another emerging alternative to organic solvents are ionic liquids (ILs), which are salts with a melting point close or below room temperature [20] that integrate characteristics of water and organic solvents in one molecule [19]. ILs had shown have been an important role in different areas of analytical chemistry [21] and their potential as environmental friendly substitutes of organic solvents are under discussion yet [22].

1.3.1 Automatic sample preparation: a tool for green analytical chemistry

Despite all the recent advances described previously, sample preparation prior to separative techniques is labour-intensive and time consuming step that could represent up to 80% of the total analysis time [5]. Hence, this analytical step is commonly the bottleneck of the analytical procedure. In the case of liquid chromatography, this fact assumes particular importance since the current state-of-art of this separative technique allows the resolution of complex mixtures in a time frame of few minutes. The recent developments in the field, driven by the evolution of the instrumental technology combined with new advances in stationary phases, drastically reduced the time required for analyte separation. However, sample treatment did not keep up with this, requiring still too much time. On other hand, the current trend is an increase in the demand for analytical results, due to the more exigent legal requirements and the emerging concern about new pollutants in the environment [23]. In this context, the automation of sample preparation prior to LC analysis represents a valuable asset for improving the performance of liquid chromatographic methodologies.

Automation is a valuable tool to reduce the human effort in the analytical procedure. Indeed, automatic sample preparation is able to provide, simultaneously, short analysis time, reduction of labour and chemicals consumption. By this way, automation answers to green analytical chemistry demands, contributing for the development of more sustainable procedures. The development of automatic methods can definitely contribute to the reduction of risks, by tackling the two factors of the hazard $\times$ exposure equation. The simplification and miniaturization of the protocols rely on the introduction of new strategies that usually use benign or less hazardous chemicals, as well in the reduction of the respective amounts used. On other hand, automatic manifolds are closed circuits with minimal human intervention, which also reduces the exposure of the operator. Hence, errors are minimized, enhancing the quality of the results.

Considering the division proposed by Valcárcel and Luque de Castro [24], automatic methodologies for chemical analysis may be divided in robotic, discontinuous and continuous. Since the robotic and discontinuous methods involved mechanical operations, they are complex and expensive, and consequently, only applied to a narrow range of analytical problems. In contrast, the continuous methods based on flow injection analysis [25] and related techniques are simple and rely on cost-effective instrumentation. Hence, they became an inexpensive and flexible tool for developing new automatic methods.

1.3.2 Automation based on flow injection analysis: from wet chemistry to sample preparation

The automation by flow injection analysis (FIA) and related techniques is based on the combination of three principles: (i) reproducible sample injection in a carrier / reagent stream, (ii) controlled dispersion of the sample zone and (iii) reproducible timing from the injection point to the detection system [26]. Since the introduction of FIA in 1975 [25], derived computer-controlled related techniques based on the same principles have been proposed (Fig. 2). Flow injection methods have been extensively applied to the automation of all kinds of chemical assays with more than 20 000 papers published in the last 35 years [27]. Nevertheless, since the early years of FIA, sample preparation has been a research topic [26], which was extensively exploited in the two monographies recently published [28-29].

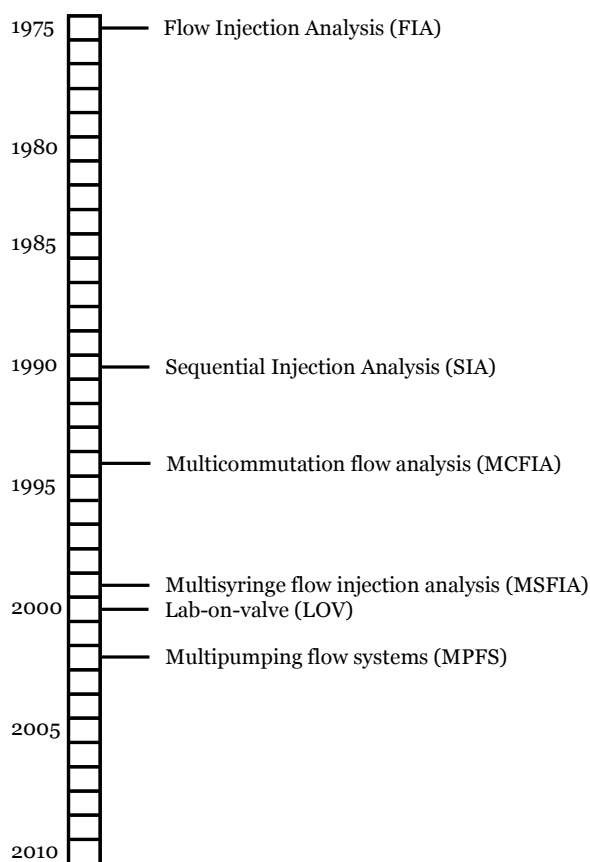


Fig. 1.2. Timeline of flow injection techniques.

Flow based manifolds are able to perform a plethora of sample preparation operations [28-30] such as dilution, filtration, digestion, derivatization and extraction. The nature and complexity of the operations carried out by the different systems were directly related with the components that could be assembled to the manifold as well by the design of the flow system, which is usually customized for a particular application. By this way, each flow based technique has different potential for handling samples that should be considered before the development of a new method.

Flow injection analysis [25] was used for treating samples taking advantage of the repeatable operation sequences ensured by the automatic system. Analyte enrichment and non-chromatographic separation techniques as liquid-liquid extraction, solid-phase extraction, gas diffusion and dialysis were applied [26]. Considering that FIA manifolds are generally manually operated and that the flow is unidirectional, the systems are usually complex and some features, such as stopped flow, and application of variable flow rates and sample volumes are difficult to implement.

Sequential injection analysis [31] inaugurated the programmable flow by introducing bi-directional flow and open architecture in the manifold. The computer-controlled manifolds are based in a multiposition selection valve that increased the versatility of the systems due to the possibility of communication with the different channels of the valve, where different devices or solutions could be accommodated [32]. Sample and reagent(s) are sequentially aspirated into a holding coil, connecting the central channel of the selection valve to a propulsion element. By flow reversal, the stacked zones are propelled through one of the lateral ports of the selection valve for further processing or analyte detection. Moreover, SIA uses preferably syringe pumps. These propulsion units are very robust and able to handle volume in the microliters range without the deterioration problem observed for the flexible tubes present in the peristaltic pumps typically used in FIA systems. By this way, when compared to FIA, SIA is able to perform extraction procedures (SPE, LLE, gas-diffusion, dialysis) using small volumes of reagents and solvents [32]. The programmable flow also made possible the time-based injection. Indeed, volumes and flow rates are controlled by the software parameters and not by the physical configuration of the manifold (injection valve loop or internal diameter of peristaltic pump tubing, respectively). Although the operation in a single channel increases the versatility of manifold, the efficiency in the mixture of the different fluidic zones is lower than in the multichannel FIA and this fact could be a limitation when several plugs have to be mixed at the holding coil.

The lab-on-valve (LOV) concept [33] is a miniaturized approach of SIA. Although based in the same principles and basic configuration (propulsion unit and multiposition selection valve), LOV is characterized by a monolithic device mounted atop of a selection valve that accommodates a detection unit. From the sample preparation point of view, LOV become an unique platform for handling solid suspensions in microscale, creating the possibility of renewable SPE in a non-robotic format that could be hyphenated with different instruments [34-35].

Multicommutation flow analysis (MCFIA) [36] and multisyringe flow injection analysis (MSFIA) [37] are computer-controlled flow techniques based on the implementation of the “flow network” concept. The use of an array of three-way commutation valves to build a customized manifold enables the access to the solutions in a very flexible way. This fact results in an enhanced ability to accommodate different flow patterns and sample preparation devices [38]. Typical MCFIA configurations would include single (syringe pump) or multichannel (peristaltic pump) propulsion units that aspirate or propel the solutions to a detection system. On other hand, MSFIA technique is based in the use of a multisyringe burette as propulsion device. This equipment is a multichannel piston pump, containing up to four syringes, driven by a single motor, with a three way commutation valve placed at the top of each syringe. This device which is also able to interface extra commutation valves in order to create the flow network. Due to its versatility (bi-directional programmable flow) and chemical resistance (ability for propelling organic solvents), MSFIA has been extensively used for the implementation of sample treatment procedures including extraction and digestion [39].

Multipumping flow systems (MPFS) [40] are also based in the “flow network” concept. In contrast with all other flow techniques, MPFS relies on the use of an array of solenoid actuated micropumps that ensure the flow management [41]. Moreover, the solenoid micropumps are able to produce a turbulent flow [42], which results in an enhanced, faster mixing between the different solutions when compared to that attained under laminar flow conditions existing in previous FIA systems. Though MPFS has been essentially a tool for the automation of chemical assays [41], some manifolds devoted to sample preparation are described in the literature [43-44]. Moreover, due to the flexibility of the “flow network” approach, the combination between different techniques (MCFIA, MSFIA and MPFS) has been used as a solution to improve the extractive performance of the methodologies [45-47].

1.4 Interfacing flow analysis and liquid chromatography

Flow analysis and liquid chromatography are related hydrodynamic techniques. Both are based in the injection of a sample in a continuous carrier stream that is sent to a detector under controlled dispersion conditions. The main operational differences between the two techniques rely on the existence of a separation column between the injection point and the detector and the operation at high-pressures, typical from LC equipments. Nevertheless, considering the interactions between mobile and stationary phase, the existence of a chromatographic column changes drastically the way how the sample travels along the carrier stream, providing different analytical abilities for each analytical approach, which were critically discussed by Ruzicka and Christian [48]. The most significant difference between the two techniques can be founded in the analytical goal [48-49]. Flow injection techniques are able only to quantify one or a few analytes, whereas LC is able to determine simultaneously several analytes. Flow injection analysis can improve the sensitivity and selectivity of the determination in pre or post-column arrangements [49]. Since the analyte concentration and matrix cleanup are two of the most frequent sample treatment procedures in liquid chromatography, these steps can be provided by the flow system. Moreover, the flow injection manifold is able manage post-column reactions, which are used to enhance the optical properties of the analytes, which are directly related with the sensitivity. On other hand, LC introduces a separative capacity in flow injection analysis and related techniques, by enhancing the selectivity and providing multi-analyte determination. By this way, the on-line coupling of flow techniques and liquid chromatography is a synergic process that combines the advantages of automation, namely simplification and miniaturization [1] with the robustness of liquid chromatography analysis.

The assertion of a manifold as a flow analysis – liquid chromatography hyphenated system is not an easy task. Some researchers consider any post-column derivatization system to be a flow injection analysis system. Taking into account the three principles of flow injection analysis [26], Luque de Castro and Valcárcel defined, in 1992, the FIA - LC systems as the manifolds that included two injection valves, with each of them assigned to FIA and LC [49]. Considering that in 1992 computer-controlled techniques were emergent, the current definition of hyphenation between flow analysis and liquid chromatography should include all configurations that are able to manage a sample plug in dispersion controlled conditions. By this way, it is possible to establish the boundary between flow injection analysis and the continuous flow approach [50-51], where the samples and / or reagents are pumped by a propulsion unit that could be similar to the

units used in the different flow techniques, but without suffering dispersion while travelling into the tubes. Considering these characteristics, it is possible to define a flow injection analysis – liquid chromatography hyphenated methodology as a method where the solution to be analyzed or the reagent is under dispersion controlled conditions during the automatic procedure.

The coupling between flow injection analysis and liquid chromatographic may assume different designs, depending on where the automatic method is placed and the degree of integration of the liquid chromatographic separation. Hence, three different hyphenation approaches could be defined (Fig. 3). The flow injection system could be placed before the separation (upstream) in a pre-column arrangement, acting as a front end to the separative method (Fig. 3 (A)). In this case, the main purpose of the system is to improve selectivity and sensitivity by preconcentration and cleanup [28]. On other hand, the flow injection manifold could act in a post-column arrangement (Fig. 3 (B)) (downstream) and used as a tool for derivatization. Finally, the last approach includes a full integration between flow injection analysis and LC. In this particular strategy, the chromatographic column is one of the manifold components, introducing separative capacities in the hybrid, low pressure flow injection system (Fig. 3 (C)).

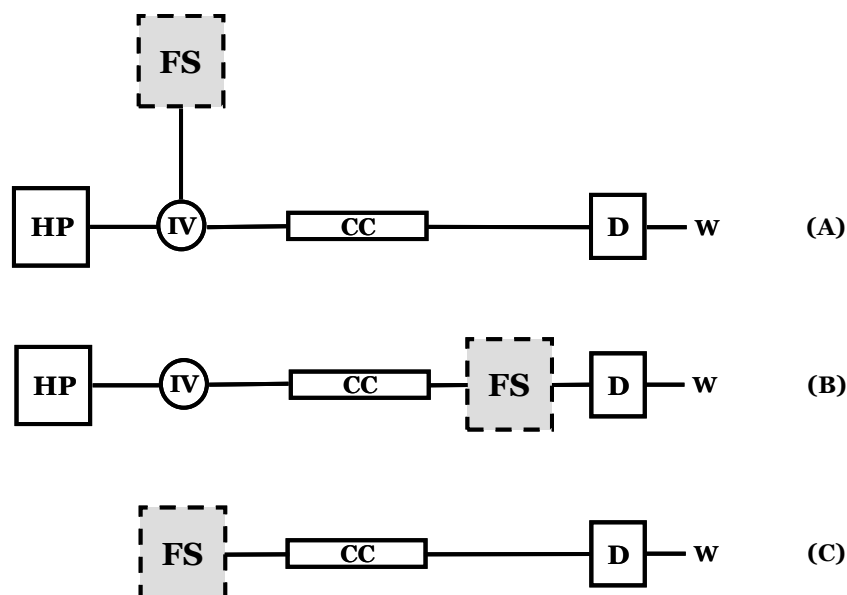


Fig. 1.3. Interface designs between flow injection analysis and liquid chromatography. (A), pre-column hyphenation; (B), post-column hyphenation; (C), hybrid system; FS, flow injection system; HP, high-pressure chromatographic pump; IV, high-pressure injection valve; CC, chromatographic column; D, detector; W, waste

1.4.1 Upstream hyphenation – pre-column arrangements

The use of flow injection analysis and derived techniques as front end to liquid chromatography is the preferential approach reported in the literature [52]. Regarding the features of these manifolds, several sample preparation operations such as SPE, derivatization, LLE, dialysis, supported liquid membrane extraction (SLME), gas-diffusion or filtration were accommodated [52].

Since preconcentration and sample cleanup are the most common goals during sample preparation, SPE has been extensively used in flow analysis manifolds [28-30]. SPE is easy to automate and it is available for a broad range of applications. Moreover, there are a large variety of commercial products and the cost is relatively low when compared with other extraction techniques. Under flow analysis format, SPE can be performed in a permanent or renewable mode. In the first case, a column filled with sorbent is assembled to the manifold while in the second one the sorbent is packed and discarded after the elution of the analytes in a fully automatic fashion [34].

Since wet chemistry comprises the majority of flow analysis applications, sample derivatization can be easily performed by flow injection analysis based manifolds, namely FIA and SIA, coupled to liquid chromatography [53]. The objectives of the chemical derivatization of the analytes are the enhancement of sensitivity and / or selectivity of the method. The sample is merged with the reagent (FIA) or mixed by flow reversal (SIA) before reaching the LC injection valve. When the sample is abundant, reverse FIA could be used as a strategy to minimize reagent consumption, considering that in this particular mode of operation the reagent is injected in a sample stream [54].

Membrane separation techniques able to perform non-chromatographic separations such as dialysis [55], gas diffusion [56], SLME [57] and LLE [58] were also carried out in flow analysis format and hyphenated to LC. Commonly, customized lab-made devices are included in the configuration. These devices are made with polymeric materials and accommodate the membrane between engraved channels corresponding to the donor and acceptor stream or organic and aqueous phase, in the case of LLE. This array of techniques provides sample cleanup, preconcentration or dilution, resulting in an improved overall performance of the methodology. The shortcomings of these methodologies are related with the intrinsic low recoveries attained for low yield mass transfer processes such as dialysis and gas diffusion, the necessity of a periodic replacement of the membrane and the poor compatibility of some materials of the different manifold parts with organic solvents. Flow injection techniques based on the “flow network” and MSFIA concept are amenable to implement membrane based non-chromatographic separations because they

combine multichannel propulsion and programmable flow control. Therefore, different flow paths and flow rates are available, resulting in an enhanced flexibility of the configuration, and consequently, of the hyphenated methodology.

The elimination of suspended solids by filtration, particularly relevant when solid samples are handled or chemical processes are monitored, can also be included in automatic flow injection systems. Membranes filters and frits, placed inside the respective holders, can be assembled to the configuration. The time between filter replacements are commanded by the amount of particles present in the sample as well as by the pore diameter of the filtration surface.

The interface between the flow injection system and the LC equipment is usually ensured by the LC injection valve (Fig. 4). After the sample preparation steps, the sample plug can be totally or partially injected for separation. The latter case, defined as heart-cut injection (Fig. 4 (A)), is the most common way to inject the sample into the LC equipment. The advantages of this approach, provided by the small volumes injected (usually up to 50 μL), are the possibility of avoiding band broadening of the peaks and sample dilution when a less concentrated zone of the plug is selected. However, when trace levels are aimed, the partial injection of the sample plug provides lower enrichment factors.

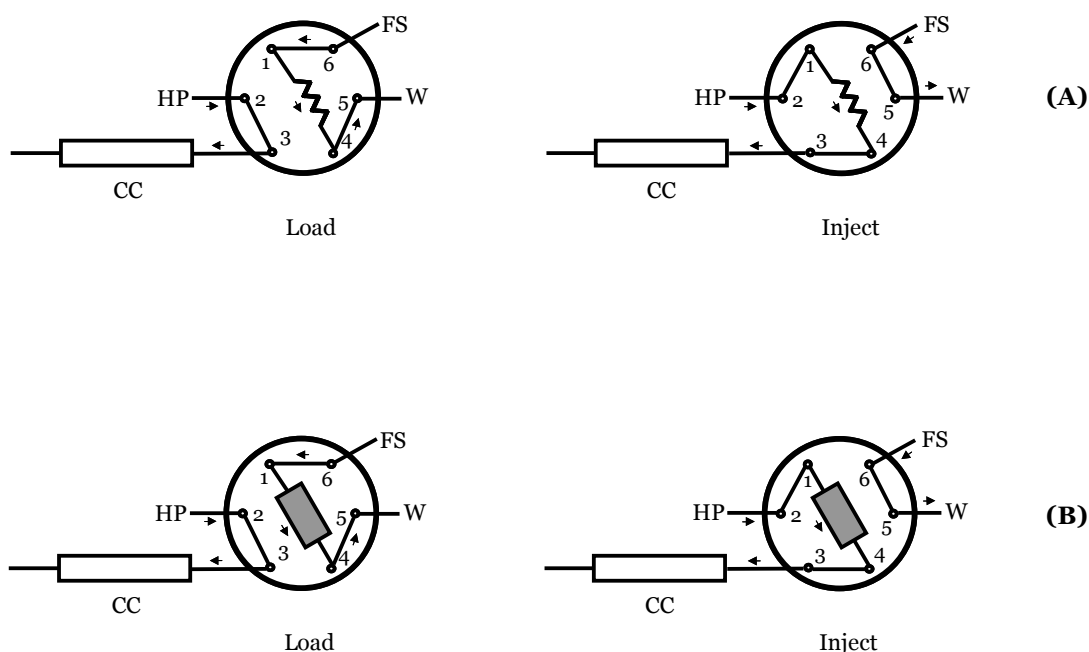


Fig. 1.4. Illustration of a pre-column interface between the outlet channel of a flow injection system and a liquid chromatograph based on a six port high-pressure injection valve, and using a heart-cut (A) or column switching (B) strategy. FS, flow injection system; HP, high-pressure chromatographic pump; CC, chromatographic column; W, waste. The connection between ports 1 and 4 of the injection valve represent an injection (volume $\leq 50 \mu\text{L}$) (A) and a SPE column (B).

Considering that SPE is one of the most popular techniques used as front end to LC [7], another possible approach, based on sample loading into a packed column placed at LC injection valve followed by its switching and elution by the LC mobile phase is the so-called column switching injection (Fig 4 (B)). Though this strategy is not very common for flow analysis – LC hyphenated schemes, it constitutes the most often used scheme for automatic sample processing for chromatographers. The advantages of this design are the simplicity and the flexibility of the system and the total compatibility between the eluate composition and LC mobile phase, since the mobile phase is the eluent. Regarding to the disadvantages, partial elution and carryover between consecutive samples are the most common problems for reversed-phase sorbents, due to weak elution strength of some mobile phases with high aqueous content. Another important limitation, typically observed in automatic SPE systems based on a permanent packed column, is the irreversible sorption of matrix components.

1.4.2 Downstream hyphenation – post-column arrangements

Post-column hyphenation between flow analysis and LC could be also an interesting way to enhance the performance of the methodologies, particularly by performing the derivatization of the separated analytes [49].

Post-column derivatization of the compounds separated by LC, usually by mixing with chromogenic or fluorogenic reagents, is an important way to increase the sensitivity of the methods. The selected reagents should be able to provide fast reactions in order to guarantee a high reaction product concentration in a short period of time with a minimum dilution of the separated analyte. The most common way to make this operation is by adding a secondary stream of the derivatizing agent by a three way junction placed between the exit of the chromatographic column and the detector. This design ensures a suitable mixture and reaction time with a minimum dilution but does not correspond to a flow injection analysis – LC hyphenation scheme, since the sample is merely merged with the reagent and not injected into a carrier or reagent stream.

Notwithstanding, the downstream hyphenation was successfully exploited for the development of interfaced methodologies that combined screening and confirmatory analysis (Fig. 5) [49, 59]. In the screening mode of operation, the selectivity was ensured by an extraction procedure or by a selective derivatizing agent and the result corresponds to the sum of individual contribution of all compounds of analytical interest. For the positive tested samples, a confirmatory step was performed by separating the target

analytes by LC. At the end of the separative step, successive aliquots could be consecutively injected into the flow system (Fig. 5 (B)) or the merging zones approach could be used, by merging the eluate from the LC system with a large reagent plug (Fig. 5 (C)) [59].

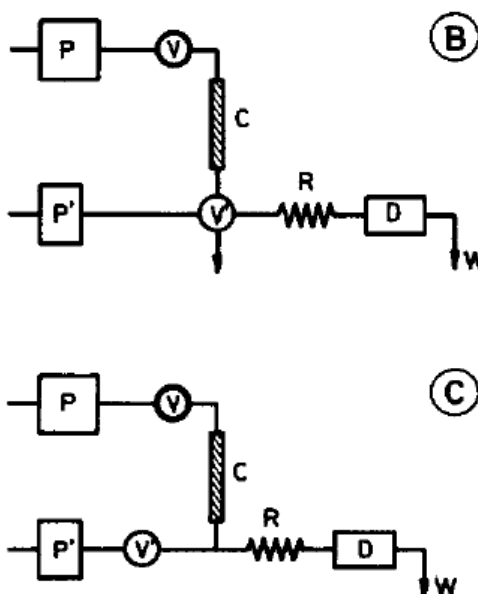


Fig. 1.5. Types of FIA-LC post-column configurations [59]. P, high-pressure pump; P', FIA pump; V, LC injection valve; R, reactor; C, chromatographic column; D, detector; W, waste. Copyright 1988 Elsevier Science Publishers.

Considering the current state-of-art, it is clear that the downstream hyphenation between flow analysis and LC has not been so attractive for the researchers as the upstream hyphenation. This fact is not strange if we consider two complementary reasons: the post-column interface between flow systems and LC is more difficult than the continuous flow approach, because it is necessary a high degree of synchronism between the two modules. In addition, post-column handling of the separated analytes does not avoid the sample preparation step, which makes these analytical methods very complex. Furthermore, the current trends in LC, which are based on the use of mass spectrometry detectors, with high sensitivity and selectivity, are creating a growing demand for new sample preparation techniques with high performance. Nevertheless, programmable flow analysis manifolds have never been used in downstream hyphenation, where they could have an interesting role. Programmable flow allows the possibility of a flexible and perfect synchronism between the LC equipment and the flow system. By this way, coupling programmable flow techniques as SIA could provide new possibilities for post-column operations, and consequently an enhanced performance of the hyphenated methodology. A possible manifold, depicted at Fig. 7, shows the potential abilities of this approach

considering that the lateral ports of the multiposition valve could easily accommodate reagents and connections to other devices or instruments. Therefore, after separation, the separated analyte(s) could be send to the LC detector or reach the flow system and processed. In the latter case, the analyte could be processed by a chemical reaction aiming a more sensitive detection or by a extraction device for a secondary sample preparation procedure. Subsequently, the separated plug could be directed to a detector or conducted to second dimension chromatographic procedure.

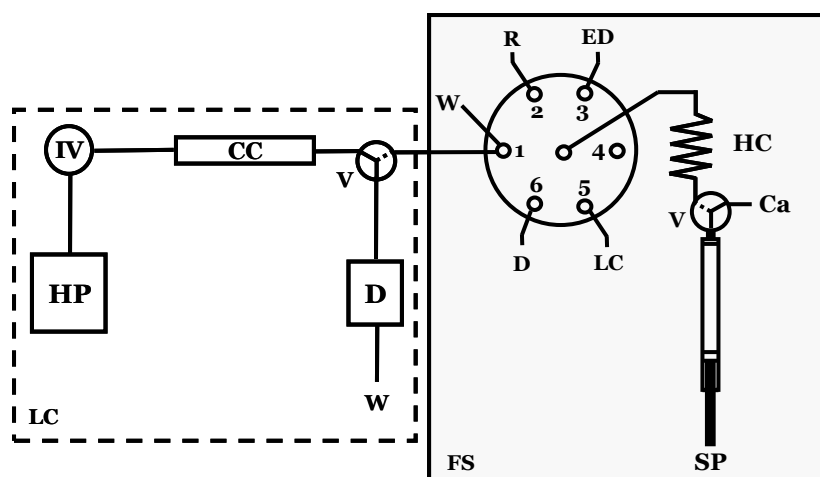


Fig. 1.6. Illustration of the potential of a SIA system (programmable flow) for downstream hyphenation with LC. LC, liquid chromatograph; FS; sequential injection analysis system; HP, high-pressure pump; IV, high-pressure injection valve; CC, chromatographic column; V, three way commutation valve; D, detector; W, waste; R, reagent; ED, extraction device; HC, holding coil; Ca, carrier; SP, syringe pump.

1.4.3 Hybrid systems – liquid chromatography in flow analysis format

The introduction of the monolithic columns in liquid chromatography [60] created new possibilities in the coupling between LC and flow analysis. The enhanced permeability properties of porous monolithic silica rods allowed operation at backpressures five to ten times lower than those attained for the conventional packed bed columns with particles of 5 – 10 μm . This operation conditions can be supported by some propulsion units present in flow systems. Hence, a short reversed-phase monolithic column was assembled to the flow injection manifold, resulting in the introduction of chromatographic separations in flow injection analysis format. Therefore, hybrid systems comprising a chromatographic column were introduced, namely, Sequential Injection Chromatography (SIC) (Fig. 8) [61] and Multisyringe Chromatography (MSC) (Fig. 9) [62], based on SIA and MSFIA,

respectively. In addition, some approaches based on the typical FIA design have also been proposed [63-64].

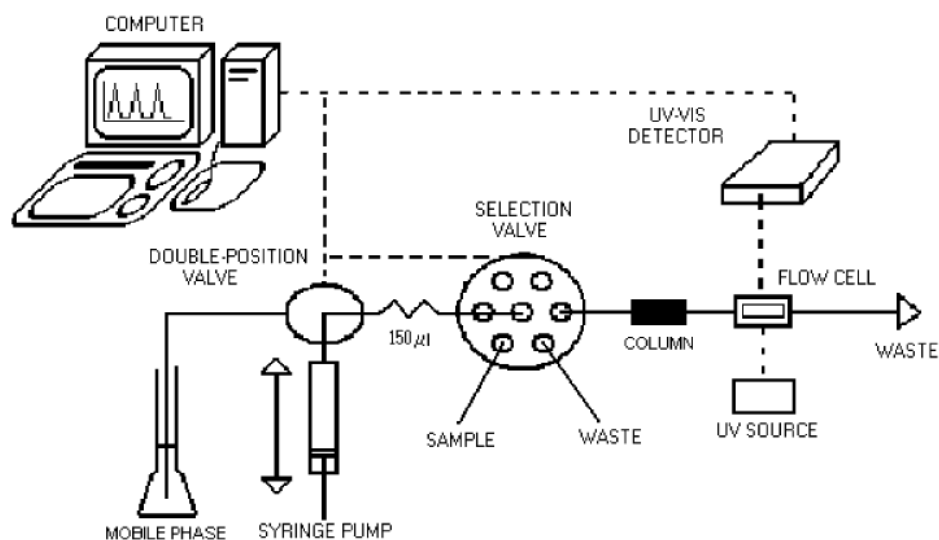


Fig. 1.7. Scheme of sequential injection chromatography set-up for the chromatographic separation and determination of sodium diclofenac, methylparaben and propylparaben [61]. Copyright 2003 Elsevier Science Publisher.

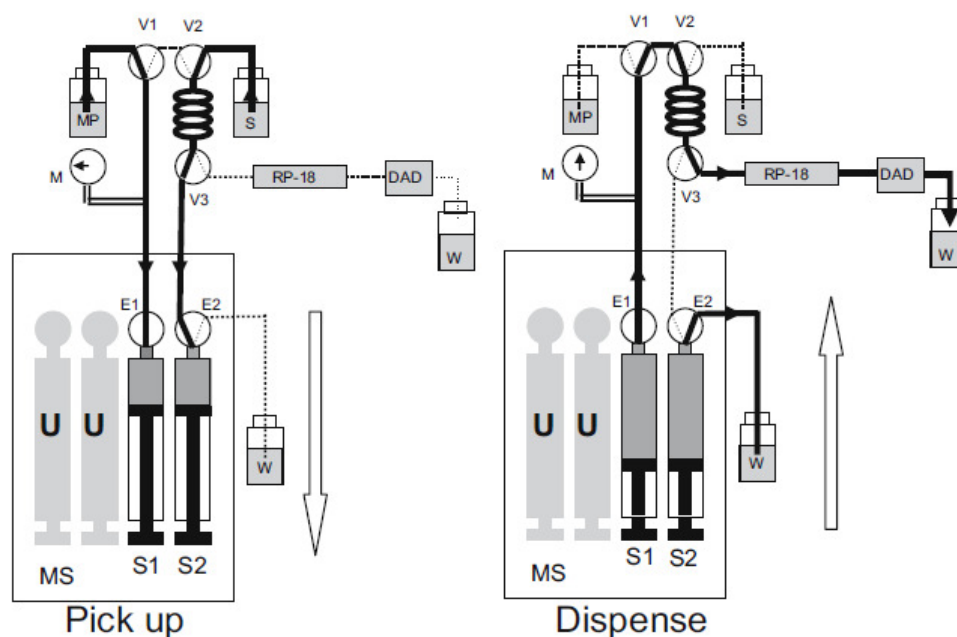


Fig.1.8. Multisyringe chromatography system used for isocratic separation of β -lactamic antibiotics [62]. MS, multisyringe burette; M, manometer; V_i , solenoid valves; MP, mobile phase; S, sample; W, waste; RP-18, monolithic column; DA, diode array detector; U, unused syringe. Copyright 2007 Springer Verlag.

Hence, chromatographic separations have been implemented with low pressure operation with an analytical performance comparable with the HPLC instrumentation [65-66]. Nevertheless, from the technical point of view, some limitations were noticed.. Gradient elution is difficult due to the absence of electronic valves and in-line mixing chambers typical from the high-pressure pumps and flow rates are limited to the maximum backpressures allowed by the propulsion devices available. Peristaltic and syringe pumps have been used, peristaltic pumps only managed 5.0×4.6 mm columns [64] while syringe pumps could propel mobile phase through 25.0 or 50.0×4.6 mm columns [65]. The use of more robust piston pumps, similar to high-pressure chromatographic units, was recently introduced. The use of these apparatus enabled the creation of elution gradients [63] or increased the maximum working pressure of the systems [67]. The efficiency of the separations is also limited by the size of the column (the most common size is 25×4.6 mm) and the separation is limited [63]. In order to circumvent these drawbacks and improve the analytical information of the novel methodologies, recent developments comprised the use of different mobile phases [64, 68] or columns [67] for the same determination, long path flow cells [69] and also chemometric tools for data analysis [70].

The inclusion of sample preparation steps in these hybrid methodologies is also reported in the literature. The manifolds of SIC and MSC have a flexible design that makes possible the assembling of sample preparation devices in the ports of the multiposition valve or in the flow network, respectively. By this way sample preparation in a fully automatic fashion is possible, and steps as solid-phase extraction [71], filtration and dilution [72] were performed in an in-line mode prior to the chromatographic separation. Moreover, the multichannel propulsion of the multisyringe burette could manage the analyte derivatization prior to the detection [71].

Taking into account the simple sample matrix and the intimate relation between LC and pharmaceutical analysis, the majority of the applications described in the literature are based on the analysis of pharmaceutical formulations [65-66]. Recently, new applications including biological and environmental samples were also reported [69-71].

1.5 Flow injection analysis based methodologies for sample preparation coupled to liquid chromatography: an overview

In the present section, the published literature related the use of flow injection analysis based methodologies as front end to liquid chromatography (Table 1.2), subject of the

present thesis, will be reviewed and discussed considering its operation conditions and analytical performance (Table 1.3). Hybrid systems, namely SIC and MSC, were not considered here.

The coupling between flow injection based methodologies and liquid chromatography for sample preparation comprises twenty five references in the literature (Table 1.2). Although the first work was published in 1990, only after 2003 a significant number of applications have been reported (Fig. 1.9), indicating the growing interest of this research topic.

Regarding the flow techniques used, about half of the papers (56%) applied FIA as flow technique (Fig. 1.10). The choice of FIA could be explained considering that it is the oldest and the most widespread flow approach, its cost is low and it is also easy to implement. However, in the latest years the applications were more frequently based on computer-controlled flow techniques, such as SIA and MSFIA. They are indeed becoming predominant, representing together approximately the other half of the publications. It is clearly that the versatility of these techniques and the robustness of syringe pumps associated with them are very attractive features for the design of new automatic manifolds for sample preparation.

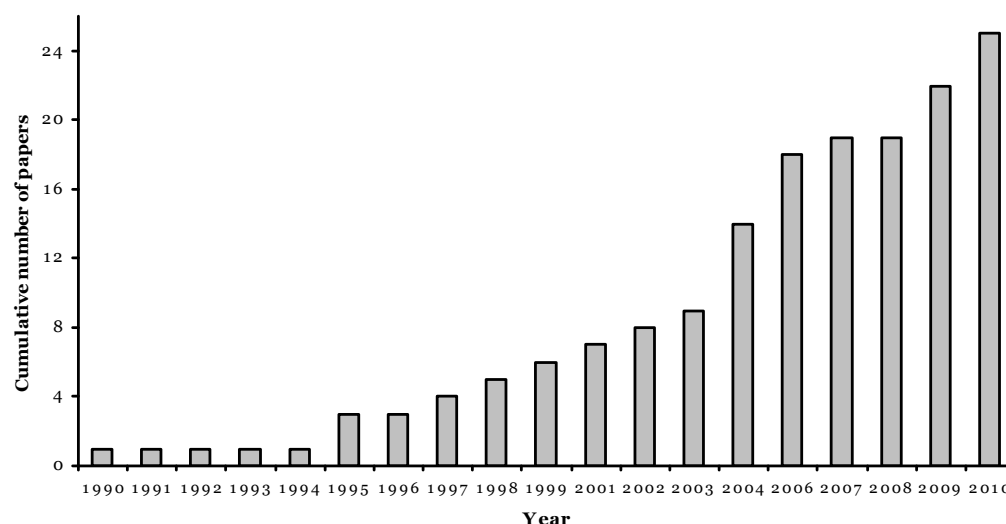


Fig. 1.9. Cumulative number of papers per year until June 2010, describing analytical methodologies based on the coupling of flow systems and liquid chromatography.

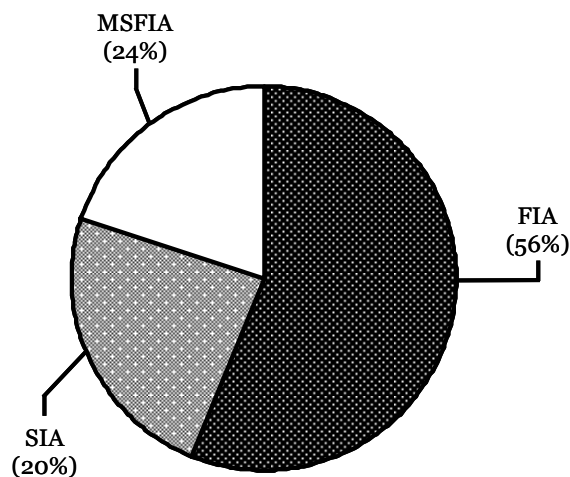


Fig. 1.10. Distribution of papers describing flow injection based methods for sample preparation coupled to liquid chromatography regarding to flow injection and derived techniques applied.

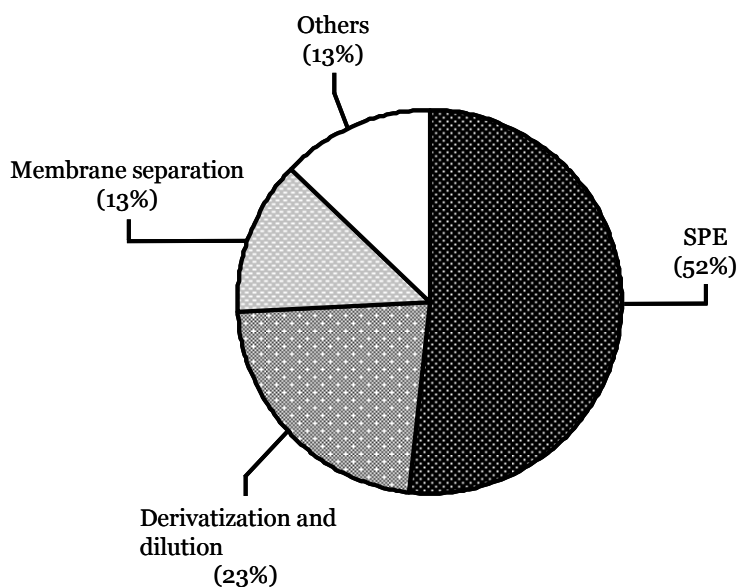


Fig. 1.11. Distribution of papers describing flow injection based methods for sample preparation coupled to liquid chromatography regarding to the type of sample preparation technique applied.

Considering the operations for treatment of the samples performed in the flow manifolds (Fig. 1.11), the majority (52%) was devoted to SPE procedures. The higher number of reports including SPE is clearly related with the ease of implementation of this extraction technique in flow analysis format. SPE could be performed by simply including a packed bed column to the manifold. Furthermore, the variety of sorbents commercially

available and the possibility of replacing the manual procedures or upgrading the classical column switching automatic approach used in LC, transform SPE under flow injection analysis format in a very interesting tool for researchers, who routinely apply SPE based manifolds to preconcentration and cleanup procedures prior to LC determination. Derivatization and dilution, reported in 23% of the surveyed methodologies, are the operations usually performed in flow injection manifolds for chemical analysis, and they were extremely useful for enhancing the sensitivity or adjusting the linear range in coupled methodologies, respectively. The extraction based on membrane techniques such as dialysis, gas diffusion and supported liquid membrane extraction were also reported for the separation and extraction of different analytes, representing about 13% of the reported methods. Furthermore, other techniques as microwave assisted extraction, liquid-liquid extraction, evaporation or filtration were also used to prepare liquid and solid samples for LC analysis.

The use of automatic flow injection based sample preparation has been applied to different analytical areas (Fig. 1.12). The largest number of applications dealt with the environmental field, comprising 43% of the papers that included seawater, river water, wastewater, soil and respective extracts as samples. Other major areas of application were food and biomedical analysis, representing 25 and 21% of the reported applications, respectively. In the food analysis field, several samples as milk, soft drinks, coffee, wine and drink water were analysed. Concerning to biomedical analysis field, the matrices processed comprised biological fluids and pharmaceutical formulations. In the section “others”, different samples (ore and black liquor from paper industry) were considered.

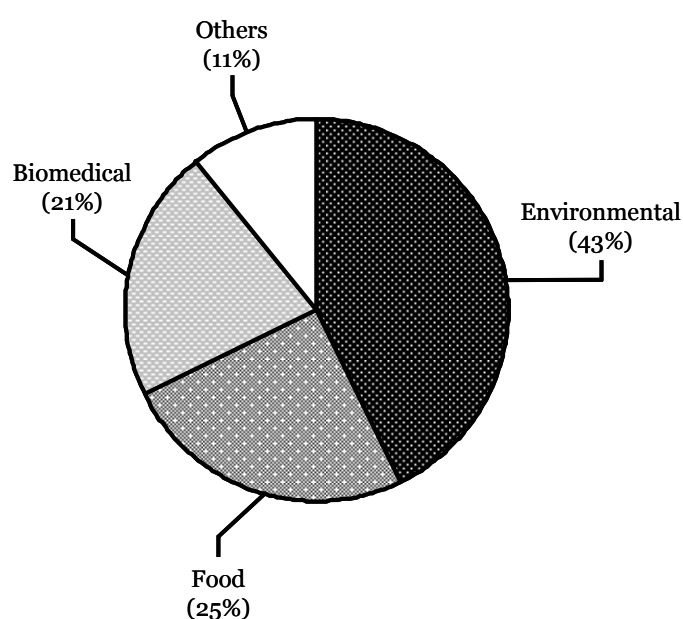


Fig.1.12. Distribution of papers describing flow injection based methods for sample preparation coupled to liquid chromatography regarding to area of application.

Table 1.2. Flow injection based and derived methodologies for sample preparation coupled to liquid chromatography determination.

Sample			Chromatographic conditions				
Flow technique	Analyte	Type	Treatment	Column	Mobile phase ^a	Detection	Ref.
FIA	Caffeine	Beverages, biological fluids	LLE	Si packed (5 μm)	ACN:dichloromethane (30:70)	UV	[58]
FIA	Zidovudine	Biological fluids	SPE	Si packed (5 μm)	ACN:dichloromethane (40:60)	UV	[58]
FIA	Ammonia and methylamines	Seawater	Gas diffusion	PS-DVB ^b packed (5 μm)	HCl 40 mmol L ⁻¹	Cond.	[56]
FIA	Levodopa and carbidopa	Deproteinated plasma	SPE	ODS packed (5 μm)	MeOH:phosphate buffer 70 mmol L ⁻¹ pH 2.8, EDTA 1 mmol L ⁻¹ , OSA 1 mmol L ⁻¹ (16:84)	Amp.	[73]
FIA	Metals	Waste water	Derivatization, SPE	ODS packed (10 μm)	MeOH:H ₂ O (80:20)	UV	[74]
FIA	Anions	Water	Dialysis	n.a.	Carbonate buffer 50 mmol L ⁻¹	Cond.	[75]

Note: ^a ratios in percentages v/v; ^b sulfonated; Amp., Amperometry; Cond., conductivity; FIA, flow injection analysis; LLE, liquid-liquid extraction; n. a., not available; SPE, solid-phase extraction; UV, ultra-violet.

Table 1.2. Flow injection based and derived methodologies for sample preparation coupled to liquid chromatography determination (continued).

Sample			Chromatographic conditions			
Flow technique	Analyte	Type	Treatment	Column	Mobile phase ^a	Detection Ref.
FIA	Anthocyanins	Wine	SPE, evaporation	ODS packed (5 µm)	ACN:formic acid 10% v/v (5:95 to 40:60)	Vis [76]
FIA	Bile acids	Biological fluids extracts	SPE	ODS packed (4 µm)	ACN:MeOH (65:35)	ELS [77]
FIA	Metals	River water	SPE	ODS packed (10 µm)	ACN:acetate buffer 3.0 mmol L ⁻¹ (pH 6.0), TBABr 6.2 mmol L ⁻¹ (37:63)	Vis [78]
FIA	PAHs	Soil	MAE, filtration, dilution, SPE	ODS packed (4 µm)	ACN:H ₂ O (75:25 to 100:0 v/v)	FL [79]
FIA	Mercury species	Seafood extracts	SPE	ODS packed (5 µm)	MeOH:ACN:H ₂ O:APDC (45:33:22:0.1)	UV [80]
FIA	Metals	Zinc ore digest	SPE	PBDMA	Tartaric acid 3.0 mmol L ⁻¹ and oxalic acid 1.0 mmol L ⁻¹	Cond. [81]

Note: ^a ratios in percentages v/v; ACN, acetonitrile; APDC ammonium pyrrolidine dithiocarbamate; Cond., conductivity; ELS, evaporative light scattering; FIA, flow injection analysis; FL, fluorescence; MAE, microwave assisted extraction; ODS, octadecyl silica; PAH, polycyclic aromatic hydrocarbons; PBDMA, polybutadiene maleic acid; TBABr, tetrabutyl ammonium bromide; UV, ultra-violet; Vis, visible.

Table 1.2. Flow injection based and derived methodologies for sample preparation coupled to liquid chromatography determination (continued).

Sample		Chromatographic conditions					
Flow technique	Analyte	Type	Treatment	Column	Mobile phase ^a	Detection	Ref.
FIA	Metals	Wastewater	Derivatization	ODS packed (10 μm)	ACN:acetate buffer (pH 6.0) TBABr 3 mmol L ⁻¹ (37:63)	Vis	[54]
FIA	Phenolic compounds	River water	SLME	ODS packed (10 μm)	MeOH:CH ₃ COOH (45:55)	UV	[57]
FIA	Organic acids	Wine	Dialysis	ODS packed (5 μm)	ACN:phosphate buffer 50 mmol L ⁻¹ (1:99)	UV	[55]
SIA	Aromatic polyhydroxy compounds	Black liquor	SPE	PS-DVB packed (10 μm)	MeOH:H ₂ O (40:60)	UV	[82]
SIA	Caffeine	Urine and beverages	SPE	n. a.	MeOH:ACN: ammonium acetate 50 mmol L ⁻¹ :CH ₃ COOH (17:12:70.4:0.6)	UV	[83]
SIA	GABA	Biological fluids	Derivatization	ODS packed (4 μm)	MeOH:ammonium acetate 15 mmol L ⁻¹ (40:60 to 100:0)	FL	[84]

Note: ^a ratios in percentages v/v; ACN, acetonitrile; FIA, flow injection analysis; FL, fluorescence; GABA, gamma aminobutyric acid; MeOH, methanol; n. a., not available; ODS, octadecyl silica; PS-DVB, polystyrene divinylbenzene; SIA, sequential injection analysis; SLME, supported liquid membrane extraction; SPE, solid-phase extraction; UV, ultra-violet; Vis, visible.

Table 1.2. Flow injection based and derived methodologies for sample preparation coupled to liquid chromatography determination (continued).

Sample			Chromatographic conditions			
Flow technique	Analyte	Type	Treatment	Column	Mobile phase ^a	Detection Ref.
SIA	Aminoacids	Pharmaceutical formulations	Derivatization	ODS packed (4 µm)	MeOH:ammonium acetate 20 mmol L ⁻¹ (30:70 to 80:20)	FL [85]
SIA	Metals	Foodstuff	Derivatization	ODS packed (5 µm)	ACN:acetate buffer 10 mmol L ⁻¹ with TBABr 3.5 mmol L ⁻¹ (30:70)	Vis [86]
SIA	Gemfibrozil	Pharmaceutical formulations	Dilution	ODS monolithic	MeOH:H ₂ O:CH ₃ COOH (75:24:1)	UV [87]
MSFIA-LOV	NSAIDs and bezafibrate	Water, urine	SPE	ODS packed (5 µm)	MeOH:H ₂ O, HCOOH 1% v/v	UV [88]
MSFIA	Phenolic compounds	Water and soil extracts	SPE	ODS monolithic	ACN:phosphate buffer 50 mmol L ⁻¹ (36:64)	UV [89]
MSFIA-LOV	UV filters	Bathing waters	SPE	ODS monolithic	ACN:CTAC 100 mmol L ⁻¹ (65:35)	UV [90]

Note: a ratios in percentages v/v; ACN, acetonitrile; CTAC, cetyltrimethylammonium chloride; FL, fluorescence; LOV, lab-on-valve; MeOH, methanol; MSFIA, multisyringe flow injection analysis; NSAID, non steroidal anti-inflammatory; ODS, octadecyl silica; SIA, sequential injection analysis; TBABr, tetrabutyl ammonium bromide; UV, ultra-violet; Vis, visible.

Table 1.2. Flow injection based and derived methodologies for sample preparation coupled to liquid chromatography determination (continued).

Sample		Chromatographic conditions					
Flow technique	Analyte	Type	Treatment	Column	Mobile phase ^a	Detection	Ref.
MSFIA-SIA	Triazine herbicides and by-products	Water and soil extracts	SPE	ODS packed (3.5 μm)	ACN:H ₂ O (20:80 to 70:30)	UV	[91]
MSFIA-LOV	Riboflavin	Foodstuff	SPE	ODS monolithic	MeOH:CH ₃ COOH: triethylamine (15:84.5:0.5), OSA 5 mmol L ⁻¹	UV	[92]

Note: ^a ratios in percentages v/v; ACN, acetonitrile; LOV, lab-on-valve; MeOH, methanol; MSFIA, multisyringe flow injection analysis; ODS, octadecyl silica; OSA, octanesulfonic acid; SPE, solid-phase extraction; UV, ultra-violet.

Table 1.3. Figures of merit of flow injection based methodologies for sample preparation coupled to liquid chromatography.

Analyte	Sample volume	Linear range	LOD	Repeatability (RSD%)	Determination frequency (h ⁻¹)	Ref.
Caffeine	Up to 1.9 mL	9 – 40 mmol L ⁻¹ ^a	2 nmol L ⁻¹ ^b	< 3	5	[58]
Zidovudine	Up to 470 µL	0.15 – 26 µmol L ⁻¹ ^c	0.038 µmol L ⁻¹ ^c	<3	3-5	[58]
		38 – 190 mmol L ⁻¹ ^d	83 nmol L ⁻¹ ^d			
Ammonia and methylamines	24.0 – 48.0 mL	Up to 2000 nmol L ⁻¹	3 – 40 nmol L ⁻¹	< 6	2 – 4	[56]
Levodopa and carbidopa	750 µL	5.0 – 2000 ng mL ⁻¹	0.3 – 1.5 ng mL ⁻¹	≤ 3.8	6	[73]
Metals	2.0 mL	40 – 1120 µg L ⁻¹	0.16 – 1.1 µg L ⁻¹	≤ 5.5	3	[74]
Anions	200 µL	1 – 50 mg L ⁻¹	0.9 – 2.23 mg L ⁻¹	n. a.	4	[75]
Anthocyanins	2.0 mL	0.6 – 60 mg L ⁻¹	0.2 mg L ⁻¹	≤ 15	1.5	[76]
Bile acids	1.0 mL	0.5 – 50 µmol L ⁻¹	0.1 – 0.2 µmol L ⁻¹	≤ 5.2	5	[77]
Metals	Up to 25.0 mL	0.5 -40 µg L ⁻¹	0.12 – 2.1 µg L ⁻¹	< 7.4	2	[78]

Note: ^a, for a sample volume of 30 µL; ^b, for a sample volume of 1.9 mL; ^c, urine samples; ^d, plasma samples; LOD, limit of detection; RSD, relative standard deviation.

Table 1.3. Figures of merit of flow injection based methodologies for sample preparation coupled to liquid chromatography (continued).

Analyte	Sample volume	Linear range	LOD	Repeatability (RSD%)	Determination frequency (h ⁻¹)	Ref.
PAHs	0.1 – 1 g	0.001 – 1.25 µg g ^{-1 e}	≤ 0.002 µg g ^{-1 e}	≤ 5.4 ^e	3	[79]
		0.005 – 1.2 µg g ^{-1 f}	≤ 0.04 µg g ^{-1 f}	≤ 4.8 ^f		
Mercury species	4.00 mL	10 – 3000 µg L ⁻¹	10 – 25 ng g ⁻¹	≤ 3.0	3	[80]
Metals	n. a.	6 – 600 µg	3–5 µg	≤ 5.1	1.5	[81]
Metals	n. a.	0.01 – 6.0 µg mL ⁻¹	0.02 – 1.00 µg mL ⁻¹	< 1	3.75	[54]
Phenolic compounds	10 mL	1 – 500 µg L ⁻¹	0.35 – 0.71 µg L ⁻¹	≤ 4.2	2.7	[57]
Organic acids	400 µL	250 – 7500 mg L ⁻¹	135 – 213 mg L ⁻¹	≤ 5.4	7.5	[55]
Aromatic polyhydroxy compounds	30 µL	0.1 – 2.5 mmol L ⁻¹	0.05 mmol L ⁻¹	< 10%	4	[82]
Caffeine	1.00 mL	0.18 – 1.80 µg L ^{-1 g} 0 – 20 µg L ^{-1 h}	n. a.	< 5.0 ^h	6	[83]
GABA	100 µL	0.01 – 3.0 mg L ⁻¹	1 µg L ⁻¹	≤ 5.0	3	[84]

Note: ^e, fluorimetric detection; ^f, spectrophotometric detection; ^g, urine samples; ^h, for beverage samples; LOD, limit of detection; n.a., not available; RSD, relative standard deviation.

Table 1.3. Figures of merit of flow injection based methodologies for sample preparation coupled to liquid chromatography (continued).

Analyte	Sample volume	Linear range	LOD	Repeatability (RSD%)	Determination frequency (h ⁻¹)	Ref.
Aminoacids	50 µL	0.5 – 10 mg L ⁻¹	10 – 26 µg L ⁻¹	≤ 7	2	[85]
Metals	200 µL	5.0 – 250 µg L ⁻¹	≤ 2 µg L ⁻¹	< 6	4.6	[86]
Gemfibrozil	100 µL	30 – 750 mg L ⁻¹	2.28 mg L ⁻¹	2.5	60	[87]
NSAIDs	18.0 / 1.0 mL	0.4 – 40 ng mL ⁻¹	0.02 – 0.62 ng mL ⁻¹ ⁱ 0.05 – 0.11 µg mL ⁻¹ ^j	≤ 8.6	3.2	[88]
Phenolic compounds	Up to 100 mL	150 – 3500 ng	< 1 ng mL ⁻¹ ^k	< 8%	4 - 10	[89]
UV filters	Up to 12 mL	60 – 1920 ng	0.45 – 3.3 ng mL ⁻¹ ^l	< 13%	6.7	[90]
Triazine herbicides and by-products	Up to 10.0 mL	0.1 – 10.0 ng mL ⁻¹ ^m 5 – 100 ng mL ⁻¹ ⁿ	0.02 – 0.04 ng mL ⁻¹ ^m 0.3 – 0.5 ng mL ⁻¹ ⁿ	< 6.0	2 ^m 1.5 ⁿ	[91]
Riboflavin	1.00 mL	0.45 – 5.00 mg L ⁻¹	0.05 and 0.12 mg L ⁻¹ ^o	< 5.5	6	[92]

Note: ⁱ, water samples; ^j, urine samples; ^k, for a sample volume of 100.0 mL; ^l, for a sample volume of 12.0 mL; ^m, for water samples; ⁿ, for soil extract samples; ^o, for infant milk formula and pig liver extract, respectively; LOD, limit of detection; RSD, relative standard deviation.

1.5.1 Hyphenated methodologies based on flow injection analysis

The first application of FIA as front end to liquid chromatography combined LLE and SPE of beverages and biological fluids samples prior to the normal phase separation and determination of caffeine and zidovudine [58]. The manifold was able perform LLE by mixing a variable volume (30 – 2000 μL) of an aqueous sample with the extraction solvent (dichloromethane) followed by the separation of the phases by one or two phase separators, depending on the analyzed sample. The organic phase was then preconcentrated in a packed column placed at the injection valve of the chromatograph. The analytes were stripped from the sorbent by the LC mobile phase for separation and UV detection. The SPE step in column switching mode was useful not only to avoid band broadening of the chromatographic peaks but also to increase the sensitivity and linear range of the method, by extracting different volumes of sample that were adjusted for each particular case. The methodology was able to quantify caffeine and zidovudine (model compounds) at low levels (nmol L^{-1} to $\mu\text{mol L}^{-1}$) in different matrices (beverages, plasma and urine). A minimum previous sample handling (dilution for beverages and mild protein precipitation for plasma samples) was nevertheless required.

Automatic normal-phase SPE was also used for the determination of anti-parkinsonian drugs in plasma samples [73]. After removal of proteins by precipitation, the plasma was injected into the manifold and extracted by an alumina column. The analytes of interest (levodopa and carbidopa) and the internal standard (methyldopa) were eluted with perchloric acid and then separated in an octadecylsilica (ODS) column ($250 \times 4.6 \text{ mm}$). The analytes were monitored in dog plasma samples after oral administration in a broad linear range ($5.0 - 250 \text{ ng L}^{-1}$ for carbidopa and $10.0 - 2000 \text{ ng L}^{-1}$ for levodopa) with a high precision ($\text{RSD} < 4\%$).

Solid-phase extraction of aqueous samples in reversed-phase mode was proposed by Criado et al. [77], who described a hyphenated methodology for the quantification of bile acids in biological fluid extracts. The manifold was designed to accommodate both screening and confirmatory determination of the analytes without any reconfiguration of the system. The protocol sequence comprised the sorption of the analytes of interest contained in biological fluids (human serum and urine) extracts followed by the matrix removal (using methanol 20%, v/v). After this step, the analysis could be conducted by two complementary approaches: in the first one (screening method), the eluted bile acids were sent directly to the evaporative light scattering detector (ELSD) and the total content of bile acids in the sample was measured. Alternatively (confirmatory method), the eluate could be separated by reversed-phase liquid chromatography and the bile acids were individually quantified. This individual assessment was only performed when abnormal or

nearly abnormal concentrations were found in the screening method. Another important aspect of this method is the effective preconcentration and cleanup of the samples. The screening analysis by using ELSD detection was only possible because a selective SPE procedure was implemented. For this, the usual analytes sorption present in an acidic solution onto a ODS sorbent was replaced by sorption of analytes present in an alkaline solution onto a column packed with 20 mg of Amberlite XAD-4, facilitating the removal of potential interferents. The six bile acids targeted by the method were quantified between $2.0 - 200 \mu\text{mol L}^{-1}$ and $0.5 - 50 \mu\text{mol L}^{-1}$, during screening and confirmatory analysis, respectively. The precision of the measurements was below 5.2% in all cases.

The same authors reported a similar strategy for the determination of polycyclic aromatic hydrocarbons (PAHs) in soil samples [79], by automatic processing the solid sample on the flow system (Fig. 1.13).

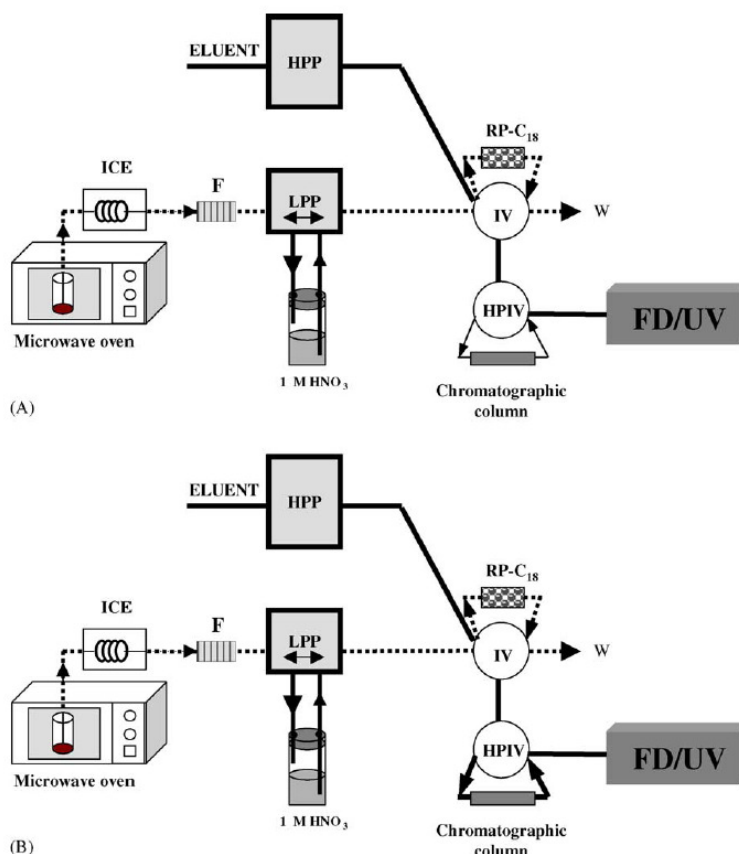


Fig. 1.13. Integrated FI-LC-FD/UV system proposed for the screening (A) and confirmation (B) of soil samples for PAHs using on-line microwave-assisted extraction [79]. HPP, high-pressure pump; F, filter; LPP, low pressure pump; IV, injection valve; W, waste; HPIV, high-pressure injection valve; FD, fluorescence detector; UV, UV-vis detector. Copyright 2004 Elsevier Science Publishers.

The soil sample (0.1 – 1 g) was mixed with acetonitrile and digested by microwave radiation during 10 minutes. Part of the organic extract, containing the analytes, was then in-line filtered and mixed with nitric acid 1 mol L⁻¹. Subsequently, the acidic mixture was extracted in a reversed-phase ODS sorbent and matrix residues were removed. After this stage, analytes were eluted with acetonitrile and directly detected due to its fluorescence properties (screening method). For the positive samples in the screening method, an individual quantitative analysis was performed. This consisted in the reversed-phase separation by an ODS analytical column of the sixteen target compounds in a gradient elution mode with fluorimetric and UV detection. Hence, the FIA-LC methodology was able to quantify PAHs with high precision (RSD ≤ 5.4%), at low levels, with LODs below 0.04 and 0.002 µg g⁻¹, for spectrophotometric and fluorimetric detection, respectively.

The derivatization of the metals prior to its reversed-phase chromatographic analysis was reported by Srijaranai et al. [54]. In order to minimize the derivatization reagent consumption, the authors proposed a reverse FIA system (Fig. 1.14). By this way, a plug of 85 µL of 4-(2-pyridylazo)-resorcinol (PAR) was injected into the standard or wastewater samples stream. The metal (Co(II), Ni (II) and Cu (II))-PAR complexes were directed towards to the loop of the LC injection valve, where 20 µL of the reaction product plug were injected and separated by ion-pair chromatography. After a chromatographic run of fifteen minutes under isocratic conditions, the peaks corresponding to the metal-PAR species and Cr(VI) were detected by visible radiation, at 530 and 440 nm, respectively. After optimization of the physical-chemical parameters by factorial design, the method demonstrated a high precision (RSD < 1%) and high sensitivity, with a linear range in the µg L⁻¹ level, which was suitable for the direct determination of the analytes in wastewater sample analysed without any enrichment procedure.

The determination of metals by a FIA-LC analyzer using ion-pair chromatographic analysis as separative technique was also the subject of the work published by Pobozy et al. [78]. Nevertheless, in this application, the metals (Co(II), Ni(II), Cd(II) and Mn(II)) contained in the standards or river water samples were preconcentrated by a FIA manifold in an ion-exchange sorbent (Chelex P). After matrix removal with deionized water, the analytes were released from the sorbent under acidic conditions (1 mL of HNO₃ 0.1 mol L⁻¹). The eluate zone corresponding to the maximum concentration of analytes was injected in the LC equipment for separation. At the chromatographic column outlet, the ions were complexed with PAR and the reaction products were detected by molecular absorption spectrophotometry. The proposed methodology was able to perform two determinations per hour at the µg L⁻¹ levels with a RSD values below 7.4%.

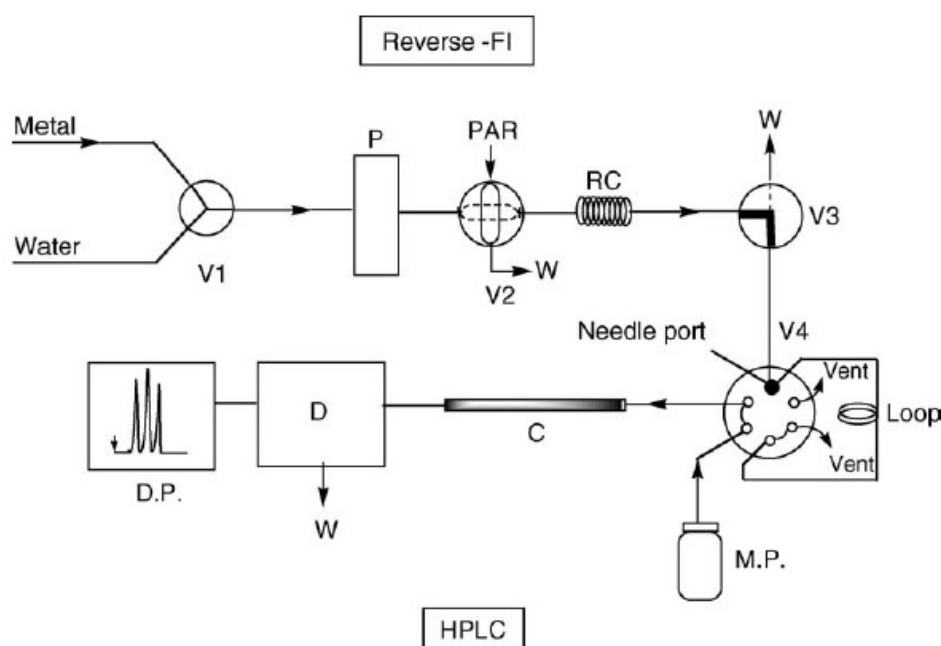


Fig.1.14. Diagram of FI-HPLC in-line derivatization system [54]: P, peristaltic pump; RC, reaction coil; V1, three way valve; V2, low pressure injection valve; V3, switching valve; V4, high pressure injection valve; C, analytical column; M.P., mobile phase; D, UV-vis detector/photodiode array detector; D.P., data processor; W, waste. Copyright 2006 Elsevier Science Publishers.

Ali et al. combined the metal complexation and SPE enrichment by FIA with reversed-phase LC for the quantification of metals in wastewater [74]. The FIA system was able to mix the analytes (Ni(II), Cu(II), Hg(II)) with the complexing agent diethyldithiocarbamate (DDC) and preconcentrate the metal-DDC complexes in a packed ODS column placed into the HPLC injection valve. After the sorbent bed washing with water, the injection valve was switched to the injection position and the analytes were eluted by the LC mobile phase (MeOH:H₂O, 80:20, v/v) and separated in a ODS analytical column. The analytical signals were recorded by a diode-array detector. The method demonstrated a good precision (RSD $\leq 5.5\%$) and the LODs were below $1.1 \mu\text{g L}^{-1}$ for the derivatization and extraction of 2.0 mL of sample containing the target analytes.

Another interesting strategy for the analysis of metallic species (Hg and organic derivatives) in seafood samples was proposed by Dong et al. [80]. The SPE-based sample preparation step relies in a displacement reaction between a presorbed Cu-pyrrolidine dithiocarbamate (PDC) complex and the target analytes. By this way, the mercury and its organic species were able to replace the copper in the complex previously trapped in a cigarette filter sorbent, which was mainly composed by cellulose acetate. The mercury-PDC chelates were eluted with methanol and subsequently separated in the chromatographic system. This approach took advantage of the chemical properties of

mercury species, and minimized the interferences of some transition metals that were usually present in the samples. High enrichment factors (75 – 85) and good precision ($RSD \leq 3\%$) were achieved for the processing of 4.00 mL of seafood extract. The minimum concentrations detected for the method were in the range 10 – 25 ng g⁻¹. A dog-fish muscle certified reference material was also analysed and the result was in agreement with the certified value.

Although the majority of the hyphenated methodologies relied on reversed-phase or ion-pairing chromatographic separation, some methodologies based on ion-chromatography (IC) are also described in the literature. The use of automatic flow based SPE as front end to IC was reported by Tannikkil et al. [81]. In this application, zinc ore extracts containing the target ions (Cd(II), Pb(II) and Zn(II)) were buffered in a continuous flow mode with 1 mol L⁻¹ ammonium acetate (pH 5.4) and trapped in an ion-exchange mini-column filled with Chelex 100. After elution with 2.0 mol L⁻¹ HNO₃, the analytes were neutralized by a 2.0 mol L⁻¹ NaOH stream and pH was then adjusted by a citrate buffer solution added in a subsequent confluence point. Part of the resulting mixture (20 µL) was injected into the IC instrument and the cations were separated during 35 minutes at a flow rate of 1.5 mL min⁻¹ and detected by conductivity. A time based sampling strategy was adopted and a global linear range of 6-600 µg was achieved (sample volume was not given). The correspondent LODs were below 5 µg for all ions under evaluation. The results obtained for the analysis of zinc ore samples were in agreement with ICP-AES and AAS reference methods.

Gas diffusion was hyphenated to ion chromatography for the analysis of ammonia and methylamines (mono-, di-, and trimethylamines) at nmol L⁻¹ levels in natural waters [56]. Gibb et al. proposed a flow injection manifold able to alkalize the sample (to achieve deprotonation of the methylamines), to prevent in-line precipitation of matrix components (by chelating Mg²⁺ and Ca²⁺ with EDTA), and to transfer the analytes of interest from the sample to the acceptor phase through a gas-diffusion membrane. After processing up to 48 mL of sample through the mass transfer device while the liquid chromatography separation of the previous sample took place, the acceptor stream was sent to IC injection valve, from which 200 µL were injected in heart-cut mode for IC analysis. Due to the low levels and the high precision aimed by the method, internal standard calibration was used and the LODs reached were in the range 3 – 5 nmol L⁻¹ for methylamines and 20 – 40 nmol L⁻¹ for ammonia. This methodology was used in a research vessel for the on-board determination of analytes in different natural water samples.

Supported liquid membrane extraction (SLME) was coupled to LC for the determination phenolic compounds (phenol, catechol, resorcinol and hydroquinone) in

spiked natural water samples [57]. The organic liquid membrane was constituted by a polymeric support (PTFE) impregnated with kerosene and tri-butylphosphate, which was placed in a lab-made device between the donor and the acceptor streams, both aqueous. The extraction was based in the weak acidic properties of the analytes. Hence, the samples or standard solutions (donor stream) were acidified ($\text{HCl } 1 \text{ mol L}^{-1}$) and the phenolic compounds were protonated, with enhanced affinity for the organic solvent placed at the membrane. In contrast, the acceptor stream was an alkaline solution ($\text{pH } 13.5$) that was able to back extract the analytes by ionizing the acidic group of the aromatic ring. A volume of $20 \text{ }\mu\text{L}$ from the acceptor stream was then injected into the LC for chromatographic determination. The use of SLME provided enrichment factors between 56 and 70 for 10 mL of preconcentrated sample with a negligible organic solvent consumption. An enhanced linear range ($1 - 500 \text{ }\mu\text{g L}^{-1}$) and precision ($\text{RSD} \leq 4.2\%$) were reported. Recoveries between 93 and 107 % were achieved during the analysis of river water samples spiked with a concentration of $5.00 \text{ }\mu\text{g L}^{-1}$ of each target compound.

On-line dilution and non-chromatographic separation by dialysis of seven ions (bromide, chloride, fluoride, nitrate, nitrite, phosphate and sulfate) contained in natural water samples prior to its determination by IC was reported by Grudpan et al. [75]. The manifold accommodated a dialysis cell and performed pH adjustment of the sample before the migration of analytes to the acceptor phase. The authors investigated a continuous flow and flow injection configurations using the same lab-made dialysis cell for both strategies. The flow injection dialysis approach was selected for analysing the drinking water samples due to the dilution factor offered (at least ten times), enhanced reproducibility, expanded linear range and lower limits of detection. Hence, the flow injection dialysis-IC method allowed the quantification of analytes at mg L^{-1} levels in water samples with an analysis time of fifteen minutes.

On-line flow injection dialysis was also implemented for the determination of six organic acids (tartaric, malic, lactic, acetic, citric and succinic) in wines [55] (Fig. 1.15). In this particular case, $400 \text{ }\mu\text{L}$ of sample or standard solution were injected into the donor stream while the acceptor stream was stopped during the dialysis period (170 s). Next, the dialysate zone was propelled in the direction of LC injection valve, from which $20 \text{ }\mu\text{L}$ were injected and separated under reversed-phase conditions. The linear range of the calibration curve was 250 to 7500 mg L^{-1} for all organic acids. The accuracy was evaluated by recovery assays with percentages of recovery in the range $84 - 104 \%$. Since the sample preparation procedure and the chromatographic run occurred in parallel within the same time frame, 7.5 determinations per hour were possible.

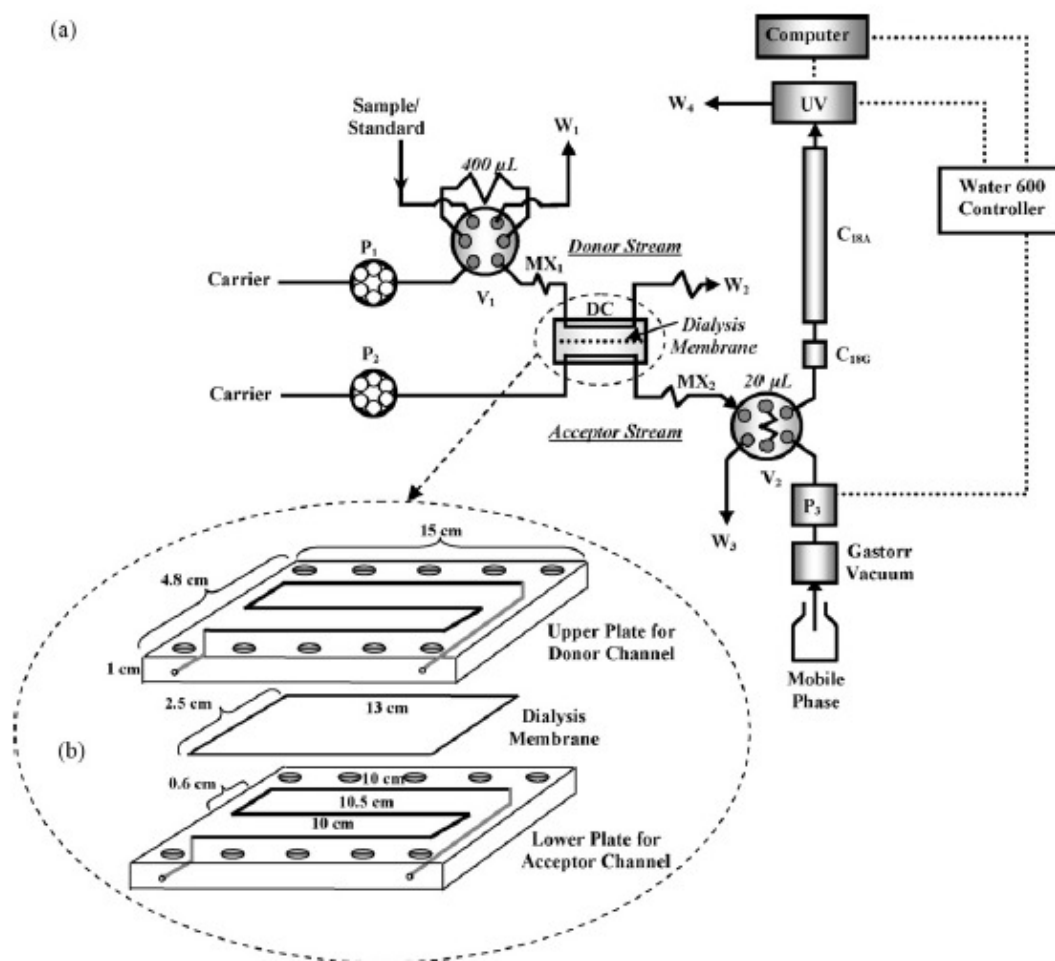


Fig. 1.15. (a) A manifold of the flow injection dialysis–LC system used for the determination of some organic acids and (b) the dialysis cell (DC) [55]; flow rate of carrier of donor and acceptor streams: 0.2 mL min^{-1} ; P1 and P2, peristaltic pumps 1 and 2; P3 HPLC pump; V1, manual-rotary injection valve; V2, HPLC manual-rotary injection valve; MX1, mixing coil 1 ($12 \text{ cm} \times 0.8 \text{ mm i.d.}$); MX2, mixing coil 2 ($7.5 \text{ cm} \times 0.8 \text{ mm i.d.}$); C18A, C18 analytical column; C18G, a C18 guard column; UV, photodiode array detector; and W1, W2, W3 and W4, wastes 1, 2, 3 and 4. Copyright 2009 Elsevier Science Publishers.

Anthocyanins, another important family of compounds found in wine samples, were quantified by a flow injection analysis-LC hyphenated methodology [76]. The new approach introduced by Mataix and Luque de Castro relied on solid-phase extraction followed by solvent evaporation and subsequent chromatographic separation with spectrophotometric detection of three anthocyanins (cyanidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside) present in wine samples at different concentration levels. The manifold included a microcolumn packed with ODS and a customized evaporation chamber. The anthocyanins contained in 2.0 mL of wine were extracted by the hydrophobic sorbent, followed by elution with 2.0 mL of acetonitrile. After five minutes of evaporation at 140°C under nitrogen stream (600 mL min^{-1}), the concentrated extract was

injected and the three anthocyanins were separated under isocratic reversed-phase mode in a chromatographic run of 40 minutes. The introduction of an in-line evaporation step allowed the adjustment of the volume injected into the LC system. This fact resulted in an expansion of the linear range of the method (when compared with an off-line approach) and, simultaneously, in the minimization of the band broadening effect, typical when large volumes of eluate are injected in the chromatographic system. LODs below 0.2 mg L^{-1} were achieved for all target analytes and the determination frequency, defined by the duration of the chromatographic run, was 1.5 determinations per hour.

1.5.2 Hyphenated methodologies based on sequential injection analysis

Sequential injection analysis was firstly applied for automatic SPE prior to LC analysis in 1995 [82]. Lukkary et al. proposed a SIA manifold to monitor aromatic polyhydroxy compounds (pyrocatechol, protocatechuic acid, pyrogallol and gallic acid) in black liquor from the kraft digestion process. The samples were collected each 15 minutes, cooled, filtered, extracted by SPE and separated by reversed-phase LC in a fully automatic fashion. This application illustrates the analytical potential of SIA as a process control tool. The system used a minimum sample volume ($30 \text{ }\mu\text{L}$) that was subsampled from a 30 mL aliquot pumped from the reactor and allowed a continuous monitoring of the kraft digestion that took approximately three hours. The method was tolerant to the major possible interfering species. Carryover effect was considered a minor problem, though the solid-phase column was replaced after twelve injections due to irreversible adsorption of the sample matrix components. The typical calibration curve was linear up to 2.5 mmol L^{-1} and the minimum concentration detected was 0.05 mmol L^{-1} for each of the four analytes.

Molecularly imprinted solid-phase extraction (MISPE) in SIA format was introduced by Theodoridis et al. [83]. The SIA-LC analyzer was able to extract selectively caffeine from soft drinks, coffee and urine by using a molecularly imprinted polymer packed in a mini-column that was incorporated in the manifold (Fig. 1.16). In MISPE, the selectivity is commanded by the strict control of the extraction protocol in order to promote the selective interactions between the target molecule(s) and the cavities of the imprinted polymer [93]. By this way, the authors demonstrated the ability of the SIA technique to implement the extraction protocol. Different solvents (aqueous and organic) placed in the lateral ports of the multiposition valve were used for sorbent conditioning, sample buffering, matrix removal and elution in a completely automatic fashion. Moreover, the implementation of mass calibration (based on the loading of different standard solution

volumes) simplified the calibration procedure. Caffeine was quantified in the different samples with high precision ($RSD < 5\%$) in a broad concentration range (up to $20 \mu\text{g L}^{-1}$).

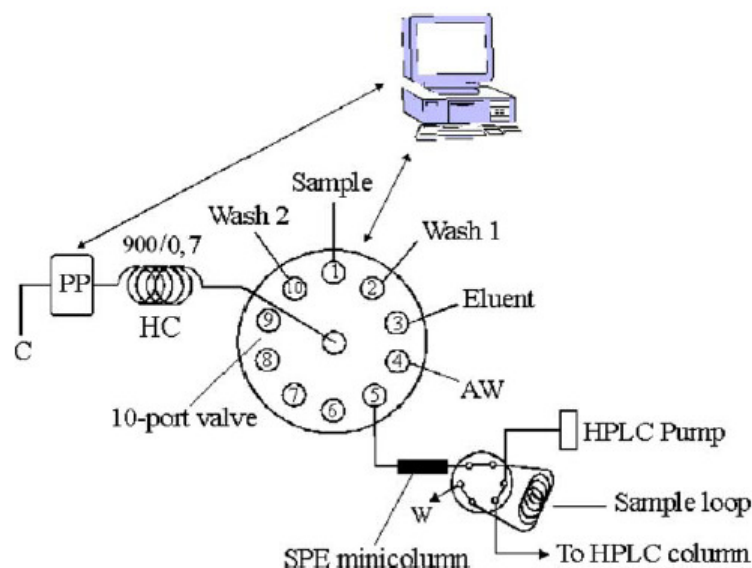


Fig. 1.16. Set-up used for the automatic MISPE of caffeine [83]. C, carrier; PP, peristaltic pump; HC, holding coil; AW, auxiliary waste; W, waste. Copyright 2004 Elsevier Science Publishers.

The chromogenic derivatization of metals (Co(II), Ni (II), Cu (II), Fe (II)) with nitro-PAPS (2-(5-Nitro-2-pyridylazo)-5-(N-propyl-3-sulfopropylamino)phenol) in foodstuff samples prior to its determination by reversed-phase LC was proposed by Burakham et al. [86] (Fig. 1.17). The samples and the reagent were sequentially aspirated in a sandwich strategy (three aliquots of sample (70, 70 and 60 μL) were intercalated with 25 μL of reagent) and mixed by flow reversal, filling the loop placed at the chromatograph injection valve. Subsequently, part of the metal-reagent complex plug (50 μL) was introduced into the chromatographic system and separated in a thirteen minutes run with spectrophotometric detection at 570 nm. The on-line approach allowed the parallel derivatization and chromatographic determination in the same time frame with a linear range between 5.0 and $250 \mu\text{g L}^{-1}$, a limit of detection below $2 \mu\text{g L}^{-1}$ and a RSD lower than 6% for all samples analyzed.

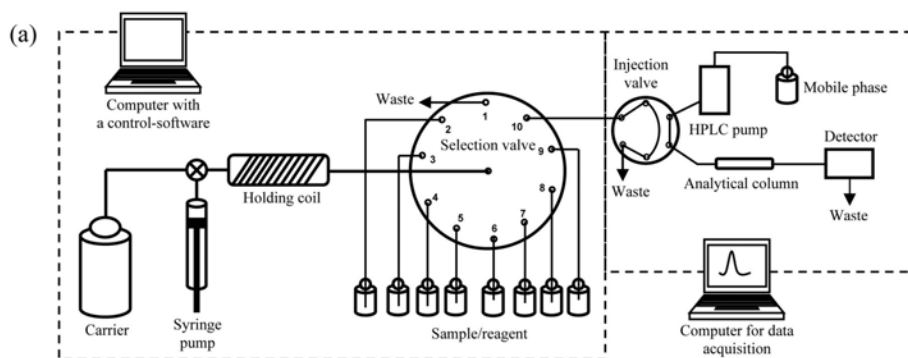


Fig. 1.17. Schematic diagram of the SIA-LC system for the derivatization and determination of metals in foodstuff [86]. Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA.

A similar procedure was adopted for the fluorimetric determination of aminoacids in pharmaceutical formulations by LC [85]. The fluorogenic reagent *o*-phthaldialdehyde (OPA) was mixed with samples or standard solutions and directed towards the LC injection valve. Part of the reagent / standard mixture filled the loop and was hold there for three minutes in order to complete the reaction before the injection and separation. After the study of the physical and chemical parameters affecting the sample preparation procedure and the reversed-phase separation, the proposed methodology allowed the separation of fourteen aminoacids in approximately thirty minutes in a gradient elution mode with a linear range comprising 0.5 to 10 mg L⁻¹. Furthermore, pharmaceutical formulations were analyzed by the proposed methodology with a satisfactory repeatability (RSD ≤ 7%).

The same authors reported a similar strategy for the quantification of γ -aminobutyric acid (GABA) in biological fluids (urine and cerebrospinal fluid) using a SIA system as front end to the LC separation [84]. Two plugs of 100 μ L, corresponding to OPA (derivatizing agent) and sample were sequentially aspirated to the holding coil and propelled in the direction of the LC injection valve. The zone containing the highest concentration of analyte was then trapped into the injection loop, followed by analyte (GABA) and internal standard (1,7-diaminoheptane) derivatives separation by a reversed-phase gradient elution. This method allowed the determination of GABA (linear range of 0.01 to 3.0 mg L⁻¹) with a minimum sample treatment and reagent consumption. Though the sample preparation procedure was completed in a short time (66 s), the method throughput was limited by the chromatographic separation. Hence, three whole analytical protocols were performed in each hour.

SIA was also proposed to perform the automatic on-line dilution of samples prior to injection into the LC equipment [87]. Dilution is an endogenous feature of flow injection systems due to the dispersion of the injected plug(s) into the carrier stream. In order to increase the dilution factor, the aspirated sample plug was partially eliminated before filling the 10 μL loop of the injection valve (Fig. 1.18). The sample volume removed by this zone sampling dilution procedure was adjusted for the different concentrations of gemfibrozil collected from a dissolution assay vessel in order to fit the linear range of the LC method. Moreover, the chromatographic determination relied on a short ODS monolithic column that provide a very short separation time. Therefore, the SIA-LC analyzer was able to process 60 samples per hour in an enhanced linear range of concentrations (30 – 750 mg L^{-1}) and high precision ($\text{RSD} < 2.5\%$). The results found were in agreement with batch reference procedures of the United States Pharmacopeia.

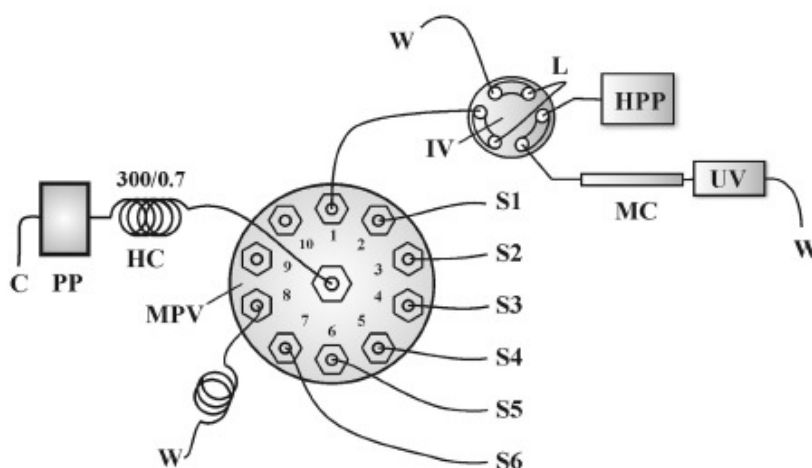


Fig. 1.18. Schematic representation of the SI-LC set-up applied to the dilution of gemfibrozil samples [87]. C, carrier (20% MeOH, v/v); PP, peristaltic pump; HC, holding coil (300 cm $\times$ 0.7 mm i.d.); MPV, multiposition valve; S1–S6, dissolution samples; I.V., high- pressure injection valve; L, injection loop (volume = 20 μL); MC, monolithic column; HPP, high pressure pump; UV, detector; W, waste. Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA.

1.5.3 Hyphenated methodologies based on multisyringe flow injection analysis

The use of multisyringe flow injection analysis as front end to liquid chromatographic separations has been exploited in the last few years essentially through the association of multisyringe burette with LOV concept, taking advantage of the combination between the

multi-channel propulsion of MSFIA and the miniaturization in sequential injection mode provided by LOV. By this way, in 2006 Quintana et al. [88] proposed a MSFIA-LOV system to perform the on-line renewable SPE of water samples in microcolumn format prior to liquid chromatography for the determination of pharmaceutical active ingredient residues in water and urine samples. All solutions and a methanolic sorbent suspension, containing a hydrophilic-lipophilic balance sorbent (Oasis HLB), were processed by the system depicted on Fig. 1.19. Hence, a microcolumn containing 4.5 mg of sorbent was packed and discarded at the beginning and the end of the analytical cycle, respectively. This manifold allowed the downscale of all procedure and the implementation of the sorbent renewal based on the bead injection concept [34].

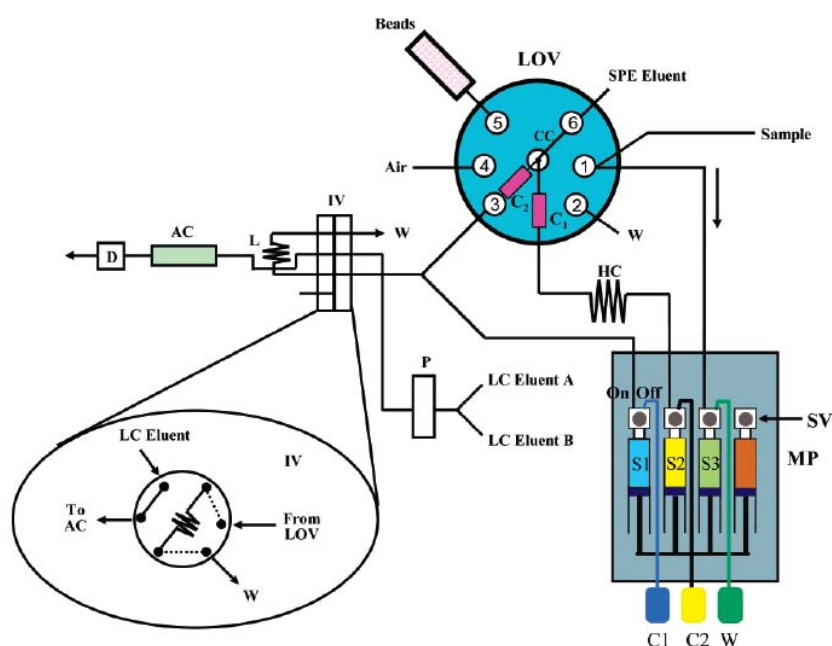


Fig. 1.19. Schematic diagram of the MSFIA-LOV manifold hyphenated with liquid chromatography for the determination of acidic drugs [88]. LOV, lab-on-valve; MP, multisyringe pump; S, syringe pump; SV, solenoid valve; HC, holding coil; CC, communication channel; C1 and C2, cavities for beads; P, HPLC pump; IV, injection valve; L, loop; AC, analytical column; D, detector; W, waste; C1, carrier (Ultrapure water) C2, carrier (10^{-2} mol L⁻¹ HCl); LC-eluent A, 80/20 (v/v) MeOH / H₂O + 0.1% (v/v) formic acid; LC-eluent B, 95/5, v/v MeOH / H₂O + 0.1%, v/v formic acid. (The injection valve is illustrated in the loading position). Copyright 2006 American Chemical Society.

Furthermore, the use of multisyringe as propulsion unit made possible the in-line dilution of the eluate with water prior to the filling of LC injection loop, matching the eluate and mobile phase compositions. Thus, band broadening effects were avoided, without any interference in the recovery values. This aspect assumed particular importance considering that the initial conditions of the gradient elution comprised the use of a high percentage of aqueous solvent, in contrast with the high percentage of the

organic solvent present in the eluate stream. The method was applied to the accurate determination with satisfactory precision ($RSD \leq 8.6\%$) of six pharmaceutical residues in environmental (natural water and wastewater) and biological (urine) matrices at ng L^{-1} and $\mu\text{g L}^{-1}$ levels, respectively.

The use of a MSFIA-LOV-LC analyzer for the screening of UV filters, considered pollutants of emergent concern, in bathing waters was recently reported [90]. The screening methodology was based in the association of a miniaturized SPE procedure with a high-throughput chromatographic method, which include a reversed-phase monolithic column and an accelerated isocratic elution mode. Hence, by percolating a variable volume up to 12 mL of sample, 60 – 1920 ng of UV filters could be trapped into the sorbent microcolumn and subsequently eluted by 95% methanol, in-line merged with water and injected by heart-cut mode into the chromatographic equipment. The repeatability, measured as RSD, was below 13% and the analysis time was 9.5 minutes, including the separation performed in parallel with the extraction of the following sample. When compared with alternative methodologies, this approach demonstrated a drastic reduction on reagent consumption and waste disposal. Moreover, analysis time could be reduced up to 33 times and enhancement factors between 51 and 140 were achieved.

Regarding the analysis of foodstuff samples, a MSFIA-LOV manifold for MISPE of riboflavin was recently proposed [92]. In this particular case, the samples were percolated through a molecularly imprinted sorptive surface aiming the selective extraction of the analyte from complex samples such as infant milk-based formulas or energetic drinks. The automatic method allowed a strict control of all steps of the extraction procedure, which is critical to promote the expected interactions between the target molecules and polymer cavities. Furthermore, the MISPE eluent (50% (v/v) methanol + 1% (v/v) acetic acid) composition was in-line adjusted by dilution 1:2 with a water stream before filling the injection loop. Hence, a final percentage of 15% (v/v) methanol was obtained and band broadening of the chromatographic peak was avoided. The linear range was 0.450 to 5.00 mg L^{-1} when 1.00 mL of sample was processed. A high precision ($RSD < 5.5\%$) and good throughput (6 det h^{-1}) were also achieved. The method was successfully applied to two certified reference materials (infant milk-based formula and pig liver). In contrast with previous methodologies [88, 90] based on the BI-LOV concept applying spherical-shaped beads, this method introduced a new strategy for handling solid suspensions composed by irregularly-shaped beads with a high precision.

Multisyringe flow injection analysis was associated with sequential injection for the development of an on-line renewable mixed mode SPE hyphenated to LC for the multiresidue determination of triazine herbicides and by-products in environmental matrices (water and soil extracts) [91]. The microscale renewable SPE procedure, based on

the bead injection concept, consisted in the automatic packing and disposal of a microcolumn that combined the two sorbents in a loop microcolumn (Fig. 1.20). The introduction of a hydrophilic-lipophilic balance sorbent (Oasis HLB) overcame the intrinsic weak sorption capacity of the MIP, and made possible the determination at the levels required by legislation. When soil extracts were analyzed, toluene was used to promote selective interactions between the analytes and the MIP cavities, contributing for the elimination of organic interferents. Hence, and for 10 mL of sample processed by the automatic SPE protocol, enrichment factors in the range 46 – 49 and LODs between 0.02 – 0.04 ng mL⁻¹ were achieved. Furthermore, this procedure demonstrated a high precision (RSD < 6.0%) and accuracy, considering that absolute recoveries were above 79% in all cases.

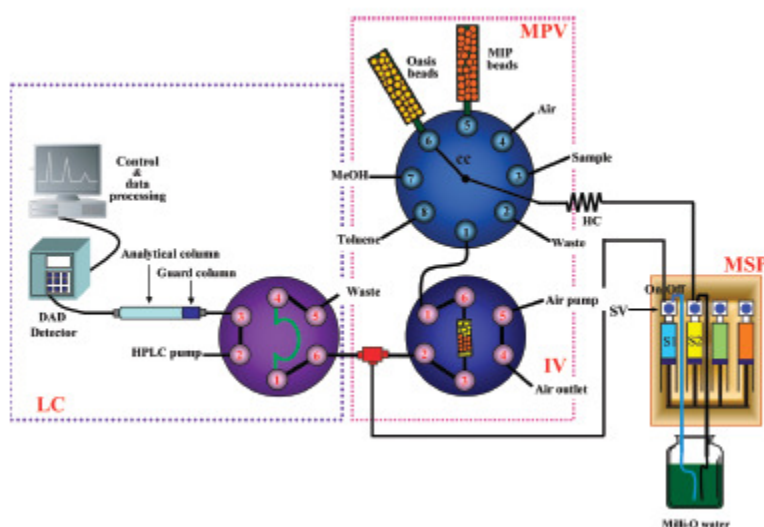


Fig. 1.20. Schematic diagram of the hybrid flow system for automatic multimodal μ SPE of trace level concentrations of chlorotriazines utilizing renewable surfaces as a front end to liquid chromatography [91]. MSP, multisyringe pump; S, syringe; SV, solenoid valve; IV, injection valve; MPV, multiposition selection valve; HC, holding coil, CC, communication channel; DAD, diode-array detector. Copyright 2010 American Chemical Society.

A MSFIA-SPE strategy for the determination of United States Environmental Protection Agency (EPA) phenolic priority pollutants in water and soil extracts was also recently reported [89]. In this particular case, large, variable volumes of sample (up to 100 mL) drawn by peristaltic pump were percolated in a non-renewable, packed SPE column assembled between two three way commutation valves present in the flow network. The eleven analytes (phenol and ten primary derivatives) under evaluation were stripped from the sorbent by methanol and injected into the chromatographic system by a heart-cut approach (injection volume of 20 μ L). This methodology provided high enhancement factors (average value of 176 for 50 mL of sample) and LODs below 1 ng mL⁻¹ (for 100 mL

of sample) for water samples. The accuracy was assessed by performing recovery assays (recoveries in the range 89 – 103 %) and also by analyzing certified reference materials (water and soil extracts). Moreover, the use of a volume-based calibration strategy associated with a separation resorting to a reversed-phase monolithic column [94], reduced the analysis time to three minutes, making this approach in a suitable tool for screening analysis. Hence, sample preparation and chromatographic analysis were carried out within the same time frame, and 4 to 10 determinations per hour could be achieved.

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2

Materials and methods

2.1 Reagents, solutions and samples

All chemicals used were of analytical-reagent grade and were used with no further purification. Ultra-pure water, obtained from a MilliQ system (Billerica, MA, USA), was used as solvent for aqueous solution. For the preparation of mobile phases for the chromatographic analysis, methanol and acetonitrile HPLC grade were used. Organic or aqueous-organic solutions used during the development of the methods were also obtained from these solvents.

The standard stock solutions were prepared by weighing the respective pure compound in an analytical balance AG 285 (Mettler-Toledo, Columbus, OH, USA) followed by dissolution in the appropriate solvent (methanol, water or buffer solution). Alternatively, standard stock solutions were prepared by diluting of certified standard (chapters 3 and 4). Working standard solutions resulted from the accurate dilution of the stock solution by using micropipettes, glass pipettes and volumetric flasks of different capacities. All volumetric glassware was class A. Micropipettes, with maximum capacities of 100, 200, 1000 and 5000 μL , were periodically calibrated.

Mobile phases were prepared by mixing the appropriate volume of the components measured previously in volumetric cylinders. Whenever possible, the aqueous and the organic components were prepared separately and mixed by the high-pressure gradient pump of the LC equipment. In all cases, the mobile phases and / or respective components were filtered through hydrophobic or hydrophilic polyvinylidene fluoride membranes with a pore diameter of 0.22 or 0.45 μm , and degassed by ultrasonic radiation during 15 minutes before use.

When necessary, pH measurements of aqueous solutions were performed using a combined glass pH electrode (ref. #52-02) and a milivoltmeter (model GLP 22), both supplied by Crison Instruments (Allela, Spain).

The sorbents used for the solid-phase extraction procedures were obtained in cartridge or bulk format. The beads were suspended in an alcohol (ethanol or methanol) in order to be processed by the flow system (chapters 5, 6 and 7), or introduced in columns (chapter 3 and 4), made of polyetheretherketone (PEEK) or stainless steel.

Water samples from different sources were obtained locally and, when necessary, they were spiked with standard mixtures containing the target analytes. Before analysis, the pH of these samples was adjusted and they were percolated through a hydrophilic filtration membrane or syringe filter with a pore diameter $\leq 0.45 \mu\text{m}$.

2.2 Manifold components and control

2.2.1 Multisyringe burette

The propulsion unit used throughout the development of the methods was a multisyringe burette BU4S (Crisson Instruments). This device, introduced by Cerdà et al. in 1999 [1], consisted in a multi-channel piston pump, containing up to four syringes, connected to a single bar that is driven by a step motor (Fig. 2.1). At the top of each syringe is placed a three way commutation valve (NResearch, Caldwell, NJ, USA) that defines two possible flow paths: the solution reservoir (position off) or the flow network (position on). Therefore, four different flow possibilities were available for each syringe, comprising the loading of the syringes (pick up operation) or the delivering of the liquids (dispense operation) in the direction of the solution reservoir or the flow network [2].

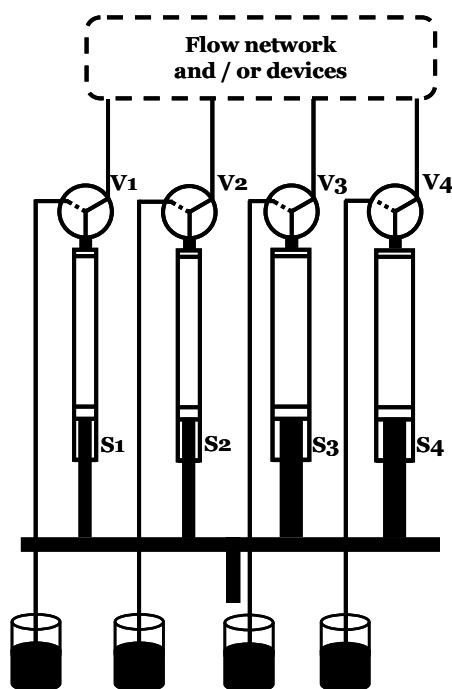


Fig. 2.1. Schematic representation of a multisyringe burette. V_i , three way commutation valves; S_i , syringes; valve position “on”, solid line; valve position “off”, dashed line.

The flow rate range of each multisyringe channel was defined by the volume of the syringe. Hence, a 5000 steps motor multisyringe equipped with syringes of 2.5, 5.0 or 10.0 mL were used, providing flow rates between 0.15 and 20.0 mL min⁻¹. The direction and

speed of piston displacement, the movement length and the position of the commutation valves were controlled by computer through a RS-232 serial port. Considering that the change in the flow direction affects the real volume dispensed or aspirated in the subsequent step [3], a “flow reversal” or “dummy” step was added to the operation protocol before the aspiration / propulsion of precise volumes whenever the flow direction was changed.

All parts of the multisyringe, including the glass syringes (Hamilton, Bonaduz, Switzerland) and the three way commutation valves, had enhanced chemical resistance to acids, bases and organic solvents. Thus, the propulsion unit was totally compatible with all solutions used throughout the experiments.

2.2.2 Lab-on-valve and multiposition valve

The propulsion unit was connected to a lab-on-valve (LOV) device [4] (chapters 5, 6, 7) that was mounted atop of an eight port multiposition valve (Crison Instruments). The LOV consisted of a monolithic structure built in a transparent polymeric material (Fig. 2.2). Channels with 1.5 mm i.d. were engraved in a block of polymethylmethacrylate (PMMA) or polyetherimide (PEI), which had different chemical resistance properties. PMMA is tolerant to methanolic solutions but is not resistant to acetonitrile. In contrast, PEI had an improved chemical resistance to both solvents, commonly used in extraction procedures prior to LC analysis. The central channel was connected to one syringe of the multisyringe module through the holding coil, while the lateral channels were available to introduce solutions and suspensions into the system or to provide auxiliary waste lines. At the exit of one of the lateral channels, a polyethylene (Supelco, Bellefonte, PA, USA, ref. #57244) or polypropylene (MoBiTec, Goettingen, Germany, ref. #2210 or ref. #2235) frit was placed to stop the beads during the microcolumn package step.

The multiposition valve (MPV) (Crison Instruments) ensured the communication between the central channel and eight lateral channels of the LOV, one at a time, by the MPV rotor, which faced the LOV surface (Fig. 2.2). The MPV channel selection was controlled by the same software used for the multisyringe control and also shared the same interface.

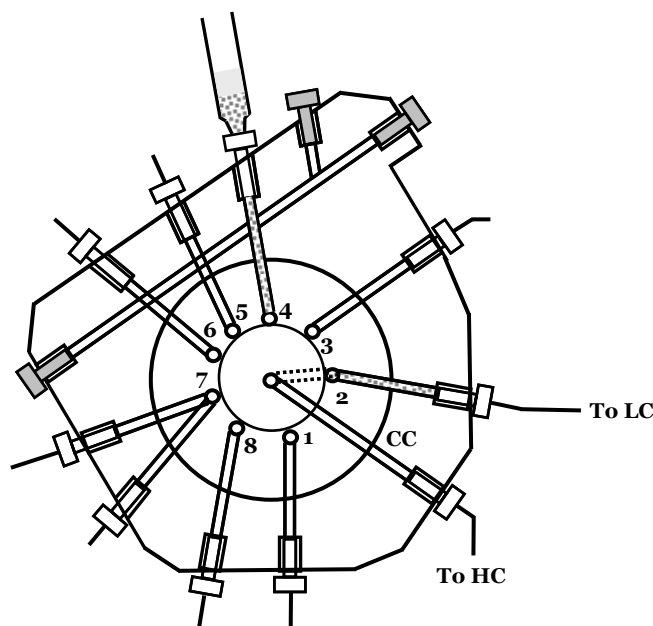


Fig. 2.2. Representation of lab-on-valve mounted atop of a multiposition valve and its connections the holding coil (HC) and liquid chromatography equipment (LC). CC, central channel; 1-8, lateral channels.

2.2.3 Other manifold components

Three way commutation valves supplied by NResearch (Caldwell, NJ, USA) (ref# 161To31 and HP225To31) were used to build the flow network in the manifold described in the chapters 3 and 4. The valves were assembled to the multisyringe module, which allowing their automatic position switching. Furthermore, in this particular configuration (chapters 3 and 4), a Gilson Minipuls 3 peristaltic pump (Villiers-le-Bel, France) was incorporated to load the sample into the extraction column. Actuation direction and speed of this auxiliary propulsion unit, as well as the position selection in the three way commutation valves, were controlled by the same lab-made software used for the control of the multisyringe burette.

The connections between the different parts of the manifolds were made with polytetrafluorethylene (PTFE) tubing with 0.8 or 1.5 mm i.d. (Omnifit, Cambridge, UK). Larger diameter tubing was used to connect the syringes to the solutions reservoir and also to assemble the holding coil (chapters 5, 6 and 7), providing lower flow resistance for aspirations at high flow rates from the solution reservoirs and an enhanced storage capacity to avoid syringe content contamination, respectively. All other connections were made with PTFE tubing with 0.8 mm i.d.. Liquid chromatography injection loops with

customized volume were made of 0.75 mm i.d. PEEK tubing. Fittings, made of different inert polymers (PEEK, polypropylene or polyoxymethylene) were used for connecting the tubing ends to other components of the manifold. T junctions manufactured in PMMA and PEI were included in the manifolds described in the chapters 5 and 7, respectively.

2.2.4 Software control and interfaces

Devices were controlled by a lab made software written in QuickBasic 4.5 (Microsoft, Redmond, WA, USA). The communication between the personal computer and the multisyringe burette was performed through a serial port, using a RS-232 – RJ-11 communication cable. Besides its own control parameters, the multisyringe also allowed the direct control of the commutation valves used to build the flow network, and also the MPV, which was connected to the multisyringe through a RJ-11 cable. In the manifold described in the chapters 3 and 4, a peristaltic pump was used. This propulsion device was interfaced by a PCL-711 card (Advantech, Taipei, Taiwan) and controlled by the same software.

2.3 Chromatographic system

All chromatographic determinations were performed on a liquid chromatography setup Merck / LaChrom 7000 series (Hitachi, Tokyo, Japan). It was composed by a high-pressure gradient pump (L-7455), a diode-array detector (L-7100) and an interface (D-7000).

The system control and data acquisition were performed by D-7000 software installed in a second CPU unit. By comparison with pure standards previously injected, the retention time and UV spectra were used for peak identification. Peak area at maximum absorbance wavelength was used as analytical signal. Calibrations curves were established by the external standard method, by plotting the peak area against the concentration or mass of analyte extracted by the flow system. The only exception was the determination of UV filters (chapter 5) where an internal standard calibration was used and the peak area ratio was calculated and applied to establish the calibration curves.

Regarding the chromatographic stationary phase, a reversed-phase ODS monolithic column (Chromolith performance 100 × 4.6 mm i.d.) connected to a guard column (5 ×

4.6 mm i.d.) of the same material. For comparison purposes, different packed columns of the same material (ODS) were used during the development of the methodology described in the chapter 5. Elution under isocratic mode was applied to all chromatographic procedures carried out during the experimental procedures.

The samples were manually injected through a Rheodyne 7725i injector (Rohert Park, CA, USA). The injection volume was adjusted for each particular situation by replacing the injection loop.

2.4 Development of automatic systems for sample preparation prior to liquid chromatographic analysis

During the performed experiments, the parameters were studied by the univariate method. This consisted in varying each parameter under study in a limited range, while keeping all the others parameters fixed. The values for the different parameters under evaluation were chosen considering the analytical performance in terms of linear working concentration range, limit of detection, determination frequency, repeatability and enrichment or enhancement factors.

The working concentration range was obtained by injecting a set of standard solutions with different concentrations and determining the range where the analytical signal was linearly related with the concentration or mass of the analyte.

Limit of detection (LOD), defined as the concentration derived from the smallest measure that can be detected with reasonable certainty for a given analytical procedure [5], was calculated as the mass of compound corresponding to the interception plus three times $s_{y/x}$ [6] (chapters 3 and 4) or as the concentration corresponding to a signal to noise ratio of 3 [7] (chapters 5 and 7).

Determination frequency, expressed as the number of determinations per hour, was evaluated for the whole analytical cycle, which included the sample preparation and the chromatographic separation. Considering that these two parts of the analytical method were carried out in parallel (chapters 3-5 and 7), determination frequency was calculated based on the time taken by the slowest part of the procedure.

Relative standard deviation (RSD) values were used to assess the repeatability (precision) of the described methodologies. The values of standard deviation were obtained by consecutive determinations of the samples ($n \geq 3$, chapters 4-7), or by the statistic $s_{y/x}$, applied as an estimate of the standard deviation [6] (chapter 3).

The preconcentration capacity was evaluated considering the enrichment or enhancement factor [8]. These values were calculated as the ratio between the slope of the calibration curve obtained after the extraction procedure and the slope of the calibration curve obtained by the direct injection of the analytes into the chromatographic system.

Besides analytical performance, green chemistry credentials [9] were also considered and variables such as waste generation, chemicals and organic solvents consumption were regarded during the development of the reported methodologies, aiming their reduction.

The accuracy of the developed methodologies was assessed by analyzing samples spiked with defined amounts of the target analyte(s) (chapters 3-5 and 7) or by analyzing certified reference materials (chapters 4 and 7).

When spiked samples were used, the standard solutions containing the analyte(s) were added but they did not represent more than 0.5% of the total sample volume. Recovery values were calculated as the ratio between the determined and the added concentration.

Certified reference materials were analyzed following the preparation procedure proposed by the manufacturers. The results obtained by the proposed methodologies were compared with the certified values, corresponding to a concentration (chapter 7) or an interval of concentrations (chapter 5 and 7).

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3

**Automatic flow system for evaluation of
polystyrene-divinylbenzene sorbents applied to
preconcentration of phenolic pollutants**

*Manuscript accepted for publication in
International Journal of Environmental Analytical Chemistry*

**Automatic flow system for evaluation of polystyrene-divinylbenzene
sorbents applied to preconcentration of phenolic pollutants**

Hugo M. Oliveira, Marcela A. Segundo* and José L. F. C. Lima

*REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia, Universidade do Porto,
Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal*

*Corresponding author. *E-mail:* msegundo@ff.up.pt; Tel: +351 222078994; Fax: +351 22202078961.

Abstract

In this work, an evaluation of commercially available polystyrene-divinylbenzene sorbents for solid-phase extraction (SPE) of eleven phenolic compounds is intended. Considering the particle size and cross-linking degree, Amberlite XAD-4 (commercial or grounded), Macronet MN-200 and Lichrolut EN were tested. The SPE protocol was performed by an automatic system, providing repeatable experimental conditions for assessment of sorbent capacity, breakthrough volume and enrichment factor (EF). A positive correlation between EF and $\log K_{ow}$ was found for Amberlite XAD-4 while a negative correlation was observed between EF and molecular weight of analyte for Macronet MN-200 and for Lichrolut EN. This indicates a prevalence of hydrophobic interactions or molecular exclusion depending upon the polymer cross-linking degree. Despite the similar repeatability ($RSD < 4.7$, $n > 6$) and recovery values attained (97.6 – 102.7 %, using 50 mL of sample) for all sorbents, Lichrolut EN is the best choice for analytical application as higher EF and lower LOD values (between 18 and 207 ng) were attained for this sorbent.

Keywords: automation, multisyringe flow injection analysis, phenolic pollutants, polystyrene-divinylbenzene, solid-phase extraction

1. Introduction

Phenolic compounds are ubiquitous pollutants generated in the production of plastics, dyes, drugs, pesticides, paper and also in the petrochemical industry [1]. Due to the intense and unpleasant organoleptic properties and toxicity at low concentration levels ($\mu\text{g L}^{-1}$), these compounds have been included in the United States Environmental Protection Agency (EPA) list of priority pollutants [2]. The determination of EPA phenolic pollutants in water samples from different sources is usually performed by separation techniques as GC-MS [3], LC coupled to ultra-violet [4] or electrochemical detection [5]. Although GC-MS provides higher sensitivity than LC-UV, the derivatisation of the analytes prior to chromatographic run is usually unavoidable. Furthermore, a previous sample preparation step comprising the preconcentration of the target analytes and matrix removal is always required for both chromatographic methodologies.

In this context, solid-phase extraction (SPE) is a suitable technique for performing the enrichment of water samples [6] because it requires low amounts of organic solvents and allows the processing of large sample volumes. Three categories of sorbents, based on silica, carbon or polymeric materials, have been used to extract phenolic compounds from water samples by a reversed-phase mechanism [7]. Besides the limited pH working range, silica sorbents have the inconvenient of providing low recoveries for the more polar compounds [8]. On the other hand, when carbon sorbents were used, poor recoveries were achieved due to difficulties in removing the trapped compounds from the sorbent surface [9]. These sorbents also have poor mechanical stability and band broadening was observed in the chromatograms [9,10]. In contrast, polymeric materials do not have limitations in the pH working range, have good mechanical properties and provide quantitative recovery of phenolic compounds [11].

Generally, polymeric sorbents comprise a polystyrene-divinylbenzene (PS-DVB) hydrophobic structure. Different particle size and cross-linking degree are available depending on the supplier. Hence, the objective of this work was to evaluate commercially available polymeric sorbents concerning their performance for SPE of EPA priority pollutants prior to their determination. Three adsorbents were chosen: Amberlite XAD-4, Lichrolut EN and Macronet MN-200. These were chosen considering the different particle size and the cross-linking grade, which results in different surface areas. Amberlite XAD-4 was used in the commercial form (300-850 μm) and as the grounded product (125-250 μm) [12]. The commercial product have a cross-linking degree lower than 16% [13] and a surface area of 750 $\text{m}^2 \text{g}^{-1}$. Macronet MN-200 is classified as a hyper cross-linking degree sorbent with

particle size in the range 300-1200 μm and a surface area of 1000 $\text{m}^2 \text{g}^{-1}$ [14]. Finally, Lichrolut EN particle distribution is in the range 40-120 μm and a surface area of 1200 m^2 was achieved for 1g of this PS-DVB sorbent with high cross-linking degree [15]. Amberlite XAD-4 and Lichrolut EN are currently applied to SPE of phenolic compounds [16,17]. Although generally used for wastewater treatment through sorption and removal of phenolics from waste waters, Macronet MN-200 was only once applied for analytical purposes to the best of our knowledge [18].

In order to attain repeatable experimental conditions, the comparison was established using a multisyringe flow injection analysis (MSFIA) system [19] to perform the concentration and elution steps of the SPE protocol in an automatic fashion. This type of automatic flow system enables the assembly of a flow network, where solutions from the different syringes can be either delivered to the flow system or returned to its own vessel, without interfering with the other channels [20]. Several elements may be incorporated into the flow network, including solid phase reactors or columns [21,22]. In the present work, the outlet of the SPE column was connected to the LC sample loop [23], which was automatically filled with eluate before each chromatographic determination, assuring repeatable conditions.

2. Experimental

2.1. Reagents and solutions

All chemicals used were of analytical-reagent grade and used with no further purification. A MilliQ system was used to obtain ultra-pure water (resistivity $> 18 \text{ M}\Omega \text{ cm}$), used for the preparation of all aqueous solution. Methanol HPLC grade (Merck, Darmstadt, Germany) was used for the preparation of all methanolic solutions and the same solvent was also used as eluent. The sorbents Amberlite XAD-4, Macronet MN-200 and Lichrolut EN were obtained from Fluka (Buchs, Switzerland), Purolite (Brasov, Romania) and Merck (Darmstadt, Germany), respectively. Amberlite XAD-4 with particle size in the range 125-250 μm was prepared as described in a previous work [24].

The phenolic compounds listed in Table 1 were obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of each compound was prepared by weighing 100 mg of the respective compound followed by dissolution in methanol in order to obtain a final concentration of 1000 mg L^{-1} . Stock solutions were rigorous diluted in methanol in order to obtain standard solutions of each individual compound or mixtures of them. For the standard

solutions submitted to the preconcentration step, the dilution was performed by using 10 mmol L⁻¹ aqueous HCl as solvent to match the pH of water samples.

Table 1 here, please

The aqueous component of the mobile phase for the chromatographic method was a 50 mmol L⁻¹ sodium dihydrogen phosphate (Fluka) solution with pH adjusted to 5.25 using a sodium hydroxide solution (6 mol L⁻¹). The organic component of the mobile phase was acetonitrile HPLC grade, obtained from Merck. The mobile phase was prepared from a 36:64 solution of the organic and aqueous components, filtered through a 0.45 µm porous membrane and degassed by ultrasounds before use.

2.2. Chromatographic determination of phenolics

The chromatographic method was performed by using a Merck/Hitachi LaChrom 7000 series (Hitachi Ltd., Tokio, Japan) equipped with a pump (L-7100), a diode array detector (L-7455) and an interface (D-7000). The monolithic analytical column used for the reversed-phase separation of the studied compounds was a Chromolith RP-18e (100 x 4.6 mm id) coupled to a pre-column (5 x 4.6 mm id) of the same material (Merck). The interface between the automatic SPE system and the chromatograph was a Rheodyne 7725i high pressure manual injector (Rheodyne, Rohnert Park, CA, USA), containing a loop with an internal volume of 20 µL. This loop was filled automatically at the end of each SPE cycle and its content was injected into the HPLC system by the same device.

The method used in this work was proposed by Cledera-Castro *et. al.* [25], allowing the isocratic separation of eleven EPA phenolic priority pollutants in less than three minutes (Fig. 1). The only modification to the original method was the temperature. Room temperature (approximately 23°C) was adopted because no significant differences in the chromatogram were observed when this temperature was compared with the original value of 36 °C.

Fig. 1 here, please

2.3. Flow injection apparatus

The manipulation of solutions inside the flow conduits of the automatic SPE system was performed by a BU4S multisyringe burette (Crison Instruments, Alella, Spain) equipped with a syringe of 10 mL in position 2 and a syringe of 5 mL in position 3 and, a Minipuls 3 peristaltic pump (Gilson, Villiers-le-Bel, France) equipped with polyvinylchloride pumping tubes. A three-way commutation valve (NRResearch, Caldwell, NJ, USA) connected to the head of each syringe of the multisyringe module allowed the optional coupling to the

manifold lines or to the solution vessel. An array of four extra commutation valves controlled by the same module completed the basis of the flow network of the present system. The commutation valves options were codified in on/off lines. For the valves placed at the head of the multisyringe the “on” line indicated the communication of the solution to the flow network whereas the “off” line was assigned to the solution reservoir.

The control of the propulsion devices was performed by a personal computer running lab-made software written in QuickBasic 4.5 (Microsoft, Redmond, WA, USA). Two different hardware channels were used. The multisyringe module control (number of steps, the direction of piston displacement and the position of the commutation valves) was performed through a serial port and the peristaltic pump control (flow direction and rotation speed) was mediated by an interface card (PCL-711, Advantech, Taipei, Taiwan).

2.4. Manifold and automatic SPE procedure

The automatic flow injection system components were arranged as shown schematically in Fig. 2. PTFE tubing with 0.8 mm id (Omnifit, Cambridge, UK) was used for all connections. The different sorbents used in this work were packed in two different columns. For trapping Amberlite XAD-4 commercial product (213 mg), Amberlite XAD-4 grounded product (194 mg) and Macronet MN-200 (188 mg), a stainless steel column 20 mm of length and 7 mm of internal diameter was used. A polyetheretherketone (PEEK) device presenting the same tubular configuration with 24 mm length and 3 mm of internal diameter was used for packing Lichrolut EN (87 mg). In both cases the sorbents were trapped inside the columns by using polypropylene filter disks supplied by MoBiTec (Goettingen, Germany) with a pore diameter of 35 μm (stainless steel column) or 10 μm (PEEK column).

Fig. 2 here, please

The complete protocol sequence for the automatic SPE procedure, including nine steps, is described in Table 2. The first command comprises the system cleaning (including the extraction column) from the previous cycle with 3500 μL of methanol followed by the sorbent bed conditioning with 2500 μL of HCl 10 mmol L^{-1} (step 2). After this, the peristaltic pump was activated at a flow rate of 4 mL min^{-1} , and a variable volume of sample was loaded into the extraction column (step 3). Afterwards, the syringes were refilled with the volume of methanol and HCl solution necessary for the next two steps (step 4). Subsequently, 2500 μL of HCl solution were propelled through the sample channels and the extraction column in order to remove residues of the compounds (step 5). After the commutation of the appropriate valves, a variable volume of methanol (dependent of the sorbent used) was sent in the

direction of the sorbent bed to elute the analytes (step 6). During this step the injection valve loop was filled and 20 μ L of the eluate were injected into the chromatographic system by rotating the HPLC injection valve to the “inject” position (step 7), beginning the chromatographic run. At last, the syringes were refilled (step 8) and the injection valve was turned to the “load” position (step 9). Thereafter, the system was ready for the next SPE cycle.

Table 2 here, please

3. Results and discussion

3.1. Design of the automatic SPE method

The SPE automatic manifold was based on a multisyringe burette that allowed a high precision in controlling the flow rates and volumes of solvents/solutions used for sorbent elution and conditioning. Furthermore, the assembling of a peristaltic pump into the manifold for propelling the sample through the extraction column enabled drastic reduction of the time necessary for the sampling step. Otherwise, the sample would be propelled by one of the burette syringes, but it would require several washing steps to prevent carryover between samples.

The inclusion of the extraction column between two commutation valves (V5 and V7) allowed that the operations of loading and elution were performed in opposite ways, avoiding the possible compaction of the sorbent particles and the consequent creation of back pressure which could result in the modification of the flow rate or in the column clogging. The commutation valve V8 provided an alternative flow path for the sample exchange without contamination of the extraction column.

The performance of the different sorbents was assessed by considering the sorbent capacity, the breakthrough volume and the enrichment factor obtained after the preconcentration step. The evaluation of the elution volume, breakthrough volume and the sorbent capacity was performed using P and 246TCP as model compounds. These compounds were chosen because they represented the extreme zones of polarity and molecular weight (Table 1) and they were also located in the extreme zones of retention time in the chromatographic run (Fig. 1).

The initial conditions for the operation of the automatic SPE system were fixed according to previous work describing on-line coupling of the PS-DVB sorbents with liquid chromatographic systems [26]. The flow rates used during the SPE cycle were chosen considering the time of contact between the solutions (washing/conditioning solution, sample and eluent) and the sorbent, but also the existence of back pressure during the operation and

the dispersion of the segment of eluate before filling the loop of the chromatographic injection valve. For these reasons flow rates of 4 mL min^{-1} for sample loading, up to 5 mL min^{-1} for sorbent system washing/conditioning and 1.5 mL min^{-1} for the elution step were used in all experiments.

The volumes used for system washing/conditioning were selected as the minimum values that avoided “memory effect” between samples and a correct conditioning of the extraction column before the sample loading step. A volume of $3500 \mu\text{L}$ of methanol was sent through the extraction column to perform the removal of all analytes which could be present into the flow line and two portions of $2500 \mu\text{L}$ of $\text{HCl } 10 \text{ mmol L}^{-1}$ were sent by S2 to the SPE device immediately before and after the sample loading in order to conditioning the sorbent and for removing sample residues before elution, respectively.

The elution volume was fixed after obtaining the elution profile of the model compounds. The experiment consisted in the extraction of $5 \mu\text{g}$ of P and 246TCP and subsequent evaluation of the peak area obtained with different elution volumes in the range $550\text{--}1250 \mu\text{L}$. Similar elution profiles were obtained for both compounds in each sorbent, indicating methanol as a suitable solvent for desorption of the analytes. The volume for which the maximum peak areas were obtained varied from $610 \mu\text{L}$ (for Lichrolut EN) to $925 \mu\text{L}$ (for Amberlite XAD-4 $125\text{--}250 \mu\text{m}$). These differences were observed due to the difference of mass of sorbent used and the bed volume of the extraction columns. The length of the connection tubing between the extraction column and the chromatograph injection valve loop (L1, Fig. 2) was the minimum possible in order to minimize the dispersion and consequently the dilution of the segment of eluate.

3.2. Sorbent loading capacity

For each sorbent a calibration curve was established by extracting 5 mL of individual standard solutions of P and 246TCP in the range $1\text{--}10 \text{ mg L}^{-1}$. The volume loaded was selected in order to guarantee that no breakthrough occurs. In all cases, no deviations in the linearity of the calibration curve were observed up to the maximum amount of P and 246TCP loaded, which corresponded to $35 \mu\text{g}$ of each compound for Lichrolut EN and $50 \mu\text{g}$ for the remaining sorbents. These results demonstrated that no overloading occurred up to the maximum amounts of the compounds extracted. The amount of $50 \mu\text{g}$ of P and 246 TCP was not tested with Lichrolut EN due to the high value of peak area (maximum peak height $> 1 \text{ AU}$) obtained for concentrations above 7 mg L^{-1} .

3.3. Breakthrough volume

The breakthrough volume corresponds to the largest sample volume that can be processed without loss of analyte and for which recovery for all sample volumes lower than the breakthrough volume will be 100% [27]. This value defines the maximum volume which allows an exhaustive extraction of the analyte from the matrix. Two classical methods have been described for the experimental measurement of the breakthrough volume [6,27]. The frontal analysis method is based on the continuous feeding of the SPE column with analyte, accompanied by continuous or discrete monitoring of the non-retained analyte by detecting the UV signal at the outlet of the sorbent bed. Another common approach to estimate the breakthrough volume is the preconcentration of different sample volumes, each containing the same amount of analytes and then measuring the analytical signal obtained after elution of the compounds. Both methods are time-consuming and may not reflect the real working conditions, especially the concentration levels used when sample analysis is performed. For these reasons, in the present work, a different experimental approach based on the enrichment of different sample volumes with a constant concentration of analyte is proposed, by taking advantage of the time based sampling of the automatic flow system. Therefore, the loading volume was defined (and changed) by computer control, after fixing the flow rate and the time during which the peristaltic pump was activated (Table 2, step 3). A mass calibration curve was established by plotting the peak area against the mass of analyte loaded into the column [28]. This experiment consisted in the extraction of sample volumes between 1 and 100 mL of a standard solution containing $500 \mu\text{g L}^{-1}$ of the model compound. This concentration was chosen in order to guarantee a maximum amount of compound that did not cause overloading of the extraction column. In the calibration curve, when a deviation from the linearity of the calibration curve was observed, it was considered that breakthrough occurred.[29] The results obtained for all sorbents (Table 3) are expressed as the volume range, defined by the two experimental points between which breakthrough occurred, or as the maximum volume loaded without breakthrough.

Table 3 here, please

Due to the hydrophobic character of the sorbents used, the values of breakthrough volume were lower for the more polar compound tested (P). For the sorbents with high cross-linking degree, as Macronet MN-200 and Lichrolut EN, no breakthrough was observed up to the maximum volume of 246TCP loaded (100 mL). The best performance for P was achieved when Amberlite XAD-4 (grounded product) and Lichrolut EN were used, with breakthrough

volumes above 75 mL. Considering the results obtained for both model compounds, the highest breakthrough volumes were achieved for Lichrolut EN.

3.4. Enrichment factors and analytical figures of merit

The enrichment factors (EF) were calculated for each sorbent by using the ratio between the slope of the calibration curves obtained after extraction of 50 mL of standard mixtures and the slope obtained by injecting directly the analytes into the chromatographic system [30]. The concentration range used was 20-100 $\mu\text{g L}^{-1}$ for each compound in the extracted mixtures and 500-8000 $\mu\text{g L}^{-1}$ in the standard mixtures used for direct injection. When Lichrolut EN was used, the concentration of the compounds in the extracted mixtures was adjusted for the range 6-30 $\mu\text{g L}^{-1}$. The results obtained in this experiment (Fig. 3) demonstrated that Lichrolut EN provided the higher values of enrichment factor (between 110 and 215), with a mean value of 176. The lower values of EF were obtained for Amberlite XAD-4 with a range between 24 and 47 and an average value of 37. These results could be explained for the differences between the two sorbents in the cross-linking degree and consequently, in the surface area available for the interactions between the analytes and the sorbent. However, for Amberlite XAD-4 with small particle size (125-250 μm) the values of EF increased (values between 54 and 67) and were higher than those obtained when using Macronet MN-200 (values between 29 and 57) that is a polymer with a very high cross-linking degree. These results show that an enhancement of the surface area by decreasing the particle size improved the capacity of the PS-DVB sorbents with low cross-linking for the retention of these phenolic compounds, resulting in performances comparable with sorbents with high cross-linking degree that by the nature of their structure have higher surface areas.

Fig. 3 here, please

Phenol and its primary derivatives listed as priority pollutants by the EPA (Table 1) have distinct physical properties according to the different groups present in the aromatic ring. Thus, the values obtained for EF were compared with the partition coefficient and the molecular weight. In the case of Amberlite XAD-4, a positive correlation ($R = 0.867$) between the enrichment factor and the $\log K_{ow}$ was observed (Fig. 4). A linear relationship was established: $EF = (10.96 \pm 7.24) \times \log K_{ow} + 12.0 \pm 17.0$, where limits of confidence are indicated for $\alpha = 0.05$, d.f. = 5. Thus, the interval of confidence for the slope value did not include the zero value and the slope value obtained was significantly different from zero ($t_{calc} = 8.705$, $t_{tab} = 2.571$, $\alpha = 0.05$, d.f. = 5), providing evidence about the linear relationship between these two variables [31]. This relationship may be explained by the reversed-phase

mechanism of interaction between the phenolic compounds and the polymer, which promotes the adsorption of the more hydrophobic compounds. A similar behavior was found for the grounded product: $EF = (6.64 \pm 4.81) \times \log K_{ow} + 45.4 \pm 11.3$, $R = 0.846$, $\alpha = 0.05$, d.f. = 5. The slope value was also significantly different from zero ($t_{calc} = 7.930$, $t_{tab} = 2.571$, $\alpha = 0.05$, d.f. = 5). Nevertheless, for grounded Amberlite XAD-4, the values of EF were higher due to the enhancement of the total surface area available for the extraction process.

Fig. 4 here, please

Although the same mechanism of interaction was present, the behavior reported for Amberlite XAD-4 was not observed with the sorbents with high cross-linking degree (Macronet MN-200 and Lichrolut EN, data not shown). Furthermore, for these sorbents, a negative correlation between the EF and the molecular weight was found (Fig. 5). A linear relationship was established for Macronet MN-200: $EF = (-0.146 \pm 0.105) \times MW + 69.3 \pm 16.4$, $R = 0.812$, $\alpha = 0.05$, d.f. = 6. Thus, the interval of confidence for the slope value did not include the zero value and the slope value obtained was significantly different from zero ($t_{calc} = -8.342$, $t_{tab} = 2.447$, $\alpha = 0.05$, d.f. = 6), providing evidence about the linear relationship between these two variables [31]. For Lichrolut EN, $EF = (-0.562 \pm 0.251) \times MW + 267 \pm 40$, $R = 0.913$, and $t_{calc} = -13.411$, $t_{tab} = 2.447$ for $\alpha = 0.05$, d.f. = 6. For these cases, the molecular size of the phenolic compound had an important role on the extraction process because the high cross-linking between the polymeric chains worked as a molecular sieve, promoting the sorption of the compounds with low molecular size. These results are in agreement with recent observations from Sychov et al. [32], indicating that the whole interior of the hypercrosslinked polystyrene particled is accessible to small analytes but not to larger molecules.

Fig. 5 here, please

Concerning the analytical figures of merit (Table 4), the determination of LOD values was based on the calibration curves established for the determination of EF and they are given as mass of compound (present in 50 mL of sample), calculated from the concentration obtained for the interception plus three times $s_{y/x}$ [31]. LOD values between 180 and 480 ng were obtained for Amberlite XAD-4, with mean values of 316 and 278 ng for commercial and grounded sorbent, respectively. Lower values were obtained for Macronet MN-200 (LOD between 83 and 368 ng, with mean value of 193 ng) and the lowest values were obtained for Lichrolut EN (LOD between 18 and 207 ng, with mean value of 82 ng). This was expected as the highest EF values were obtained for this sorbent.

Table 4 here, please

The linear calibration range was 20 – 100 $\mu\text{g L}^{-1}$ for a preconcentration volume of 50 mL, except for Lichrolut EN. For this sorbent, the linear range attained was 6 – 30 $\mu\text{g L}^{-1}$ for the same sample volume. Repeatability was assessed through the statistic $s_{y/x}$, applied as an estimate of the standard deviation ($n = 6$ or 12) [31]. Values lower than 4.5% were obtained for all sorbents tested (Table 4), accounting for the repeatable conditions attained by the automation of the SPE procedure. Recovery studies were performed using 50 mL of MilliQ water fortified with 80 $\mu\text{g L}^{-1}$ of each phenolic compound (or 24 $\mu\text{g L}^{-1}$ for Lichrolut EN). Similar results were attained for all sorbents, with recovery values between 97.6 and 102.7%. For Lichrolut EN further analytical application was developed [23], providing statistically comparable results when certified reference material was analyzed. In fact, for samples RTC-QCI-032 and U-QCI-076 (Promochem, LGC Standards), the total phenolics content was in agreement with the certified value [23]. Moreover no matrix effect was observed for mineral, tap and seawater samples, with mean recovery values ranging from 89-103% for all analytes tested.

Compared to previously described on-line SPE methods for chromatographic determination of phenolics [9,10,23,33-36], the sorbents studied here presented better or similar performance (Table 5). In fact, organic solvent consumption was similar (4-5 mL) and effluent production was lower (9.5 mL compared to 20-28 mL) in this work. The method proposed by Wissiack et al [34] provided better performance regarding these aspects, but the concentration working range was higher and narrower.

4. Conclusions

In the present work, the utilization of an automatic flow system provided repeatable conditions for comparison of different SPE sorbents. For this, the implementation of different flow directions for loading and elution operations was essential, because it prevented the compaction of the sorbent bed and avoided the creation of preferable flow paths. Compared to previous alternatives in this area, the multisyringe equipment was an advantageous choice as it was compatible with organic solvents because only glass syringes and PTFE valves were in contact with the manipulated fluids. Moreover, the hyphenation with the LC equipment through its injection valve was successful while the computer control of the SPE protocol

avoided unnecessary reagent (conditioning solution, eluent) consumption during the chromatographic run.

Concerning the performance of the sorbents, higher breakthrough volumes were obtained for Lichrolut EN for both model analytes studied (P and 246TCP). Higher values were also obtained for Lichrolut EN, followed by Amberlite XAD-4 (grounded particles), Macronet MN-200 and Amberlite XAD-4 (commercial particles), regarding the enrichment factors obtained under the same experimental conditions. As a consequence, lower values of LOD were also attained when applying Lichrolut EN. Nevertheless, repeatability and recovery values were similar for all sorbents tested. These results indicate Lichrolut EN as the best choice for analytical applications because lower concentrations can be determined by using this sorbent, fostering the determination in samples at low ng mL^{-1} levels using conventional HPLC-DAD technique [23].

Concerning the molecular interactions between the analytes and the sorbent, it was observed a positive correlation between the EF values and the hydrophobic character of the phenolic compound (expressed as $\log K_{ow}$) for Amberlite XAD-4, justified by the enhanced retention of more hydrophobic molecules due to the prevalence of the reversed-phase mechanism of interaction between the analytes and this sorbent. For the sorbents with higher cross-linking degree, this was not observed. In fact, a negative correlation between the EF values and the molecular weight of the analyte was verified, indicating that the structure of these sorbents may act like a molecular sieve, granting or restricting access to inner surfaces according to the analyte size. This fact may have consequences not only in the analytical application of these sorbents but also on their application for removal of phenolic pollutants in effluents. In both situations, larger molecules will be less retained despite the enhancement of surface area brought by the higher cross-linking degree.

Acknowledgements

Hugo M. Oliveira thanks Fundação para a Ciência e Tecnologia (FCT) and FSE (III Quadro Comunitário) for the grant SFRH/BD/22494/2005. We acknowledge Neoquímica for the gift of Macronet MN-200 sorbent.

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Fig. 1 Typical chromatogram obtained under the conditions described in the text by direct injection of the compounds. Concentration of each phenolic compound: $10 \mu\text{g mL}^{-1}$. Peak labels: 1) 24DNP, 2) 46DNOC, 3) P, 4) 4NP, 5) 2CP, 6) 2NP, 7) 24DMP, 8) 4C3MP, 9) 24DCP, 10) PCP, 11) 246TCP. Mobile phase; acetonitrile:phosphate buffer (36:64 vol.); flow rate, 4.00 mL min^{-1} .

Fig. 2 (A) MSFIA-SPE manifold for the automatic preconcentration of phenolic compounds, connected to the chromatographic system through the injection valve, represented on “load” position. (B) Schematic representation of the injection valve configuration on “inject” position. MS, multi-syringe; Si, syringe; Vi, commutation valves; EC, SPE column; IV, injection valve; PP, peristaltic pump; W, waste; L1, connection tubing ($300 \times 0.8 \text{ mm id}$); S2, syringe 10 mL ; S3, syringe 5 mL ; C, carrier ($\text{HCl } 10 \text{ mmol L}^{-1}$); EL, eluent (methanol); St, sample or standard solution; HP, high-pressure pump; MC monolithic chromatographic column; MSFIA, preconcentration flow system; CP, closed port. In the commutation valves, the position “on” is represented by a solid line while the position “off” is represented by a dotted line. The needle port and connection to waste in IV are also represented by dotted lines.

Fig. 3 Enrichment factors (EF) for 50 mL of standard mixtures with concentrations in the range $6\text{--}80 \mu\text{g L}^{-1}$ using the different sorbents (dotted bar, Amberlite XAD-4 (commercial product); white bar, Amberlite XAD-4 ($125\text{--}250 \mu\text{m}$); black bar, Macronet MN-200; striped bar, Lichrolut EN).

Fig. 4 Relation between enrichment factor (EF) and the partitioning coefficient ($\log K_{ow}$) of phenolic compounds after SPE using Amberlite XAD-4 (commercial product ($\circ$)) and grounded particles ($\square$)).

Fig. 5 Relation between enrichment factor (EF) and the molecular weight (MW) of phenolic compounds obtained after SPE using the solid-phases Lichrolut EN (Δ) and Macronet MN-200 ($\square$)).

Table 1

Detection wavelength (λ), molecular weight, partitioning coefficients octanol / water ($\log K_{ow}$) and dissociation constants (pK_a) of EPA phenolic priority pollutants [25,26]

Phenolic compound	λ (nm)	MW (u)	$\log K_{ow}$	pK_a
2,4-Dinitrophenol (24DNP)	360	184.1	1.53	4.09
2-Methyl-4,6-dinitrophenol (46DNOC)	375	198.1	2.12	4.34
Phenol (P)	215	94.1	1.50	9.99
4-Nitrophenol (4NP)	315	139.1	1.90	7.16
2-Chlorophenol (2CP)	195	128.6	2.15	8.55
2-Nitrophenol (2NP)	210	139.1	1.78	7.21
2,4-Dimethylphenol (24DMP)	195	122.7	2.42	10.6
4-Chloro-3-methylphenol (4C3MP)	195	142.6	3.10	9.55
2,4-Dichlorophenol (24DCP)	200	163.0	2.08	7.85
Pentachlorophenol (PCP)	220	266.3	5.01	4.93
2,4,6-Trichlorophenol (246TCP)	200	197.5	3.69	7.42

Table 2

Procedure for the automatic solid-phase extraction of phenolic compounds prior to its chromatographic analysis.

Step	Instrumentation	Protocol	Description
1	Multisyringe piston pump	Dispense 3500 μL at 3 mL min^{-1} with heads V2 off, V3 on, V5 on, V6 off, V7 on, V8 off, IV load	Washing of sorbent bed and injection valve loop with methanol
2	Multisyringe piston pump	Dispense 2500 μL at 5 mL min^{-1} with heads V2 on, V3 off, V5 off, V6 on, V7 off, V8 off, IV load	Sorbent conditioning with HCl, pH 2.0
3	Peristaltic pump	Propel X μL at 4 mL min^{-1} with heads V2 off, V3 off, V5 off, V6 off, V7 off, V8 off, IV load	Sample loading (variable volume up to 100 mL)
4	Multisyringe piston pump	Pickup 3720-4350 μL at 15 mL min^{-1} with heads V2 off, V3 off, V5 off, V6 off, V7 off, V8 off, IV load	Piston bar adjustment
5	Multisyringe piston pump	Dispense 2500 μL at 5 mL min^{-1} with heads V2 on, V3 off, V5 off, V6 on, V7 off, V8 off, IV load	Matrix removal and tubing cleanup with HCl, pH 2.0
6	Multisyringe piston pump	Dispense 610-925 μL at 1.5 mL min^{-1} with heads V2 off, V3 on, V5 on, V6 off, V7 on, V8 off, IV load	Elution of the analytes using methanol
7	HPLC injection valve	Rotate IV to inject position	Start of chromatographic run
8	Multisyringe piston pump	Pickup 9500 μL at 15 mL min^{-1} with heads V2 off, V3 off, V5 off, V6 off, V7 off, V8 off, IV inject	Piston bar adjustment
9	HPLC injection valve	Rotate IV to load position	Connect IV loop to MSFIA-SPE system

Table 3

Breakthrough volume range (mL) obtained for the different sorbents after extraction of volumes between 1-100 mL of P or 246TCP standard solutions with a concentration of $500 \mu\text{g L}^{-1}$.

Sorbent	P	246TCP
Amberlite XAD-4 (commercial product)	$40 < V < 50$	$75 < V < 100$
Amberlite XAD-4 (grounded particles)	$75 < V < 100$	$50 < V < 100$
Macronet MN-200	$25 < V < 50$	$V > 100$
Lichrolut EN	$V > 75$	$V > 100$

Table 4

Analytical figures of merit obtained for each sorbent tested

compound	Amberlite XAD 4 (commercial)					Amberlite XAD 4 (125-250 µm)					Macronet					Lichrolut				
	LOD / ng	tested conc. / µg L ⁻¹	RSD ^a	recovery % ^b	LOD / ng	tested conc. / µg L ⁻¹	RSD ^a	recovery % ^b	LOD / ng	tested conc. / µg L ⁻¹	RSD ^a	recovery % ^b	LOD / ng [23]	tested conc. / µg L ⁻¹	RSD ^a	recovery % ^b				
24DNP	289	85.0	4.1	97.6±1.6	262	85.0	2.2	99.7±1.8	368	85.0	2.7	98.7±3.5	207	25.5	1.2	100.3±1.1				
46DNOC	480	81.2	1.7	100.0±0.5	437	81.2	3.5	98.8±1.1	321	81.2	1.9	100.4±0.3	103	24.4	2.8	102.1±1.1				
P	358	82.4	2.9	98.5±2.3	290	82.4	2.6	99.6±3.8	163	82.4	1.4	100.2±0.4	157	24.7	2.1	98.1±1.4				
4NP	286	92.8	2.3	98.9±2.0	300	92.8	3.0	98.3±0.3	83	92.8	1.4	99.6±0.4	110	27.8	1.4	99.2±0.4				
2CP	226	86.2	1.9	99.2±1.6	180	86.2	1.9	99.3±1.2	209	86.2	1.9	99.5±1.2	31	25.9	1.7	99.2±1.3				
2NP	317	81.1	2.6	98.1±1.2	224	81.1	1.5	99.5±1.2	86	81.1	1.6	99.7±1.7	87	24.3	2.3	101.6±1.6				
24DMP	293	84.1	1.3	99.5±0.1	297	84.1	2.9	98.4±1.6	171	84.1	1.6	99.1±0.5	30	25.2	1.6	99.1±0.7				
4C3MP	292	79.9	1.4	99.5±0.3	224	79.9	1.2	99.8±1.5	98	79.9	1.4	99.7±0.4	18	24.0	2.0	100.9±3.4				
24DCP	305	77.4	1.3	99.1±0.4	287	77.4	1.2	99.9±1.6	204	77.4	1.9	100.1±0.3	39	23.2	1.9	99.8±3.2				
PCP	381	42.9	4.5	99.3±3.9	NA	NA	NA	NA	227	42.9	1.9	100.6±2.4	66	12.9	2.2	100.2±5.0				
246TCP	244	83.9	1.6	100.6±0.9	NA	NA	NA	NA	NA	83.9	1.6	102.7±2.7	55	25.2	5.1	NA				

^a calculated using the statistics $s_{y/x}$ as estimate of standard deviation [33] (n = 6 or n = 12) divided by the tested concentration.^b n = 3

NA = not available

Table 5.
Analytical performance of on-line SPE chromatographic methods for determination of pollutant phenolics

Methodology	Working range / $\mu\text{g L}^{-1}$	LOD / $\mu\text{g L}^{-1}$	Sorbent	Sample volume / mL	Determination frequency / h^{-1}	Organic solvent / mL	Effluent ^a / mL	Reference
LC-DAD	20-100	4.5-9.6	XAD-4 (commercial)	50	4	4.1-4.4 ^c	9.5	Present work
LC-DAD	20-100	3.6-8.7	XAD (125-250 μm)	50	4	4.1-4.4 ^c	9.5	Present work
LC-DAD	20-100	1.7-7.4	Macronet MN-200	50	4	4.1-4.4 ^c	9.5	Present work
LC-DAD	6-30	0.4-4.1	Lichrolut EN	50	4	4.1-4.4 ^c	9.5	Present work
LC-DAD	1.5-140	0.3-2	Lichrolut EN	25-100	4-10	4.1 ^c	9.1	[23]
LC-ED	0.01-10	0.001-0.075	PLRP-S	1.0-4.0	2.6	16 ^c	28	[33]
LC-MS	100-300	n. a.	Several ^b	≤ 100	2	3 ^c	8	[34]
SFC-DAD	3-25	0.4-1.9	PLRP-s	20	1.5	10 ^c	20	[35]
LC-UV	0.01-25	0.01-0.7	Lichrolut EN	100	1.6	5 ^c + 5 ^d	12.5	[9]
LC-UV-FL	1.5-100	0.2-0.6	Modified PS-DVB	≤ 100	0.93-2	12 ^c	24	[36]
LC-UV-ED	0.05-20	0.035-0.07	Modified PS-DVB	≤ 20	2	12 ^d	22	[10]

^a excluding sample volume; ^b comparative study between C18 HD, PLRP-S, Hamilton-PRP-1, Hysphere GP, Hysphere SH and Oasis HLB; ^c methanol; ^d acetonitrile; SFC, supercritical fluid chromatography, LC, liquid chromatography; DAD, diode-array detection, ED, electrochemical detection, MS, mass spectrometry detection; UV, ultra-violet detection; FL, fluorimetric detection; n. a., not available.

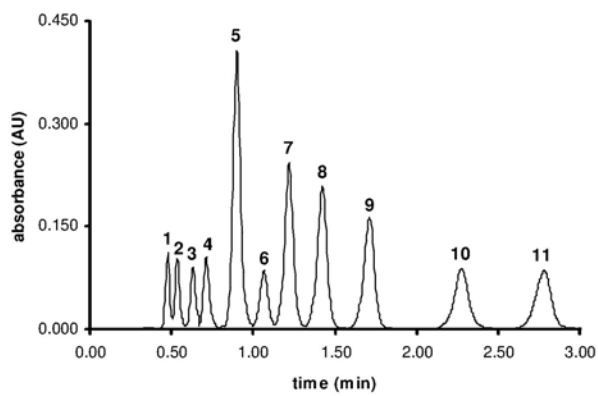


Figure 1

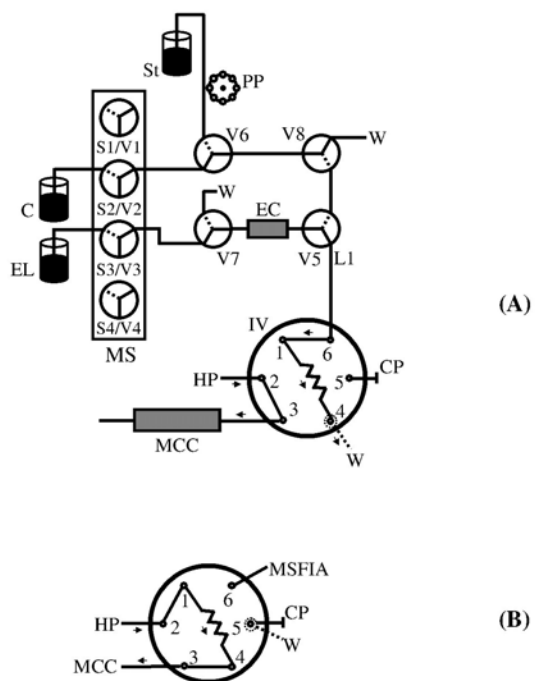


Figure 2

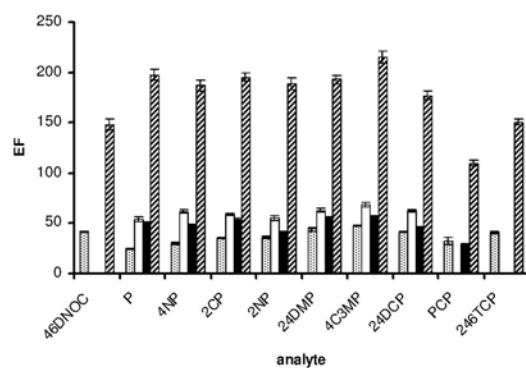


Figure 3

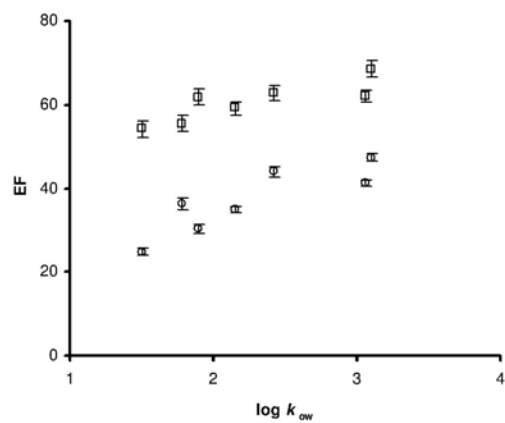


Figure 4

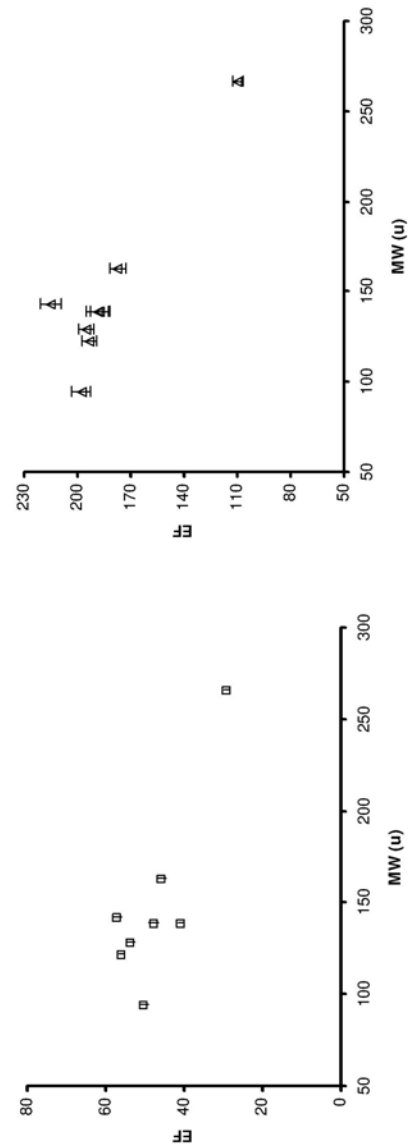


Figure 5

4

**Multisyringe flow injection system for
solid-phase extraction coupled to liquid
chromatography using monolithic column for
screening of phenolic pollutants**



Multisyringe flow injection system for solid-phase extraction coupled to liquid chromatography using monolithic column for screening of phenolic pollutants

Hugo M. Oliveira^a, Marcela A. Segundo^{a,*}, José L.F.C. Lima^a, Victor Cerdà^b

^a REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal

^b Department of Chemistry, Faculty of Sciences, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Illes Balears, Spain

ARTICLE INFO

Article history:

Received 26 May 2008

Received in revised form

18 September 2008

Accepted 21 September 2008

Available online 30 September 2008

Keywords:

Phenolic pollutants

Automatic solid-phase extraction

Water analysis

On-line preconcentration

Multisyringe flow injection analysis

ABSTRACT

In this work a fast, automatic solid-phase extraction procedure hyphenated to HPLC-UV is proposed for screening of priority phenolic pollutants in waters at ng mL^{-1} levels. A flow through column, containing polystyrene-divinylbenzene sorbent, was incorporated to a multisyringe flow injection system (MSFIA), where the sample loading and analyte elution were carried out after computer control. The MSFIA system also directed the eluent to fill the injection loop of the chromatograph, coupling the sample preparation to its determination. High enrichment factors were attained for phenol and ten of its derivatives (mean value 176 for 50 mL of sample), with LOD values lower than 1 ng mL^{-1} for the maximum volume of sample used (100 mL). For all analytes, mean recoveries between 89 and 103% were obtained for different water matrices. Certified reference material and a contaminated soil (RTC-CRM 112) were also tested successfully. The determination frequency was $4\text{--}10 \text{ h}^{-1}$, providing an automatic, fast and reliable tool for water quality and environmental monitoring.

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1. Introduction

Phenolic compounds are widespread aquatic pollutants. Considering their toxicological and organoleptic effects, the United States Environmental Protection Agency (EPA) has listed phenol and several derivatives as priority pollutants [1]. Their input into the ecosystems results directly from the human activity or indirectly from the transformation of natural or synthetic chemicals and they are often found in waters from different sources [2–5]. The screening of phenol and its derivatives in risk areas is essential for public health protection in order to spot timely illegal discharges from industry or contamination by pesticides, even at low levels.

Different strategies have been described for the quantification of phenolic compounds in water samples, including separation techniques such as liquid chromatography (LC) [6–8] or gas chromatography (GC) [9–11] coupled to different detectors. Due to the low volatility character of phenolic compounds, LC is employed more often than GC because analyte derivatization is avoided. Furthermore, with the advent of monolithic columns, fast analysis is possible [12], allowing the determination of several phenolic

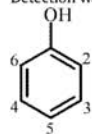
derivatives in about 3 min [13,14]. However, sample preparation is frequently required in order to attain analyte enrichment and matrix removal.

Despite the available fast chromatographic methods, the sample preconcentration/clean up step restricts the sample throughput, conditioning the number of samples that can be processed in environmental surveillance schemes. In this context, automation of the sample preparation step is relevant. In fact, some alternatives resorting to robotic systems have been described [15,16]. However, the hyphenation to the chromatograph is not easy, requiring dedicated, expensive equipment. Other alternatives using flow-systems with on-line column switching schemes have also been described for simpler hyphenation between sample treatment and determination [17,18].

Flow injection systems have been clearly underexploited for this task [19], especially the more recent, computer controlled techniques. Multisyringe flow injection analysis (MSFIA) [20] is one of them and its features (flow network design, multi-channel operation and total compatibility of the manifold with organic solvents) allow the assembling and operation of solid-phase extraction (SPE) devices [21]. Therefore, the objective of the present work is the development of a fast MSFIA-SPE procedure coupled to LC-UV determination for screening of priority phenolic pollutants in waters and environmental samples at ng mL^{-1} levels.

* Corresponding author. Tel.: +351 222078994; fax: +351 222078961.
E-mail address: msegundo@ff.up.pt (M.A. Segundo).

Table 1
Detection wavelength, log K_{ow} , pK_a and structures of EPA phenolic priority pollutants.



Phenolic compound	λ /nm	log K_{ow} ^a	pK_a ^a	Structure				
				C ₂	C ₃	C ₄	C ₅	C ₆
2,4-Dinitrophenol (24DNP)	360	1.53	4.09	NO ₂		NO ₂		
2-Methyl-4,6-dinitrophenol (46DNOC)	375	2.12	4.34	CH ₃		NO ₂		NO ₂
Phenol (P)	215	1.50	9.99					
4-Nitrophenol (4NP)	315	1.90	7.16			NO ₂		
2-Chlorophenol (2CP)	195	2.15	8.55	Cl				
2-Nitrophenol (2NP)	210	1.78	7.21	NO ₂				
2,4-Dimethylphenol (24DMP)	195	2.42	10.6	CH ₃		CH ₃		
4-Chloro-3-methylphenol (4C3MP)	195	3.10	9.55		CH ₃	Cl		
2,4-Dichlorophenol (24DCP)	200	2.08	7.85	Cl		Cl		
Pentachlorophenol (PCP)	220	5.01	4.93	Cl	Cl	Cl	Cl	Cl
2,4,6-Trichlorophenol (246TCP)	200	3.69	7.42	Cl		Cl		Cl

^a From references (1) Dean, J.R.; Tomlinson, W.R.; Makovskaya, V.; Cumming, R.; Hetheridge, M.; Comber, M., Solid-phase microextraction as a method for estimating the octanol–water partition coefficient. *Anal. Chem.* 1996, 68, (1), 130–133; (2) Li, N. Q.; Lee, H. K., Trace enrichment of phenolic compounds from aqueous samples by dynamic ion-exchange solid-phase extraction. *Anal. Chem.* 1997, 69, (24), 5193–5199.

2. Material and methods

2.1. Reagents and solutions

All solutions were prepared with water from MilliQ system (Millipore, Bedford, MA, USA) (resistivity $> 1.8 \times 10^5 \Omega \text{ cm}$) and chemicals of analytical-reagent grade quality. Methanolic solutions were prepared with methanol HPLC grade (Merck, Darmstadt, Germany). This organic solvent was also used as SPE eluent. The SPE sorbent Lichrolut EN (cross linked styrene-divinylbenzene) was obtained from Merck.

The phenolic compounds (Table 1) used were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were the following: phenol (P), 2-nitrophenol (2NP), 4-nitrophenol (4NP), 2,4-dinitrophenol (24DNP), 2-methyl-4,6-dinitrophenol (46DNOC), 2-chlorophenol (2CP), 2,4-dichlorophenol (24DCP), 2,4,6-trichlorophenol (246TCP), pentachlorophenol (PCP), 4-chloro-3-methylphenol (4C3MP), and 2,4-dimethylphenol (24DMP). The stock solution of each phenolic compound was prepared by accurately weighing the appropriate mass and by dissolving it in methanol in order to obtain a final concentration of 1000 mg L^{-1} . The working standard solutions (containing either mixtures or individual compounds) were obtained by rigorous dilution of the stock solutions in methanol (for direct injection into HPLC system) or in 0.010 mol L^{-1} HCl (for the preconcentration step).

For accuracy assessment, recovery assays were performed using analytical grade standards of each compound obtained from Supelco (Bellefonte, PA, USA). All standards were purchased in 1 mL methanol solution with the concentration of 5000 mg L^{-1} , except for 4C3MP, whose concentration was 500 mg L^{-1} . For the preparation of working standard solutions the same procedure described for the standard solutions was adopted, except for the solvent, which was replaced by water from different sources. In this case, before spiking the water samples with the mixture of analytes, they were filtered through a $0.45 \mu\text{m}$ membrane filter and the pH was adjusted to 2 with concentrated HCl (12 mol L^{-1}). The sea water matrix was prepared after the APHA/AWWA/WEF procedure [22]. Furthermore, certified reference material from LGC-Standards (Middlesex, UK), ref. U-QCI-760 and RTC-QCI-032, was also applied for accuracy assessment.

For the HPLC methodology, HPLC grade acetonitrile was purchased from Merck. Sodium dihydrogen phosphate (Sigma, St.

Louis, MO, USA) solution was prepared by dissolving 6.8 g of the salt in 1000 mL of water, and the pH of this solution was adjusted to a final value of 5.25 using a NaOH solution (6 mol L^{-1}). All solutions were filtered through a $0.45 \mu\text{m}$ membrane filter and degassed using ultrasound before use.

2.2. Apparatus

Chromatographic determinations were conducted on a Merck/Hitachi-LaChrom 7000 series (Hitachi Ltd., Tokio, Japan) equipped with a diode array detector (L-7455), a pump (L-7100) and a Chromolith RP-18e ($100 \text{ mm} \times 4.6 \text{ mm i.d.}$) column with pre-column ($5 \text{ mm} \times 4.6 \text{ mm i.d.}$) (Merck). The chromatographic system was controlled by an interface (D-7000) and the D-7000 software. The eluate of the preconcentrated sample was injected by using a Rheodyne 7725i manual injector (Rheodyne, Rohnert Park, CA, USA) equipped with $20 \mu\text{L}$ loop. This device was also used as interface between the chromatographic and the automatic MSFIA-SPE system.

Solutions were propelled in the preconcentration system by a multisyringe burette (Crison Instruments, Allela, Spain) and a Minipuls 3 peristaltic pump (Gilson Villiers-le-Bel, France) equipped with polyvinylchloride pumping tubes. In this work, the multisyringe module was equipped with a syringe of 10 mL in position 2 and a syringe of 5 mL in position 3. Besides the three-way commutation valve (NResearch, Caldwell, NJ, USA) connected to the head of each syringe, four extra commutation valves were connected and controlled through the burette. For all valves, the exchange options were classified in on/off lines.

A personal computer, running lab-made software written in QuickBasic 4.5 (Microsoft, Redmond, WA, USA), controlled the multisyringe operation (number of steps and direction of piston displacement, position of all commutation valves) by a serial port. The peristaltic pump control (flow direction and rotation speed) was performed by the same software using a PCL-711 interface card (Advantech, Taipei, Taiwan).

2.3. Manifold and MSFIA procedure

The different components of the flow-system were disposed as shown schematically in Fig. 1. All connections were made with 0.8 mm i.d. polytetrafluorethylene (PTFE) tubing (Omnifit, Cam-

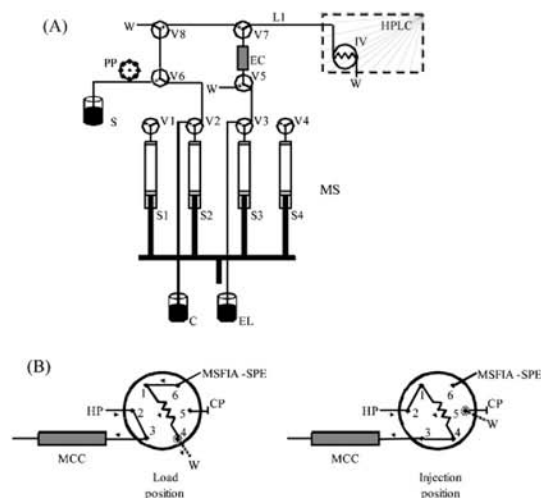


Fig. 1. (A) MSFIA-SPE manifold for the automatic pre-concentration of phenolic compounds coupled to LC equipment. (B) Detailed representation of the connection between MSFIA-SPE manifold and the chromatographic system through the injection valve on "load" and "inject" positions. MS, multisyringe; S_i, syringe; V_i, commutation valves; EC, SPE column; IV, injection valve; PP, peristaltic pump; W, waste; L1, connection tubing (30 cm); S₂, syringe 10 mL; S₃, syringe 5 mL; C, carrier (HCl 0.010 mol L⁻¹); EL, eluent (methanol); S, sample or standard solution; HP, high-pressure pump; MCC, monolithic chromatographic column; MSFIA-SPE, pre-concentration flow-system; CP, closed port. In the valves, the position "on" is represented by a solid line while the position "off" is represented by a dotted line. Needle port and connection to waste in the injection valve are also represented by dotted lines.

bridge, UK). A lab-made polyetheretherketone (PEEK) extraction column was used. This column presented a tubular configuration with 24 mm of length and 3 mm of internal diameter, containing 87 mg of sorbent. The resin was trapped inside the column by using filter disks from MoBiTec (Goettingen, Germany) with pore diameter of 10 μ m (ref. #2210).

Table 2

Protocol sequence for the automatic solid-phase extraction of phenolic compounds from water.

Step	MS operation	Chromatograph	Position of the commutation valves ^a								MS operation	
			1	2	3	4	5	6	7	8	Volume/ μ L ^b	Time/s
1	Sorbent bed is washed with methanol	Chromatographic run (sample X-1); injection loop is washed with methanol	-	F	N	-	N	F	N	F	7000	70
2	HCl solution is sent through the column for sorbent conditioning	Chromatographic run (sample X-1)	-	N	F	-	F	N	F	F	2500	30
3	Syringes are refilled and sample X is loaded to the pre-concentration column ^c where analytes retention take place	Chromatographic run (sample X-1) finishes	-	F	F	-	F	F	F	F	3720	14.8–750
4	HCl solution is sent through the column for matrix removal	-	-	N	F	-	F	N	F	F	2500	30
5	Methanol is sent through the sorbent, eluting the retained analytes	The loop of the injection valve is filled by eluent	-	F	N	-	N	F	N	F	1220	24.4
6	-	The segment of eluate trapped in the loop is injected and the chromatographic run starts	-	-	-	-	-	-	-	-	-	-
7	Syringes are refilled	Chromatographic run (sample X); the injection valve is returned to "load" position	-	F	F	-	F	F	F	F	9500	38

^a N and F represent position "on" and "off", respectively.

^b The indicated values for volume refer to syringe 2 (10 mL).

^c The peristaltic pump is activated (8 mL min⁻¹) during the time necessary for propelling the defined sample volume (up to 100 mL).

The protocol sequence adopted for the automatic solid-phase extraction of EPA phenolic compounds is described in Table 2. The complete sequence included six steps. A volume of 3500 μ L of methanol was propelled through the extraction column and the chromatographic injection loop in order to clean the system (step 1). After commutation of the appropriate valves, 2500 μ L of 0.010 mol L⁻¹ HCl solution were used for conditioning the sorbent (step 2). Afterwards, with all valves in "Off" position, the peristaltic pump was activated and a variable sample volume up to 100 mL was direct towards the extraction column at flow rate of 8.0 mL min⁻¹. Simultaneously, the syringes were filled (step 3). Subsequently, the extraction column was washed with 2500 μ L of HCl solution in order to remove the sample matrix (step 4). The analytes retained were eluted with 610 μ L of methanol and direct to the chromatograph, filling the IV loop (step 5). Next, 20 μ L of the eluate were injected into the chromatographic system by rotating the injection valve to the "inject" position (step 6), beginning the chromatographic run. Finally, the syringes were refilled and the injection valve was returned to the "load" position (step 7).

2.4. Soil sample

A certified soil sample (ref. RTC-CRM 112, LGC Standards) was analyzed. Five grams of soil were weighted and added to 100 mL of water. After 19 h of storage in the dark, the mixture was passed through a 0.22 μ m filter. The filtrate was acidified to pH 2 by addition of concentrated HCl and then processed by the MSFIA system.

3. Results and discussion

3.1. Chromatographic analysis

The chromatographic method used in this work was adapted from that proposed by Cledera-Castro et al. [13]. The utilization of a monolithic column (100 mm $\times$ 4.6 mm i.d.) allowed the separation and quantification of eleven EPA priority pollutants in approximately 5 min with an analytical performance comparable to that obtained by using conventional microparticulate reversed-phase columns. All the chromatographic conditions (mobile phase

composition, flow rate and sample volume) were maintained with exception of temperature. Room temperature (approximately 23 °C) was applied instead of 36 °C because no significant differences were observed in the performance of the method using either value.

3.2. Development of the automatic MSFIA-SPE system

The use of a MSFIA flow based manifold allowed the precise control of the flow rate and volume of the solutions used for the sorbent conditioning and respective elution. The glass syringes and PTFE valves that constitute the multisyringe burette and all remaining components of the manifold were also compatible with organic solvents. A peristaltic pump was assembled to the flow manifold in order to load sample volumes up to 100 mL. By using this device the necessity of filling a holding coil before sending the sample to the extraction column with the correspondent time-consuming repositioning movements of the piston bar [23] was avoided, allowing a drastic reduction in the time necessary to perform this step during the protocol sequence.

Some operational parameters as the carrier solution, volumes and flow rates used were fixed according to reported data [24] and our previous experience [25]. The retention of the target analytes was performed at pH 2.0, using HCl 0.010 mol L⁻¹ as carrier. Samples and standards were also acidified to this pH. Taking into account the reversed-phase interactions between the sorbent and the analytes, this pH was necessary to prevent the ionization of all priority phenolic pollutants, which pK_a is in the range 4–11 (Table 1). A volume of 3.5 mL of methanol was used for removing the analytes not injected into the HPLC system in the beginning of each protocol sequence. This procedure allowed a complete cleaning of the extraction column and the HPLC connection tubing, eliminating carryover between consecutive samples. Two portions of 2.5 mL of carrier solution (HCl 0.010 mol L⁻¹) were fed into the extraction column immediately before and after the sample loading step, performing the sorbent conditioning and the sample matrix cleanup. This volume was fixed considering the volumes of the sorbent bed, connection tubing and commutation valves, guaranteeing an operation without contamination by consecutive injections of samples. In order to prevent the formation of a liquid gap before the sorbent bed top with a consequent pressure build-up, the sample loading and the elution steps were performed in opposite ways. Therefore, the extraction column was placed between two commutation valves (Fig. 1, V5–V7). The length of the connection tubing between the exit of the extraction column and injection valve (Fig. 1, L1) was made as short as possible to prevent dispersion of the extracted analytes. The introduction of the commutation valve V8 (Fig. 1) in the flow path between the sample commutation valve (V6) and the extraction column (V7–V5) was necessary to avoid the contamination of the extraction column when the sample or standard solutions were changed.

The hyphenation between the MSFIA-SPE manifold and the chromatograph also required a precise positioning of the eluent plug in order to assure the insertion of the maximum concentration of phenolic compounds into the injection loop. Therefore, the volume of eluent is a critical aspect on attaining the highest enrichment factor and good precision in the chromatographic determination. As depicted in Fig. 2, the elution profile was assessed for eluent volumes between 550 and 750 µL. P and 246 TCP were used as model compounds because they present polarity values placed at extreme zones, considering the *K*_{OW} range of the target analytes (Table 1). It was observed that the maximum value for the analytical signal was obtained for 610 µL of methanol, which was chosen for further experiments. The similar elution profile obtained for both compounds (Fig. 2) indicated that methanol was a suitable

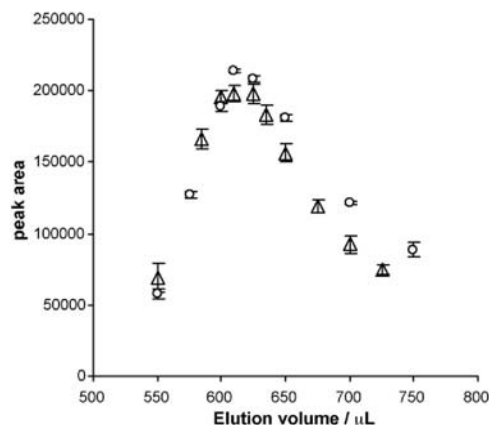


Fig. 2. Elution profile, corresponding to the peak areas obtained for different volumes of methanol used for the elution of 5 µg of P (Δ) or 246TCP (○) contained in 1 mL of HCl 0.01 mol L⁻¹.

eluent for the simultaneous desorption of all phenolic compounds from the sorbent. It was also observed that a degassing step before use and the store temperature of the eluent during the working day had a crucial role in the repeatability of the automatic SPE procedure. In the experiments, at room temperature without methanol degassing, a relative standard deviation (RSD) of 10% was obtained for 14 consecutive extractions of 10 mL of 0.5 µg mL⁻¹ of phenol. This impaired precision was caused by formation of very small gas bubbles at the surface of the sorbent bed, which appeared in the water/methanol interface when the eluent was fed into the extraction column. This source of error was minimized by degassing the eluent periodically with helium and by keeping it on ice during analysis. With these precautions, the RSD achieved for the same experimental set was lower than 3%.

The flow rate of the sample loading step is a relevant parameter due to the possibility of handling large volumes in a short period of time. Besides the determination frequency, this factor influences directly the enrichment capacity and subsequently the limit of detection and the sensitivity of the method. A study to evaluate the influence of the flow rate in the retention of the analytes was carried out by loading a volume of 50 mL of a standard mixture containing 15 ng mL⁻¹ of each phenolic compound at flow rates in the range 2–8 mL min⁻¹. Flow rates above 8 mL min⁻¹ were not used because compaction of the bed particles was observed, creating high back pressures and resulting in the clogging of the extraction column. For lower flow rates, the results showed no difference in the adsorption of all analytes into the extraction column as peak area values were similar for all flow rates tested. Therefore, the flow rate of 8 mL min⁻¹ was adopted to perform the retention of the analytes from the water sample into the sorbent bed, minimizing the time required for this step.

3.3. Analytical performance and application to water samples

The analytical performance of the MSFIA-SPE system was evaluated considering the application range, limit of detection (LOD), enrichment factor (EF), determination frequency, repeatability and accuracy.

Using the MSFIA-SPE system it was possible to obtain mass-based calibration curves, by plotting the peak area obtained for each compound against the mass of analyte loaded into the extraction column. This calibration was performed by loading different vol-

Table 3

Linear dynamic range, calibration curve parameters, values for limit of detection (LOD) and enrichment factors (EF) obtained for the proposed method.

Calibration curve ^a							
Compound	Linear range/ng	Slope/area units ng ⁻¹	Interception	r ²	LOD/ng	LOD ^b /ng mL ⁻¹	EF ^c
24DNP	350–3500	32.9 ± 0.6	1813 ± 818	0.995	207	2	n. a.
46DNOC	350–3500	31.9 ± 0.5	830 ± 674	0.998	103	1	148 ± 5
P	300–3000	38.7 ± 0.8	424 ± 924	0.996	157	2	198 ± 5
4NP	225–2250	52 ± 1	–116 ± 949	0.997	110	1	187 ± 6
2CP	200–2000	214 ± 5	1243 ± 888	0.999	31	0.3	195 ± 4
2NP	200–2000	57 ± 1	–431 ± 891	0.994	87	0.9	189 ± 6
24DMP	200–2000	236 ± 5	1106 ± 982	0.999	30	0.3	193 ± 4
4C3MP	200–2000	147 ± 3	835 ± 286	0.996	18	0.2	215 ± 6
24DCP	150–1500	164 ± 3	644 ± 947	0.998	39	0.4	177 ± 4
PCP	200–2000	57 ± 1	–979 ± 780	0.996	66	0.7	110 ± 3
246TCP	150–1500	96 ± 2	–421 ± 1070	0.995	55	0.6	151 ± 3

^a For 25 mL of standard mixtures.^b For sample preconcentration volume of 100 mL.^c For sample preconcentration volume of 50 mL. n. a., not available.

umes of standard, resulting in a dynamic calibration range [25,26]. Besides using a single or a small number of standard mixtures to calibrate the system, it was also possible to adjust the loaded volume to the concentration of the target analytes for each sample.

Calibration curves (Table 3) were established by extracting 25 mL of a set of standard mixtures containing a defined amount of each compound. The linear dynamic range varied from 150–1500 ng (24DCP and 246TCP) to 350–3500 ng (24DNP and 46DNOC).

The LOD was calculated as the mass of compound corresponding to the interception plus three times $S_{y/x}$ [27]. The values obtained varied between 18 ng for 4C3MP and 207 ng for 24DNP. Considering the maximum volume of sample used in this work (100 mL) LOD values ≤ 1 ng mL⁻¹ was attained for nine of the eleven target analytes (Table 3).

The enrichment factor for each analyte was calculated by the ratio between the slope of the calibration curves obtained after SPE and the slope of the calibration curve obtained after direct injection of the analytes into the chromatographic system [28]. For the pre-concentrated volume of 50 mL the average EF was 176 and only for 24DNP and PCP the value was lower than 150 (Table 3). Higher values of EF would be easily attained by increasing the sample volume used in the extraction step.

The time required for a complete analytical cycle included the time necessary for performing the SPE protocol followed by the chromatographic run. As these two steps were performed in parallel, the total analysis time was defined by the SPE procedure when the sample volume was higher than 19.2 mL (144 s in step 3, Table 2) or by the chromatographic run (that takes about 5 min) for lower

sample volumes. For screening purposes at low $\mu\text{g L}^{-1}$ concentration levels, sample volumes between 25 and 100 mL must be applied, thus sample throughput was limited by the SPE protocol. This procedure took between 6 (for 25 mL of sample) and 15 min (for 100 mL of sample), hence the determination frequency was 4–10 h⁻¹.

Regardless of the sample volume processed, it should be emphasized that a reduced amount of reagents and solvents were necessary to perform the sample preparation. For each SPE procedure only 4.1 mL of methanol and 5 mL of HCl 0.010 mol L⁻¹ were required. Furthermore, the sorbent was reutilized up to 100 times, without loss of performance.

The accuracy and repeatability of the MSFIA-SPE method were assessed by analysing spiked water samples from different sources and certified reference material. The samples (MilliQ water, mineral water, tap water and sea water) were spiked with a mixture of certified standard solutions. An amount of 500 ng of each compound was added to 50 mL (10 ng mL⁻¹) and to 100 mL (5 ng mL⁻¹). The results provided by the MSFIA-SPE method expressed as percentage of recovery are listed in Table 4. Mean recoveries between 89 and 103% were obtained for the different samples and more than 90% of the determinations presented a recovery between 85 and 110%. The results demonstrated that the performance was not affected by the saline content or the ionic strength of the sample due to the similar results for the different matrices analyzed. Moreover, the similarity founded in the results obtained using different sample volumes indicated that no breakthrough occurred up to 100 mL. Repeatability was estimated by calculating RSD of 5 consecutive

Table 4Values of percentage of recovery obtained for the preconcentration of 50 and 100 mL of spiked water sample containing 500 ng of each phenolic compound. The listed values correspond to a mean of five determinations $\pm$ standard deviation.

Compound	Milli Q water		Mineral water		Tap water		Sea water	
	50 mL	100 mL	50 mL	100 mL	50 mL	100 mL	50 mL	100 mL
24DNP	90 ± 4	87 ± 5	83 ± 7	86 ± 6	n. a.	n. a.	107 ± 4	98 ± 3
46DNOC	119 ± 9	117 ± 5	93 ± 10	94 ± 6	99 ± 5	108 ± 3	124 ± 5	118 ± 5
P	96 ± 4	82 ± 3	96 ± 10	96 ± 9	88 ± 2	84 ± 5	95 ± 3	87 ± 3
4NP	98 ± 3	90 ± 5	88 ± 10	93 ± 7	85 ± 6	88 ± 4	100 ± 3	92 ± 3
2CP	91 ± 3	84 ± 3	90 ± 8	95 ± 7	86 ± 4	90 ± 5	93 ± 4	89 ± 4
2NP	112 ± 8	106 ± 5	90 ± 9	95 ± 7	88 ± 3	93 ± 1	97 ± 3	91 ± 5
24DMP	100 ± 5	93 ± 5	91 ± 10	94 ± 6	n. a.	n. a.	107 ± 4	99 ± 4
4C3MP	118 ± 8	106 ± 6	102 ± 11	114 ± 9	89 ± 6	95 ± 4	109 ± 4	105 ± 5
24DCP	112 ± 7	103 ± 4	90 ± 8	99 ± 6	89 ± 5	97 ± 4	96 ± 4	93 ± 5
PCP	86 ± 6	93 ± 2	n. a.	n. a.	n. a.	n. a.	94 ± 8	94 ± 5
246TCP	110 ± 9	126 ± 9	86 ± 10	108 ± 10	88 ± 4	103 ± 9	116 ± 10	122 ± 3
Mean recovery	103	99	91	98	89	95	103	99

n. a., not available.

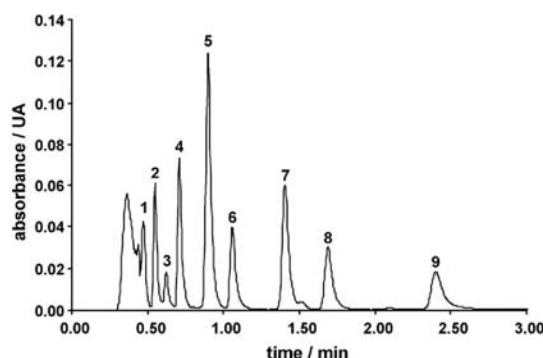


Fig. 3. Chromatogram obtained for aqueous extract of soil sample RTC-CRM 112. Sample volume = 4 mL (1) 24DNP, (2) 46DNOC, (3) P, (4) 4NP, (5) 2CP, (6) 2NP, (7) 4C3MP, (8) 24DCP, (9) PCP.

determinations of each spiked sample. For all determined analytes a RSD $\leq 8\%$ was obtained.

Concerning the analysis of reference standard material, values of 106 ± 5 , 94 ± 4 , 45 ± 2 and 67 ± 4 ng mL⁻¹ were attained for 24DNP, P, 2CP and 24DCP in sample RTC-QCI-032 (sample volume = 10 mL). The total content of phenolics was 312 ± 8 ng mL⁻¹, which is in agreement with the gravimetric certified value of 310 ng mL⁻¹. For sample U-QCI-076, values of 77 ± 3 , 54 ± 3 , 114 ± 5 and 55 ± 2 ng mL⁻¹ were attained for 24DNP, P, 2CP and 24DCP (sample volume = 7 mL). The total phenolic content was 300 ± 6 ng mL⁻¹, which is also in agreement with the certified value (309 ± 3 ng mL⁻¹) and within the advisory range for this sample (138 – 340 ng mL⁻¹). Finally, an aqueous extract of a certified soil sample (RTC-CRM 112), contaminated with phenols from a wood treatment site, was also analyzed (Fig. 3). The phenolic compounds present in the soil were quantified (Table 5) and results within the certified confidence interval were obtained for 4NP, 2NP and PCP. Values lower (P, 4C3MP, 24DCP) or higher (46DNOC, 2CP) than the confidence limits were also obtained, probably due to the different extraction conditions applied here, where water was used instead of an organic solvent.

3.4. Comparison to previous techniques

Compared to previous work for fast screening of phenolics [14], the system proposed here is faster, requiring at most 15 min for sample extraction compared to the 200 min reported before. Furthermore, the automatic SPE protocol developed here was directly coupled to the LC determination, making the whole procedure less dependent of the operator when compared to other methods [29].

Table 5

Results obtained for the analysis of a certified soil sample (RTC-CRM 112), based on its aqueous extract.

Compound	Present method/mg kg ⁻¹	Reference value/mg kg ⁻¹	Confidence interval/mg kg ⁻¹
24DNP	4.62 ± 0.19	n. a.	n. a.
46DNOC	7.49 ± 0.29	4.75	2.35–7.15
P	1.25 ± 0.06	2.45	1.35–3.55
4NP	7.08 ± 0.21	5.66	2.56–8.76
2CP	4.62 ± 0.17	2.38	1.35–3.41
2NP	2.87 ± 0.13	4.33	2.40–6.27
4C3MP	2.34 ± 0.12	4.94	3.04–6.84
24DCP	1.53 ± 0.07	2.53	1.69–3.37
PCP	4.48 ± 0.12	5.05	2.12–7.98

n. a.: not available.

Other chromatographic methods hyphenated to automatic on-line SPE for the determination of EPA priority phenolic pollutants based on the “column switching” approach have also been proposed [30–34]. When compared with the present work, these methods have the inconvenient of requiring high-pressure devices and gradient elution in the chromatographic run to prevent peak broadening. Some attempts to circumvent the major drawbacks of these systems were made, using Supercritical Fluid Chromatography [31] to reduce the chromatographic run time or by performing the derivatization with an ion-pair reagent before the SPE step [30] to increase the breakthrough volume of the more polar compounds. However, the time necessary for the complete analytical procedure was about 30 min in all cases. In the present work the determination rate was enhanced up to 5 fold and this is a worthwhile aspect due to the possibility of applying this method on screening analysis. Moreover, the proposed method presents improved or similar analytical characteristics concerning the application range and limit of detection. Considering the organic solvent consumed and the effluent produced in the sample preparation step, the proposed MSFIA-SPE system is a “greener” tool, with lower values for both of them. Regarding other automatic strategies proposed for the same determination and not based on SPE hyphenated schemes, [6,35] the present method is undoubtedly more suitable for screening purposes, attaining also a higher determination frequency.

4. Conclusions

In conclusion, this is first time that a hyphenated methodology comprising the repeatable flow conditions of MSFIA systems for SPE and the high throughput offered by monolithic columns is described. Thus, a fast and reliable tool for water quality monitoring and pollutant screening is available with improved performance when compared to the conventional “off-line” and “on-line” solid-phase extraction of phenolic compounds followed by liquid chromatographic determination. This was accomplished due to the high enrichment factors attained for phenol and ten of its derivatives and the low LOD values achieved (<1 ng mL⁻¹ for 100 mL of sample). Finally, the applicability of the proposed system as a screening tool was corroborated by the recovery values attained (89–103%) when different water matrices were processed. Good results were obtained for an aqueous extract from contaminated soil, indicating the suitability for determination in soil lixiviates.

Acknowledgements

Hugo M. Oliveira thanks Fundação para a Ciência e Tecnologia (FCT) and FSE (III Quadro Comunitário) for the PhD grant SFRH/BD/22494/2005. This work was partially supported by Spanish Ministry of Education (MEC) through project CTQ2007-64331 (Plan Nacional de Ciencias y Tecnologías Químicas).

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5

**On-line renewable solid-phase extraction
hyphenated to liquid chromatography for the
determination of UV filters using bead
injection and multisyringe-lab-on-valve
approach**



On-line renewable solid-phase extraction hyphenated to liquid chromatography for the determination of UV filters using bead injection and multisyringe-lab-on-valve approach

Hugo M. Oliveira^a, Marcela A. Segundo^{a,*}, José L.F.C. Lima^a, Manuel Miró^b, Victor Cerdà^b

^a REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal

^b Department of Chemistry, Faculty of Sciences, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Illes Balears, Spain

ARTICLE INFO

Article history:

Received 27 October 2009

Received in revised form 12 March 2010

Accepted 23 March 2010

Available online 27 March 2010

Keywords:

UV filters

Automation

On-line solid-phase extraction

Lab-on-valve

Bead injection

ABSTRACT

For the first time, an automatic sample pre-treatment/detection method is proposed for the multiclass determination of UV filters (namely, benzophenone-3, ethylhexylmethoxycinnamate, butylmethoxydibenzoylmethane and homosalate) in environmental samples. The new methodology comprises in-line solid-phase extraction (SPE) of the target analytes by exploiting the bead injection (BI) concept in a mesofluidic lab-on-valve (LOV) format, with subsequent determination by liquid chromatography (LC). The proposed microanalytical system, using a multisyringe burette as propulsion unit, automatically performed the overall SPE steps, including the renewal of the sorbent in each analytical cycle to prevent sample cross-contamination and the post-extraction adjustment of the eluate composition to prevent chromatographic band broadening effects. In order to expedite the LC separation, a C₁₈ monolithic column was applied and an accelerated isocratic elution was carried out by using a cationic surfactant as mobile phase additive. The LOV-BI-LC method was proven reliable for handling and analysis of complex matrices, e.g., spiked swimming pool water and seawater, with limits of detection ranging between 0.45 and 3.2 µg L⁻¹ for 9 mL sample volume. Linear calibration was attained up to 160 µg L⁻¹ for homosalate and up to 35 µg L⁻¹ for the other target analytes, with good reproducibility (RSD < 13%, for 5 different SPE columns). The hyphenated scheme is able to process a given sample simultaneously and within the same time frame than the chromatographic separation/determination of the formerly pre-treated sample, providing concentration values every 9 min. Hence, the sample throughput was enhanced up to 33 times when compared with previously reported off-line SPE methods. A drastic reduction in reagent consumption and effluent production was also attained, contributing to the development of an environment-friendly analyzer.

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1. Introduction

Organic UV filters comprise a wide range of chemical compounds that have absorptive properties of the solar radiation. The growing concern about the pathological effects of sun exposure, namely skin cancer, originated a widespread use of these compounds not only in sunscreen products but also in a wide range of personal care products used daily by millions of people. Despite being present in cosmetics at low concentrations, usually comprised between 0.1 and 10%, their physical and chemical properties, associated with the high potential for input into surface waters, introduced an additional concern about their eventual accumulation and toxicological effects [1]. Therefore, UV filters are presently

considered contaminants of emerging concern [2,3] and reviews focused on this novel problem have been recently published [1,4,5].

Although the quantification of UV filters in cosmetics is well established [6], the determination in environmental and biological matrices is complex, because analyte enrichment and matrix clean-up are necessary to detect the analytes at trace levels [7]. Efforts have been made in order to achieve sensitive and selective methods to cover a wide range of target analytes, combining manual sample preparation with chromatographic separations. For sample preparation, liquid-liquid extraction [8], cloud-point extraction [9], solid-phase extraction (SPE) [10–14], solid-phase micro extraction (SPME) [15,16] and stir bar sorptive extraction (SBSE) [17,18] were proposed, followed either by gas chromatography (GC) coupled to mass-spectrometry (MS) [9,10,12–18] or by liquid chromatography coupled to diode array (DAD) [9,10] or MS [11,12] detection. Actually, these methods allowed the detection of UV-sunscreen chemicals at µg L⁻¹ or ng L⁻¹ levels but at the same time they are time-consuming and labour intensive. Moreover, the information

* Corresponding author. Tel.: +351 222078994; fax: +351 222078961.
E-mail address: msegundo@ff.up.pt (M.A. Segundo).

about the presence of UV filters in surface waters or biological fluids is scarce and the analytical methods reported so far are not amenable to use in monitoring protocols. Hence, automation through on-line coupling of extraction procedures to chromatography would provide a suitable tool for environmental research [19].

In this context, straightforward and cost-effective methodologies can be implemented by using flow injection analysis and related techniques prior to column separation systems [20]. The progress in the miniaturization resulted in the introduction of the third generation of flow injection analysis, the so-called lab-on-valve (LOV) [21]. Besides the downscaling in the amount of reagents used, the microchannels engraved on the LOV module allowed the accurate handling of bead material in a fully automatic fashion, defined as bead injection (BI) [22]. This mesofluidic technique was applied to sorptive beads, allowing the implementation of new microSPE protocols for a wide range of analytes [22,23]. The hyphenation of BI to LC has been merely reported once for multiresidue determination of pharmaceutical compounds using a particulate LC column [24]. In this case, the BI-LOV approach was associated to the multisyringe flow injection (MSFI) analysis [25,26], providing a multi-channel propulsion unit, which can handle different solutions and solvents simultaneously at the low μL level [26,27].

The objective of the present work was the development and validation of an MSFI-BI-LOV system for on-line coupling of microSPE with LC for multiclass determination of UV filters in environmental samples. The target analytes were chosen in order to represent four different families of the most used UV-sunscreen agents, that is benzophenones (benzophenone-3, BP3), dibenzoylmethanes (butylmethoxydibenzoylmethane, BMDDBM), cinnamates (ethylhexylmethoxycinnamate, EHMC) and salicylates (homosalate, HMS). Due to the high range of polarity of these families of compounds (Electronic Supplementary material, Table S1), it would not be possible to attain a good resolution and a short analysis time by resorting to previously described reversed-phase chromatographic methods [9,10]. Hence, we propose for the first time an ultra fast mesofluidic LOV-BI method coupled to fast monolithic C_{18} column separation in surfactant media for automatic determination of sunscreen agents in different matrices.

2. Material and methods

2.1. Reagents and solutions

Ultra pure water (resistivity $>18\text{ M}\Omega\text{ cm}^{-1}$) obtained from a MilliQ (Millipore, Bedford, MA, USA) system was used for the preparation of all aqueous solutions. Methanolic solutions were prepared with HPLC grade methanol (Merck, Darmstadt, Germany). A 95% (v/v) MeOH/ H_2O was used as eluent and solution for sorbent conditioning. All glassware was periodically washed with methanol at least once a week.

Two reversed-phase co-polymeric bead sorbents with hydrophobic/hydrophilic moiety balance and of spherical shape were used: Oasis HLB (30 μm , Waters, Milford, MA, USA), and Focus (44 μm , Varian, Palo, CA, USA). Bead suspensions were prepared by mixing approximately 200 mg of sorbent with 2 mL of ethanol.

The stock standard solutions of UV-sunscreen chemicals, benzophenone-3 (BP3), butylmethoxydibenzoylmethane (BMDDBM), ethylhexylmethoxycinnamate (EHMC) and homosalate (HMS) (Merck), were prepared by accurately weighing the appropriate mass of solid or liquid and making it to a final concentration of 500 mg L^{-1} with methanol. These solutions were stored at 4°C in the darkness. Working standard solutions were prepared by

diluting the stock solution with 10 mmol L^{-1} HCl (for preconcentration) or pure methanol (for experiments with direct injection). The same procedure was adopted for benzylcinnamate (BZC) and dibenzoylmethane (DBM) (Sigma–Aldrich, St. Louis, MO, USA), both used as internal standard for chromatographic analysis.

For the chromatographic assays, sodium dodecyl sulfate (SDS), cetyltrimethylammonium chloride (CTAC) and dioctyl sulfosuccinate (DOSS) (Sigma–Aldrich) were used as mobile phase additives. Solutions with concentrations in the range of 25–100 mmol L^{-1} were prepared by dissolving the appropriate amount of SDS or DOSS in water or by dilution of a commercial solution of 25% (w/v) CTAC. Acetonitrile (ACN) (Merck) was employed as organic modifier of the mobile phase. All mobile phase components were filtered through a 0.45 μm filter and degassed before use by ultrasound radiation during 15 min.

2.2. Chromatographic analysis

The chromatographic assays were performed on a Merck/Hitachi liquid chromatography equipment (LaChrom 7000 series, Hitachi Ltd., Tokyo, Japan), composed of an interface (D-7000), a high-pressure pump (L-7455) and a diode array detector (L-7100). Reversed-phase separation was performed in the isocratic mode with a mobile phase containing 65% of ACN and 35% of aqueous solution of 100 mmol L^{-1} CTAC. The analytical column consisted of a Chromolith RP-18e (100 $\text{mm} \times 4.6\text{ mm i.d.}$) connected to a guard column of the same material (5 $\text{mm} \times 4.6\text{ mm i.d.}$) both from Merck. A Rheodyne (Rohnert Park, CA, USA) 7725i six-port rotary valve equipped with a 400 μL loop (i.d. 0.75 mm) was employed as interface between the flow system and the LC equipment. All the chromatographic data were automatically recorded by resorting to D-7000 software. For comparison purposes, two conventional 5 μm C_{18} -particulate columns with dimensions of 150 $\text{mm} \times 3.9\text{ mm i.d.}$ (XTerra, Waters) and 250 $\text{mm} \times 4.6\text{ mm i.d.}$ (Allosphere, Alltech, Deerfield, IL, USA) were evaluated.

For each sample, retention time, spectra, and peak area at maximum absorbance wavelength (see Table S1, Electronic Supplementary material) were compared with those obtained from standards, in order to identify and quantify the target analytes. BP3, BMDDBM, EHMC, and HMS were quantified at 290, 360, 305, and 235 nm, respectively. DBM (55 $\mu\text{g L}^{-1}$) was used as internal standard for sample analysis, except for swimming pool water where degradation by free chlorine was detected. For this type of sample, BZC was added as internal standard at the same concentration level. These compounds were detected at 345 and 280 nm, respectively. Limits of detection (LOD) and quantification (LOQ) were estimated from the analysis of untreated water as the minimum concentration of a given species for which the signal-to-noise ratios are ≥ 3 and 10, respectively [24].

2.3. Flow manifold

The proposed flow system for the preconcentration, separation and determination of UV filters is depicted in Fig. 1. It comprises a multisyringe burette (Crison Instruments, Allela, Spain) as propulsion unit equipped with two syringes (Hamilton, Bonaduz, Switzerland) of 5000 μL (syringes S1 and S2) and one of 2500 μL (syringe S3). A three-way commutation valve (NRResearch, Caldwell, NJ, USA) was placed at the head of each syringe, allowing the access to the solution reservoir (position off) or the flow network (position on). The multisyringe module was connected to a lab-on-valve unit made of poly(methylmethacrylate) (PMMA) containing 8 peripheral ports (with channels of 1.5 mm i.d.), which was mounted on the head of an eight-port multi-position selection valve (MPV, Crison Instruments). The central port was connected

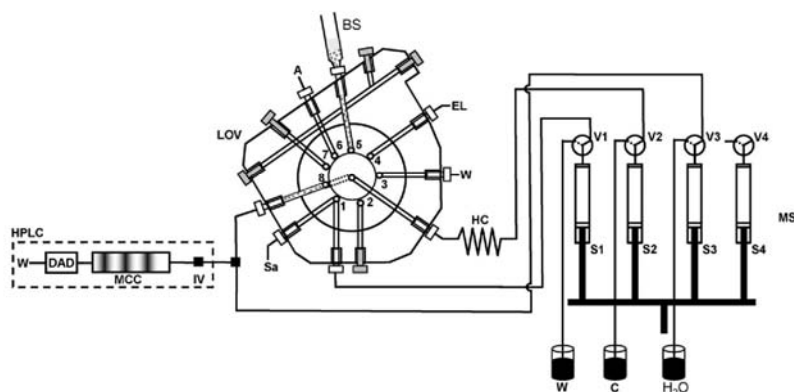


Fig. 1. Representation of the MSFI-BI-LOV system hyphenated to liquid chromatography for the determination of UV filters. MS: multisyringe; V_i: three-way commutation valve; S_i: syringe; C, carrier solution (10 mmol L⁻¹ HCl); EL, eluent (95% v/v, MeOH/H₂O); Sa, sample or standard solution; BS, bead suspension; W, waste; LOV, lab-on-valve mounted on 8-port multi-position valve; HC, holding coil; A, air; HPLC, liquid chromatograph; MCC, monolithic chromatographic column; IV, high-pressure injection valve; DAD, diode array detector. For simplicity, the HPLC high-pressure pump was not illustrated. Closed ports at LOV module are represented by grey fittings.

to syringe S2 and also to any of the eight ports of the MPV, one at a time, allowing the aspiration of the sample (port 1), eluent (port 4), bead suspension (port 5) or air (port 6) into the holding coil (HC). In order to hold the beads into the cavity of channel 8, a 1-mm thick polypropylene frit with pore diameter of 10 µm (MoBiTec, Goettingen, Germany, ref. #2210) was placed between the exit of the channel and the fitting. The MPV channel 3 was used as waste. The special configuration of the dual channel 1 (Fig. 1), one of them being connected to syringe S1, enabled the exchange of samples or standards without aspiration of the solution into the HC, minimizing the risk of carryover between consecutive solutions loaded into the system. The manifold was built with 0.8 mm i.d. polytetrafluorethylene tubing (Omnifit, Cambridge, UK). The exception was the holding coil, with 5 mL of capacity, and the connections between syringes and reservoir flasks, which were built with 1.5 mm i.d. tubing. A three-way confluence, built in PMMA, was incorporated into the manifold, connecting both MPV channel 8 and syringe S3 to the injection valve (IV) of the LC equipment. The tubing length between MPV and the confluence was 8 cm while the connection between the confluence and the injection valve was 44 cm long.

The analytical procedure was executed and controlled by personal computer running software written in-house using Quick Basic 4.5 (Microsoft, Redmond, WA, USA). The software was designed to control the position of commutation valves, the speed and direction of piston movement on the multisyringe apparatus, and also the selection of ports on the MPV.

2.4. Protocol sequence

Automatic SPE and chromatographic analysis were fully synchronized, that is, one sample was processed by the chromatograph, while the microSPE procedure was applied in parallel to the next sample. The automatic SPE procedure comprised the four main steps designated as (1) packing and conditioning of the extraction microcolumn, (2) sample loading, (3) elution and filling of the injection valve loop and (4) bead removal (see Electronic Supplementary material, Table S2). An empty SPE cartridge of 1 mL of capacity was mounted onto the port 5 of LOV by using a polyetheretherketone (PEEK) connector. Before beginning the analytical procedure, the cartridge was filled with an ethanolic suspension of beads. When the beads settled at the bottom of channel connecting to port 5, the system was ready for operation.

A brief description of the automatic MSFI-BI-LOV method is given below:

- (1) **Packing and conditioning of the extraction column.** After filling S2 with 1320 µL of carrier (10 mmol L⁻¹ HCl) and the HC with 650 µL of 95% (v/v) MeOH/H₂O, 30 µL of bead suspension was collected through port 5 at a flow rate of 0.3 mL min⁻¹. The sorbent was packed within the cavities of LOV by forward flow pumping of the beads plus methanolic solution. The conditioning procedure was completed by perfusing 1000 µL of extra acidic carrier over the sorbent bed at a higher flow rate.
- (2) **Sample loading.** When new sample is to be analyzed, the connection tubing between LOV port 1 and the sample container was washed by aspirating 500 µL of liquid by S1 through the outlet of port 1. Thereafter, S2 performed the sequential aspiration of an air segment (250 µL) and sample (4000 µL). Next, 3000 µL of HC content were dispensed through port 8, the sample perfused through the sorbent and the target analytes were retained. Discarding the remaining sample and the air plug, and the washing of HC as well were ensured by pumping 4750 µL of carrier to waste (port 3). These operational steps were repeated up to 3 times, resulting in 9 mL as total volume of loaded sample. The sample loading step was finished by removing the non-retained species with 750 µL of carrier. Simultaneously, 375 µL of water were fed into the LC injection loop to avoid contamination by sample dispersion into the tubing connecting the confluence point to S3.
- (3) **Elution of analytes.** First, an air segment (200 µL) was aspirated through port 6 to prevent the dispersion of the eluent into the carrier solution, followed by the aspiration of 700 µL of 95% (v/v) MeOH/H₂O. After refilling the syringes and reversing the direction of piston movement, the elution was performed by propelling a nominal volume of 600 µL of 95% (v/v) MeOH/H₂O at 0.75 mL min⁻¹ through the sorbent bed, which were subsequently merged with 300 µL of water (nominal) at the T junction (Fig. 1). The mixture that filled the loop of the injection valve (267 µL of eluate + 133 µL of water) was immediately injected into the chromatographic system, whereupon the analytical run was started. Thereafter, the air and remaining 95% (v/v) MeOH/H₂O were removed from the HC towards waste by 1650 µL of carrier. Finally, the rotary valve was switched to the "load" position.

Table 1
Retention factors (k') for different CTAC concentrations using mobile phases with 70 or 65% of acetonitrile.

Mobile phase composition (ACN:aqueous surfactant)	CTAC concentration (mmol L ⁻¹)	Retention factor			
		BP3	BMDBM	EHMC	HMS
70:30	0	0.99	5.90	6.20	8.20
	5	0.92	5.59	5.85	7.81
	25	0.83	4.98	5.25	7.08
	100	0.51	3.25	3.56	5.02
65:35	0	1.32	9.30	9.80	12.60
	5	1.20	8.47	8.84	11.61
	25	1.10	7.46	7.86	10.47
	100	0.64	4.28	4.73	6.74

(4) *Discarding of beads.* The packed SPE microcolumn was wetted with 20 μ L of 95% (v/v) MeOH/H₂O whereupon the used beads were aspirated back into HC. The former column content was backflushed and disposed to waste (port 3) with carrier solution (1350 μ L) followed by rinsing of the tubing connecting LOV (port 8) with the injection valve (1250 μ L carrier). After this step, the system was ready for processing the next sample.

2.5. Samples

Bathing water samples, namely swimming pool water (SPW) and seawater (SW), were collected from a public swimming pool and Matosinhos beach in Porto (Portugal), respectively. To simulate water contamination from personal care products during recreational bathing activities (especially of children in small swimming pools), a volunteer applied a commercial sunscreen preparation and immersed his feet and ankles into an inflatable child swimming pool containing 10 L of seawater for a period of 30 min, after which a sample was collected. After collection, all samples were immediately acidified to pH 2 and filtered through 0.22 μ m membrane filter (Millipore). The free chlorine content of SPW samples was determined by resorting to the standard method based on N,N diethyl-*p*-phenylene-diamine [28].

3. Results and discussion

3.1. Chromatographic method

Considering that screening methods should be fast, chromatographic assays of UV filters should be tailored to take the lowest time possible. To this end, we evaluated the usage of a monolithic column under isocratic elution conditions for separation. It should be borne in mind that isocratic separations do not require equilibration of the column after the analytical run and that the implementation of monolithic columns fosters a significant decrease in running time when compared with conventional particulate columns [29].

Isocratic LC methods reported in the literature [9,10] included the addition of surfactant molecules to the mobile phase for acceleration of the chromatographic runs as a result of the so-called "gradient effect", where the most hydrophobic molecules are shifted to decreased retention times [30]. This strategy has been successfully applied using reversed-phase (RP) packed columns. However, to the best of our knowledge, this effect has not been exploited in RP separations using monolithic columns. Hence, the influence of a cationic (CTAC) and two anionic (SDS and DOSS) surfactants in the retention of the target compounds was evaluated. The flow rate was fixed at 1.5 mL min⁻¹ and 20 μ L of a standard solution containing 5 mg L⁻¹ of each compound was injected into the LC system. Preliminary experiments were performed using mobile phase containing 80% acetonitrile:20% aqueous surfactant

solution. However, the resolution attained was poor for BMDMB and EHMC. Hence, the organic modifier content was decreased to 60–70% (v/v) using an aqueous solution containing 100 mmol L⁻¹ of surfactant. The retention was evaluated by calculating the capacity factor (k') [30] for each analyte (Electronic Supplementary material, Fig. S1).

The experimental results evidenced a decrease in retention times for the suite of analytes tested when compared to the values obtained for mobile phase without surfactant. Considering the concentration of surfactant (100 mmol L⁻¹) and the content of organic modifier (60–70%, v/v), the chromatographic separation takes place in conditions that can be defined as "high submicellar" [31]. In this case, the stationary phase is partially coated with surfactant molecules, where the hydrophobic tail is associated to the alkyl chains of the stationary phase and the polar head groups are oriented away from the surface, that is, the C₁₈ column becomes eventually more hydrophilic. Furthermore, as micelle existence is unlikely at acetonitrile contents above 30%, surfactant monomers which compete with the stationary phase for the uptake of the target compounds will prevail in the bulk mobile phase [32], decreasing the retention time of the most hydrophobic compounds.

The reduction of k' was dependent on the additive and followed the order CTAC > DOSS > SDS. In fact, when comparing k' in the absence of surfactant a decrease between 41 and 60% was verified for k' upon the usage of CTAC regardless of the ACN/surfactant proportion. For DOSS and SDS the decrease was smaller (27–53% and 20–44%, respectively). Considering the absence of amine moieties and the presence of oxygen containing groups (carbonyl and hydroxyl) in the target analytes, the electrostatic interactions between the analytes and the cationic surfactant in solution would most likely explain the experimental results.

The effect of the concentration of CTAC on the retention times was also assessed. Because of the fact that the lowest k' was obtained with mobile phases of less water content (Electronic Supplementary material, Fig. S1) and that the higher the water content the better was the peak resolution, mobile phases containing either 30 or 35% of aqueous CTAC were tested (Table 1). The k' for all UV filters decreased by 25–30% when using 70% ACN compared to 65% ACN. The same trend was observed upon increasing CTAC concentration, showing a decrease of k' around 50% at 100 mmol L⁻¹ CTAC in relation to that of the mobile phase without surfactant. Hence, a mobile phase containing 65:35 (v/v) ACN/100 mmol L⁻¹ CTAC was selected as a compromise between run time and resolution. Using these conditions, BP3, BMDMB, EHMC, and HMS were quantified at 290, 360, 305, and 235 nm, respectively (see Electronic Supplementary Information Fig. S2). Though BMDMB and EHMC peaks eluted at close retention times, the detection at different wavelengths allowed their correct quantification.

The performance of the monolithic column was compared to that of conventional 5 μ m C₁₈-particulate columns (column A, 250 mm $\times$ 4.6 mm i.d., and column B, 150 mm $\times$ 3.9 mm i.d.). Calibration curves were established by injecting standard solutions

containing 2–10 mg L⁻¹. The mobile phase composition was 65:35 (v/v) ACN/CTAC, except for the larger column, where the organic content was increased up to 80% following the recommendations by Giokas et al. [10]. The mobile phase was pumped at a flow rate of 1.5 mL min⁻¹ for the monolithic column and column B or at 1 mL min⁻¹ for column A. Monolithic column and column B showed a similar performance in terms of parameters of the calibration curves, with a maximum slope variation of 10%. On the other hand, the sensitivity of the assays was better with column A, with differences between 20 and 30% for BMDMB, EHMC and HMS, and 40% for BP3. The improvement in the separation efficiency was a consequence of the larger dimensions of this column [29]. Nevertheless, concerning run time and system backpressure, the monolithic column fostered the separation of target compounds in 9 min with a backpressure of 23 bar, in contrast with 12 min/160 bar of column B and 34 min/105 bar of column A. By the same token, the flow rate of the mobile phase in monolithic column separation can be in principle increased without loss of performance [33]. Actually, flow rates up to 3 mL min⁻¹ were tested in this work, which gave rise to the decrease of run times up to 4.5 min with no significant deterioration of the separation efficiency and the operation of the chromatograph at a backpressure <50 bar. Hence, the usage of the monolithic column was proven particularly suited to the development of a screening method for monitoring purposes.

3.2. Design of the MSFI-BI-LOV-LC system

The implementation of microscale SPE based on the bead injection concept using the LOV format requires a precise control of volume delivering and flow rates applied, in order to attain excellent performance in all the steps of sample preparation, especially for bead trapping, sample handling and elution of analytes. These features can be provided by the MSFI module which might be furnished with syringes of varied volumes, adapted to the operational steps of the analytical procedure. In the present work, we exploited this option by using one main syringe (S2) for handling of fluids from and to the HC (bead suspension, sample, eluent and carrier) and two auxiliary syringes that performed the clean-up of the sample channel (S1) and the adjustment of the eluent composition (S3) before injection into the LC.

An important modification of previously described BI setups [24,34] was the mode of trapping the beads into the LOV microchannels. Usually, one or two pieces of PEEK tubing are placed inside the engraved LOV channels [24,34]. This strategy reduces the channel diameter and traps the beads for SPE. However, it also promotes a significant increase in backpressure, limiting the perfusion flow rates through the packed microcolumn [24]. In the present work, we replaced the PEEK stopper by a polypropylene frit (Fig. 1, port 8). Hence, an effective barrier for holding the beads without clogging the channel was available, fostering the implementation of ultra fast percolating schemes, as performed in this work, where flow rates up to 8 mL min⁻¹ were used during the sample loading step. Additionally, by removing the piece of PEEK tubing, an extra portion of sorbent can be loaded, increasing the sorbent capacity available for analyte enrichment compared to earlier reported LOV-BI analyzers [34–36].

As to the choice of sorbent material for microSPE of UV filters, two factors were considered. First, it should possess reversed-phase sorptive capacity in a broad spectrum of polarity (log *K_{ow}* between 3.79 and 6.16 for the target analytes). Second, the physical characteristics of beads are also relevant for adequate handling within the LOV platform, requiring primarily spherical format, uniformity in size distribution and water-wettable properties. Two commercial available sorbents fulfilling these requirements were tested: Oasis HLB, a divinylbenzene-co-N-vinylpyrrolidone poly-

mer and Focus, a patented product described as a polar enhanced styrene-divinylbenzene resin. For evaluation of their performance, the most polar analyte (BP3) was used. In both sorbents, readily and reproducibly in-valve column formation and withdrawal was accomplished and a maximum breakthrough volume of 24 mL was achieved when a standard solution containing 3.0 mg L⁻¹ of BP3 was percolated through the SPE microcolumn with constant monitoring of the effluent with a UV detector. The loading flow rate was varied within the range 3.0–8.0 mL min⁻¹ for the preconcentration of 10 mL of 0.25 mg L⁻¹ BP3 using the two sorbents and no flow backpressure was detected in either of the flow rates assayed. Nevertheless, the repeatability, measured as relative standard deviation (RSD) for five consecutive analysis of 10 mL of 0.25 mg L⁻¹ BP3 at 8.0 mL min⁻¹ (using the same SPE column) was <3% for Oasis HLB and <8% for Focus. Hence, Oasis HLB was chosen for further experiments.

Considering the weakly acidic character of BP3, BMDMB and HMS and the reversed-phase mechanism of interaction with the SPE sorbent, 10 mmol L⁻¹ HCl was used as carrier and sample medium for the preparation of the overall standard solutions. The same held true for the real samples. The elution of the compounds from Oasis HLB bed was carried out with a mixture of 95:5 (v/v) MeOH/H₂O. This eluent was chosen attending its capability to promote the desorption of the analytes, the negligible bubble evolution within the flow network as a result of the addition of a low percentage of water and the chemical compatibility with the material (PMMA) from which the LOV piece was built. However, band broadening of the most polar UV filters was detected whenever 200 µL of eluate were injected in the chromatograph. This situation is commonly observed in on-line SPE when the eluent composition is significantly different from that of the mobile phase of the chromatographic separation [37]. To circumvent this problem, the multi-propulsion capability of the MSFI system was exploited for in-line dilution of the eluate with water (2:1), thereby providing a chemical composition similar to that of the mobile phase (namely, 65:35, v/v, ACN/aqueous phase). Dilution took place immediately before the injection valve loop by simultaneous activation of syringe S2 and S3. A “heart-cut” mode for injection of the eluate into the LC was utilized. Hence, the volume of the injection valve loop was increased to 400 µL, leading to enhanced method sensitivity. The nominal volume of 95% (v/v) MeOH/H₂O solution feeding the SPE microcolumn was adjusted to maximize peak areas taking into account the dead volume between the LOV apparatus and the injection valve loop. It has been proven that ≥414 µL of 95% (v/v) MeOH/H₂O (actual volume, propelled by S2) should be delivered to fill the injection valve loop with 400 µL of solution (267 µL of eluate + 133 µL of water added from S3). Nominal volumes of 95% (v/v) MeOH/H₂O solution ranging between 500 and 750 µL were tested, with maximum responses for the overall compounds at 600 µL. For all compounds, except HMS, peak areas were 75, 88, 95, 85, and 70% of that attained for 600 µL, considering nominal volumes of 500, 550, 650, 700 and 750 µL, respectively. The elution profile for HMS was however sharper as evidenced by peak areas that were 84, 84, 83, 70, and 52% of the maximum value (600 µL), considering the nominal volumes mentioned above.

The total time required for performing the automatic SPE protocol was 9 min, using a sample volume of 9 mL. Hence, the mobile phase flow rate for the chromatographic separation was fixed to 1.5 mL min⁻¹ to synchronize sample preparation with chromatographic analysis.

3.3. Analytical performance of the MSFI-BI-LOV hyphenated to LC

The analytical performance of the proposed methodology was assessed taking into account the dynamic linear range, breakthrough volume, enhancement factor (EF), and precision (Table 2).

Table 2

Analytical performance of the MSFI-BI-LOV method coupled to LC for determination of UV-sunscreen compounds.

Compound	Linear range (ng) ^a	Sensitivity (L µg ⁻¹) area units ^{b,c}	Breakthrough volume (mL)	Enhancement factor ^b	Reproducibility (% RSD, 5 columns, n = 4)
BP3	60–480	2895 ± 68	24	140 ± 7	2.5
BMDBM	60–420	1455 ± 39	12	51 ± 3	13
EHMC	60–420	2044 ± 55	12	63 ± 2	12
HMS	240–1920	1825 ± 38	12	140 ± 5	2.1

^a R² > 0.995.^b Calculated for a sample volume of 12 mL.^c Using 4 standards, 3 replicates (n = 12).

The use of a time-based strategy for introducing the sample into the manifold [38] allowed the implementation of mass-based calibration curves [39,40], by plotting the peak area or relative peak area against the mass of compound perfused through the SPE microcolumn. Thus, the sample volume to be processed can be adjusted according to the concentration of the analytes expected in the different real-life matrices. Calibration curves, with correlation coefficients above 0.995, were obtained by extracting amounts between 60 and 480 ng of each compound contained in a volume up to 12 mL, except for HMS. For this compound, the linear range comprised 240–1920 ng because the sensitivity of the chromatographic assay was lower than that of the other UV filters. In fact, using mobile phase as solvent, the molar absorptivity of HMS at $\lambda = 235$ nm was 0.0333 cm⁻¹ mg⁻¹ L⁻¹, which is, for example, ca. 40% the value attained for EHMC at $\lambda = 305$ nm (namely, 0.0800 cm⁻¹ mg⁻¹ L⁻¹).

Mass-based calibration was also used for the determination of analyte breakthrough volumes via the preconcentration of variable volumes over the range of 3–24 mL of a standard mixture containing 20 µg L⁻¹ of BP3 and BMDMB, 9 µg L⁻¹ of EHMC and 80 µg L⁻¹ of HMS. A multi-standard was applied in order to simulate the conditions that would be found in samples, where several UV filters may be present simultaneously. The combination of volumes and analyte concentrations provided mass values that were within the linear range previously established by a fixed-volume calibration using 9 mL of standards with different concentrations. Hence, by plotting the peak area against the volume percolated through the microcolumn (which is directly proportional to the mass of analyte), it was possible to identify the volume at which loss of linearity of the analytical signal occurred. From this volume and onwards the loaded analytes were pre-eluted from the LOV microcolumn by the standard/sample solvent itself. For all compounds, excepting BP3, the breakthrough volume was 12 mL. In the case of BP3, no breakthrough was observed up to the maximum volume loaded (24 mL), which is in agreement with the former results obtained by frontal analysis during the sorbent selection experiment. These results may also seem puzzling at a first glance because the more polar analyte (BP3) presented the highest breakthrough volume. However, it should be pointed out that the concentration applied for each target compound was not the same. In fact, for the same volume percolated through the sorbent, the mass of HMS (less polar analyte) was 4 times higher than that of BP3 (more polar analyte). Hence, at the breakthrough volumes established, the mass of HMS (present in 12 mL) was twice the mass of BP3 (present in 24 mL). Concerning the other two analytes (BMDMB and EHMC), the concentrations applied were closer to that of BP3. The better retention of BP3 could be explained by electrostatic interactions between the more polar analyte and the hydrophilic monomer of N-vinyl-pyrrolidone that appears at high percentages in Oasis HLB, conferring a mix-mode character to this sorbent.

The enhancement factor [41] was calculated as the ratio between the slopes of the calibration curves constructed by subjecting the sample to the BI protocol and direct injection (20 µL)

of the compounds into the chromatographic system. This factor represents the improvement in sensitivity that was attained by combining the on-line SPE procedure with the injection of a larger volume, which is only possible due to the on-line adjustment of eluate composition. Furthermore, as a heart-cut approach was adopted, it was not possible to calculate the enrichment factor, as only part of the eluate was introduced into the loop of the injection valve. The enhancement factors obtained using a sample volume of 12 mL were between 51 and 140 (see Table 2). The 3-fold lower enhancement factors obtained for BP3 and HMS when compared to those of the BMDMB and EHMC might be a consequence of the larger molecular size of the latter two molecules. Differences in sorption distribution over the SPE column are expected to yield different elution profiles, which would affect the amount of analyte introduced into the LC via the “heart-cut” injection mode.

The repeatability and reproducibility of the LOV-BI method were calculated as the % RSD of four replicate injections of 9 mL mixture containing 20 µg L⁻¹ of BP3, BMDMB and EHMC, and 90 µg L⁻¹ of HMS, using either a permanent SPE sorbent or five renewable microcolumns, respectively. Using a reusable packed microcolumn, the RSD values ranged from 17 to 29%, while RSDs for renewable sorbent microcolumns comprised the range of 2–13%. In the first case, the high RSDs were attributed to sample carryover effects. The strong adsorption of the most hydrophobic UV filters (log *K_{ow}* > 4) onto polymeric materials has been reported earlier by Rodil et al. [11]. The disposable SPE mode implemented here overcomes this problem by offering automatically a new sorbent aliquot for each individual sample and replicates to be processed. The better RSDs for BP3 and HMS (see Table 2) are attributed to a more reproducible and efficient “heart-cut” injection for both compounds, which concurs with the data of enhancement factors given above.

3.4. Application to environmental samples

Bathing waters (seawater (SW) and swimming pool water (SPW)), which represent the most important pathways of direct introduction of UV filters in the aquatic ecosystems, were processed by the proposed methodology for reliability and accuracy assessment.

As to swimming pool water samples, UV filters are usually not detected or detected at very low concentrations due to the reaction with free chlorine in the sample, which has an important role on the degradation of these compounds [42]. Hence, in order to evaluate the influence of Cl₂ in the assays, SPW samples were fortified at different concentration levels of the target analytes and free chlorine as well, and immediately processed by the MSFI-BI-LOV mesofluidic setup (Table 3; Electronic Supplementary material, Fig. S3). Recoveries between 86 and 112% were obtained for all compounds in the raw sample, where the Cl₂ content was <0.05 mg L⁻¹, excepting BMDMB, for which recovery was 55% at the lowest spiked concentration. When Cl₂ concentration was increased to 0.1 mg L⁻¹, recoveries for both BP3 and BMDMB were <51% but ≥96% for the highest concentration level of EHMC and both con-

Table 3

Concentrations of UV filters ($\mu\text{g L}^{-1}$) found in swimming pool water^a containing different levels of free chlorine. Limits of detection (LOD) and quantification (LOQ) for this type of sample are also listed.

	BP3 ^b		BMDMB ^b		EHMC ^b		HMS ^b	
Spike level ($\mu\text{g L}^{-1}$)	17.6	35.2	14.5	29.1	15.5	31.1	68.2	136.5
SPW ($<0.05 \text{ mg L}^{-1} \text{ Cl}_2$)	15.1 ± 1.6	37.0 ± 3.1	7.90 ± 0.04	26.5 ± 3.4	13.6 ± 0.8	n.a.	73.4 ± 3.4	151.2 ± 6.4
SPW ^c ($0.1 \text{ mg L}^{-1} \text{ Cl}_2$)	7.1 ± 0.5	17.9 ± 2.8	<LOD	12.5 ± 1.8	<LOD	29.8 ± 6.5	77.7 ± 4.1	161.9 ± 7.2
SPW ^c ($0.5 \text{ mg L}^{-1} \text{ Cl}_2$)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	71.4 ± 4.8	146.7 ± 7.8
LOD ^a ($\mu\text{g L}^{-1}$)		0.81		0.45		0.90		3.2
LOQ ^a ($\mu\text{g L}^{-1}$)		2.7		1.5		3.0		10.6

^a Sample volume = 9 mL, $n = 3$.

^b Values calculated by external calibration.

^c Samples were immediately processed after addition of free chlorine.

n.a., not available.

concentrations of HMS. At a concentration level of $0.5 \text{ mg L}^{-1} \text{ Cl}_2$, a complete degradation of the compounds was observed, with the exception of HMS that was still detected in the sample with recoveries as high as 104–107%. This experiment was also performed with concentrations of 1.0 and $2.0 \text{ mg L}^{-1} \text{ Cl}_2$ and, at both levels, the overall target compounds were quantitatively degraded. These results are in agreement with previously reported data [42] that indicate the fast formation of halogenated by-products in the presence of free chlorine for some UV filters, e.g., BP3. Although it was spiked at a rather high concentration level, the elevated tolerance of HMS to oxidation must be emphasized. This fact, associated to its low polarity ($\log K_{ow} = 6.15$) reveals the potentially high persistence of this UV-sunscreen agent in the aquatic environment.

Limits of detection (LOD) between 0.45 and $3.2 \mu\text{g L}^{-1}$, and limits of quantification (LOQ) within the range 1.5 – $10.6 \mu\text{g L}^{-1}$ were attained for the SPW sample whenever a sample volume of 9 mL was processed (Table 3). The reproducibility, expressed as RSD, was $<15\%$ ($n = 5$) for the suite of SPW samples utilizing the bead injection mode for microscale SPE.

Concerning seawater samples, the average recovery percentage at the same spike levels than those used in SPW samples was 104% with deviations $<16\%$ (Electronic Supplementary material, Table S3). Thus, the high concentration levels of salts in seawater did not pose any problem for accurate measurements.

Aimed at evaluating the putative migration of UV filters from sunscreen lotions during recreational bathing, a sample aliquot was collected 30 min after immersion of a skin area where a commercially available sunscreen preparation had been applied. The experiment was performed in a child swimming pool containing 10 L of seawater. BMDMB and EHMC, which were labelled as ingredients of the sunscreen lotion, were found in the bathing water sample (290 ± 16 and $198 \pm 12 \mu\text{g L}^{-1}$, respectively). For quality control purposes, the sample was also spiked with 19.4 ng mL^{-1} of each of these two analytes (Fig. 2). An average recovery of $101 \pm 2\%$ was found for both UV filters with RSDs $<5\%$ ($n = 5$). LODs and LOQs for BMDMB and EHMC were 1.0 and $3.3 \mu\text{g L}^{-1}$, and 0.7 and $2.4 \mu\text{g L}^{-1}$, respectively. These results indicate the suitability of the proposed methodology for monitoring the eventual migration of UV filters to bathing waters.

3.5. Comparison with previously described methods

Previously reported methods for the determination of UV filters in environmental matrices involved LC–UV [9,10], LC–MS [11,12], or GC–MS [9,10,12–18] following extraction/preconcentration of the target analytes. The figures of merit (linear range, precision, LOD and LOQ) in the proposed MSFI-BI-LOV mesofluidic system are in general terms comparable with those of the reported LC–UV methods [9,10] and also with a GC–MS method [15]. As expected, concentration values for linear working range, LOD and

LOQ were higher than those reported in LC–MS methods [11,12]. These methods however require prior manual sample preparation based on exhaustive (SPE) or non-exhaustive analyte enrichment via SPME or SBSE. In many instances, multiple-step separation procedures comprising the conditioning of the sorbent material, sample loading, matrix removal followed by elution, evaporation and reconstitution of the extract are called for. These are time-consuming tasks that may take altogether more than 100 min per sample. Moreover, a long drying step is often mandatory and, for MS detection, a derivatization protocol has proven necessary to improve the performance of the method [11]. In contrast, the automatic microSPE procedure presented here did not take more than 9 min for the preconcentration of 9 mL of sample, providing enrichment factors similar, and in some cases better, than those encountered when extracting up to 200 mL of sample [11]. As a result of downscaling the SPE procedure, waste disposal and both solvent and sorbent consumption were minimized. Regardless the sample volume, 11.1 mL of effluent were generated and merely 1.55 mL of methanol were used. In contrast to earlier SPE methods, a decrease of 60% in waste generation and more than 90% in solvent consumption was accomplished in the LOV-BI system. A 10-fold saving in the amount of sorbent was also obtained with respect to a batchwise SPE procedure prior to LC–MS, where 60 mg Oasis HLB cartridges were employed [11].

Non-exhaustive enrichment techniques, such as SPME and SBSE [15–18], feature the minimization or even avoidance of solvents. However, large sample equilibration times within the

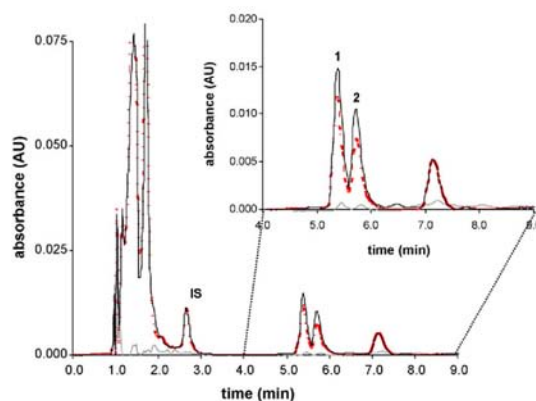


Fig. 2. Chromatograms recorded in the analysis of seawater following topical application of a sunscreen preparation and immersion of the test site for 30 min (dotted line), sample blank (grey solid line) and spiked sample (black solid line). The sample volume processed was 3 mL. (IS) Internal standard, DBM ($\lambda = 345 \text{ nm}$); (1) BMDMB ($\lambda = 360 \text{ nm}$); (2) EHMC ($\lambda = 305 \text{ nm}$).

range of 45–300 min are frequently called for to warrant appropriate method sensitivity. This leads to a 5–33-fold lower sample throughput than that of the proposed LOV-BI method, making them inappropriate for fast screening of UV filters in environmental samples.

4. Conclusions

For the first time, an automatic flow-based analytical procedure hyphenating sample pre-treatment and chromatographic determination is proposed for fast quantification of UV filters in environmental samples. The novel methodology capitalizes on the combination between flexibility, miniaturization and simplification in sample preparation integrated within the LOV mesofluidic platform and the high throughput of the chromatographic assays supported on the use of a monolithic column. This leads to cost-effective analytical procedures in terms of labour and reagent expenses, contributing to the development of miniaturized robust methods with “Green Chemistry” credentials.

The proposed methodology was successfully applied to screening of four analytes in spiked samples of recreational waters (swimming pool and seawater), chosen as model compounds representative of different UV filter families (benzophenone, dibenzoylmethane, cinnamate and salicylate). In this context, it should be emphasized that the UV–vis detection system employed here does not allow discrimination between structurally similar compounds, such as the following pairs: HMS/ethylhexyl salicylate, EHMC/isoamyl methoxycinnamate and BP3/benzophenone-1. The individual quantification of these compounds could be attained by using MS detectors or by adequate tailoring of the chromatographic separation.

Finally, considering the results obtained for the fast interaction between the UV filters tested and Cl_2 , future work should be focused on studying the kinetics and products involved in such reactions, which would provide better understanding about UV filters’ fate in the environment.

Acknowledgements

This work was financially supported by FCT through project PTDC/AAC-AMB/104882/2008, by Spanish Ministry of Education and Science through project CTQ2007-64331, and by Integrated Action n° E-48/09 and HP2008-0045. Hugo M. Oliveira thanks Fundação para a Ciência e Tecnologia (FCT) and FSE (III Quadro Comunitário) for the Ph.D. grant SFRH/BD/22494/2005.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.03.035.

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Electronic Supplementary information

On-line renewable solid phase extraction hyphenated to liquid chromatography for the determination of UV filters using bead injection and multisyringe-lab-on-valve approach

Hugo M. Oliveira⁽¹⁾, Marcela A. Segundo^{(1)*}, José L. F. C. Lima⁽¹⁾, Manuel Miró⁽²⁾, and Victor Cerdà⁽²⁾

⁽¹⁾ *REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal*

⁽²⁾ *Department of Chemistry, Faculty of Sciences, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Illes Balears, Spain*

*Corresponding author. Tel: +351 222078994. Fax: +351 222078961. E-mail: msegundo@ff.up.pt

Table S1. Structure, molecular weight, log K_{ow} , pKa, and maximum absorbance wavelength of organic UV filters analyzed.

Table S2. Protocol sequence for the determination of UV filters in environmental and biological samples.

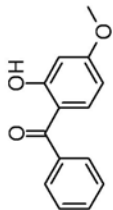
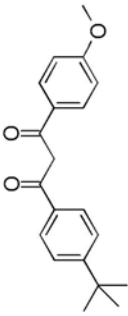
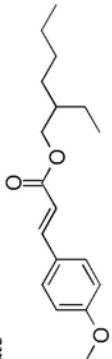
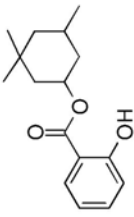
Table S3. Concentration of target UV filters ($\mu\text{g L}^{-1}$) found in spiked seawater samples.

Fig. S1. Influence of different surfactants and mobile phase composition (acetonitrile:aqueous component (water or surfactant solution at the 100 mmol L^{-1} level) (v/v)) on retention factor (k') of target UV filters

Fig. S2. Chromatogram recorded in the analysis of a standard solution prepared in methanol containing 6.0 mg L^{-1} of each analyte. For the same run, data was acquired at different wavelengths according to the target compound. Injection volume = 20 μL .

Fig. S3. Chromatograms recorded in the analysis of swimming pool water.

Table S1. Structure, molecular weight, log K_{ow} , pK_a , and maximum absorbance wavelength of organic UV filters analyzed.

compound	structure	molecular weight (Da)	log K_{ow}^a	pK_a	λ_{max} (nm)
Benzophenone-3 (BP3)		228.24	3.79	7.56	290
Butylmethoxydibenzoyl methane (BMDBM)		310.39	4.51	9.74	360
Ethylhexylmethoxycinnamate (EHMC)		290.4	5.80	---	305
Homosalate (HMS)		262.35	6.16	8.09	235

^a from Rodil et al. [11]

Table S2. Protocol sequence for the determination of UV filters in environmental and biological samples.

Description	S2	S3	S1	vol ^a (μL)	flow rate ^a (mL min ⁻¹)	LOV (position)	HPLC IV (position)
1. Packing and conditioning of extraction column							
a) filling syringes with solutions	off/aspirate	*	*	1320	15	1	load
b) aspiration of conditioning solution into HC	on/aspirate	*	*	650	5	4	load
c) collection of beads	on/aspirate	*	*	30	0.3	5	load
d) in-valve formation and rinsing of SPE microcolumn	on/dispense	*	*	1000	1.5	8	load
e) conditioning of SPE microcolumn	on/dispense	*	*	1000	6	8	load
2. Sample loading							
a) washing of sample tubing	*	*	on/aspirate	500 (S1)	3 (S1)	--	load
Start loop:							
b) aspiration of air into HC	on/aspirate	*	*	250	4	6	load
c) aspiration of sample	on/aspirate	*	*	4000	5	1	load
d) sample loading	on/dispense	*	*	3000	8	8	load
e) refilling of syringes	off/aspirate	*	*	3500	15	--	load
f) elimination of air/sample and washing HC	on/dispense	*	*	4750	15	3	load
End loop: repeat 3 times							
g) refilling of syringes	off/aspirate	*	*	750	15	--	load
h) matrix removal and washing of tubing connecting S3 to IV	on/dispense	on/dispense	*	750 (S2)	8 (S2)	8	load
				375 (S3)	4 (S3)		

Table S2. Protocol sequence for the determination of UV filters in environmental and biological samples (continuation).

Description	S2	S3	S1	vol ^a (μL)	flow rate ^a (mL min ⁻¹)	LOV (position)	HPLC IV (position)
3. Elution of analytes							
a) refilling of syringes	off/aspirate	*	*	950	15	--	load
b) aspiration of air into HC	on/aspirate	*	*	200	5	6	load
c) aspiration of 95% (v/v) MeOH/H ₂ O	on/aspirate	*	*	700	4	4	load
d) reversion of piston direction	off/dispense	*	*	100	10	3	load
e) elution and filling of the injection loop	on/dispense	on/dispense	*	600 (S2) 300 (S3)	0.75 (S2) 0.375 (S3)	8	load
f) eluate injection and activation of LC equipment	--	--	--	--	--	--	inject
g) elimination of air and washing of HC	on/dispense	*	*	1650	10	3	inject
h) switching of IV to load position	--	--	--	--	--	--	load
4. Discard of beads							
a) aspiration of carrier	off/aspirate	*	*	2170	15	--	load
b) aspiration of 95% (v/v) MeOH/H ₂ O	on/aspirate	*	*	200	6	4	load
c) wetting beads with eluent	on/dispense	*	*	20	6	8	load
d) aspiration of beads back to HC	on/aspirate	*	*	250	2	8	load
e) withdrawal of used beads	on/dispense	*	*	1350	10	3	load
e) washing of tubing connecting LOV to IV	on/dispense	*	*	1250	8	3	load

* the direction of displacement is the same for all syringes, with commutation valves in position off.

^a volume and flow rate refer to syringe S2, except when value is followed by (Si).

Table S3. Concentration of target UV filters ($\mu\text{g L}^{-1}$) found in spiked seawater samples.

compound	concentration spiked	concentration found ^a
BP3	17.6	15.1 $\pm$ 1.4
	35.2	32.1 $\pm$ 5.8
BMDBM	14.5	14.6 $\pm$ 1.8
	29.1	35.6 $\pm$ 2.7
EHMC	15.5	17.9 $\pm$ 1.1
	31.1	33.7 $\pm$ 4.3
HMS	68.2	70.7 $\pm$ 2.3
	136.5	147 $\pm$ 11

^a average value $\pm$ standard deviation ($n = 3$).

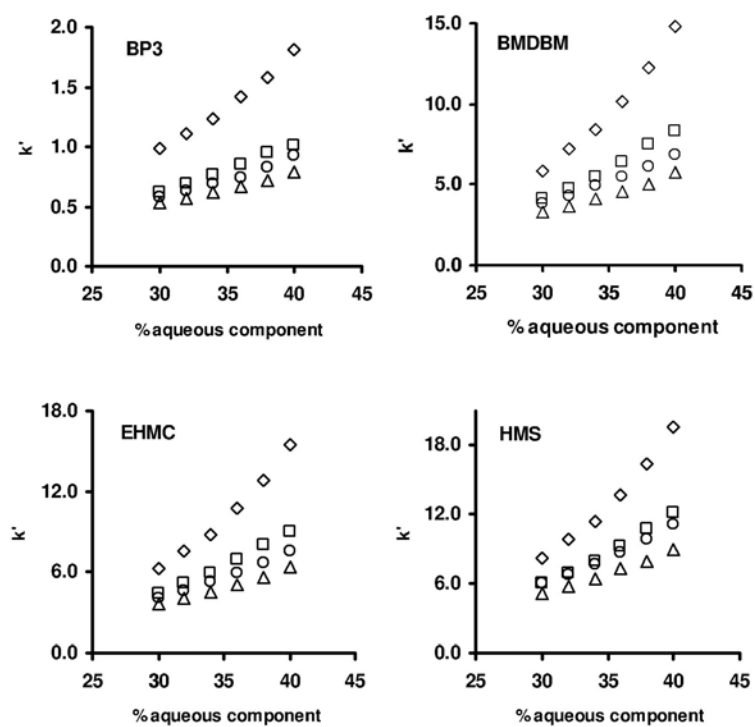


Fig. S1. Influence of different surfactants and mobile phase composition (acetonitrile:aqueous component (water or surfactant solution at the 100 mmol L⁻¹ level) (v/v)) on retention factor (k') of target UV filters. $\diamond$, water; $\square$, SDS; $\circ$, DOSS; Δ , CTAC.

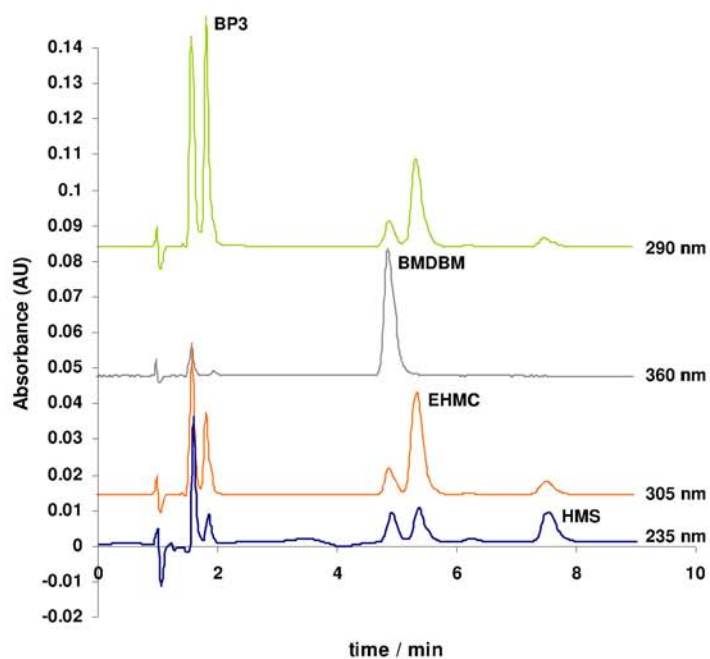


Fig. S2. Chromatogram recorded in the analysis of a standard solution prepared in methanol containing 6.0 mg L^{-1} of each analyte. For the same run, data was acquired at different wavelengths according to the target compound. Injection volume = $20 \text{ }\mu\text{L}$.

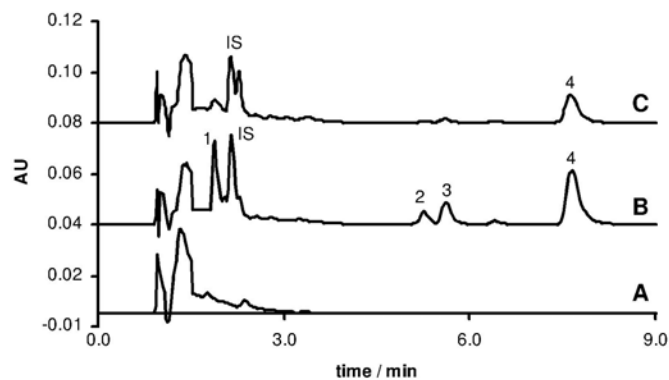


Fig. S3. Chromatograms recorded in the analysis of swimming pool water. A, sample containing $[\text{Cl}_2] \leq 0.05 \text{ mg L}^{-1}$; B, sample A spiked with UV filters (Table 4, highest spike level); C, sample containing $[\text{Cl}_2] = 0.5 \text{ mg L}^{-1}$ spiked with UV filters (Table 4, lowest spike level). Peak labels: 1, BP3; 2, BMDDBM; 3, EHMC 4, HMS; IS, internal standard (BZC).

6

**Universal approach for mesofluidic handling
of bead suspensions in lab-on-valve format**

Manuscript submitted for publication

Universal approach for mesofluidic handling of bead suspensions in lab-on-valve format

Hugo M. Oliveira¹, Manuel Miró², Marcela A. Segundo^{1,*}, José L. F. C. Lima¹

¹ *REQUIMTE, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal*

² *Department of Chemistry, Faculty of Sciences, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Illes Balears, Spain*

*Corresponding author. Tel: +351 222078994. Fax: +351 222078961. E-mail: msegundo@ff.up.pt

ABSTRACT

In the present report, new protocols are introduced for the mesofluidic handling of irregularly-shaped and non-uniformed sized bead suspensions under the lab-on-valve (LOV) format. To this end, two alternative strategies comprising the direct aspiration of beads suspended in methanol from the container or only after a resuspension step, and subsequently packed in a LOV microchannel, were evaluated and discussed. Moreover, for direct aspiration, homogeneous suspensions of the materials were prepared by increasing the viscosity of the suspension milieu with 75% (w/w) glycerol. The bead injection protocols were applied to four reversed-phase sorbent materials with different sorptive surfaces: Oasis HLB, SupelMIP β -receptors, Lichrolut EN and Discovery DSC-MCAX, and the mass of sorbent packed in each microcolumn was assessed. For the direct aspiration of methanolic solutions, lump formation and clogging of the channels were observed, resulting in a source of results with poor precision (RSD 3.9 – 68.2%). The use of glycerolic suspensions was effective for repeatable packing of Oasis HLB and SupelMIP β -receptors, though for the other two sorbents bead deposition was observed along the time. The resuspension strategy was able to handle all the materials tested with acceptable precision (RSD 1.5 – 13.9%). Furthermore, when the sorbent bed was restricted to the volume of the LOV microchannel an enhanced precision was attained (RSD < 4%). For both strategies, different volumes of suspension aiming a target mass of sorbent of 10 mg were successfully handled (RSD 3.1 – 13.9%), showing the robustness of the technique at different bead suspension volumes. The proposed bead handling protocols were applied to the analytical micro solid-phase extraction of propranolol from aqueous solutions by SupelMIP β -receptors and Discovery DSC-MCAX with high precision (RSD < 6%) and recoveries between 69 and 74%.

Keywords: lab-on-valve, bead injection, solid-phase extraction, automation

INTRODUCTION

In the recent years, flow injection analysis and related techniques have been a tool for new developments in the analytical chemistry field [1,2]. In this context, the handling of solid suspensions in a fully automatic fashion, where the solid-phase (in form of micrometric beads) could be renewed for each analytical cycle, and defined as bead injection (BI) [3], emerged as a disruptive concept in the automation of chemical assays and sample preparation [4]. In BI, the solid-phase suspended in a given solvent is a dynamic part of the system that is treated as a homogeneous solution, in a deep contrast with the classical use of solid-phase reactors in flow analysis, where the packed microcolumn is viewed as a permanent part of the manifold that should be replaced occasionally. This concept stated a new era for the automation based on flow analysis, making possible the development of new methodologies based in solid-liquid interaction that overcome the decrease in performance caused by surface deterioration along the time. Furthermore, BI allows the simultaneous monitoring of both effluent and solid phase in real time, which definitely enhances the analytical information provided in each single assay. The development of BI technique is associated with the introduction of programmable flow; firstly by sequential injection analysis (SIA) [5], where flow cells with special configurations as jet-ring cell [3,6] and magnetic flow-through cell [7-9] were assembled to the manifold, and more recently, with the introduction of lab-on-valve (LOV), the so-called third generation of flow injection analysis [10].

As a result of the progress of SIA technique towards miniaturization, the LOV module comprises a monolithic structure with microconduits machined in a polymethylmethacrylate or polyetherimide block, which is mounted atop a

multiposition valve. Due to the mesofluidic scale of the assays, the integrated detection and compatibility with real-world samples [11], LOV is becoming an attractive analytical tool, with a particular impact in the bioanalytical field [12,13]. The open architecture and simple design, associated with the flexibility provided by programmable flow operation, transformed LOV in the preferential way to develop new BI protocols, though effective alternatives are also feasible [14-17]. Despite the wide range of BI-LOV applications reported, the use of renewable sorptive surfaces was particularly exploited for methods involving color development and spectrophotometric measurements [18,19], and also solid-phase extraction (SPE) of environmental samples [18,20]. The use of BI-LOV as front end to chromatographic techniques is also a current topic of growing interest [21-26].

For the implementation of BI protocols, sorptive materials with different physical-chemical properties have been used. The manipulation of bead suspensions is the major challenge in BI because it affects the reproducibility of the microcolumn insertion, and subsequently, the precision and accuracy of the method. Previous works about this topic [3,4,18,27] are consensual about the requirements that should be fulfilled by the bead materials in order to obtain homogeneous suspensions, usually prepared in an aqueous, hydro-alcoholic or alcoholic solvent [27]. Spherical shape, uniform size distribution and water-wettability (for reversed-phase materials) have been identified as imperative characteristics. By this way, the materials commonly used in BI have their backbone structures based on polystyrene-divinylbenzene (PS-DVB), polyvinylpyrrolidone, or agarose, which fulfill the described requirements. Nevertheless, even when spherical-shaped and uniform-sized materials were used, problems in handling bead suspension were still observed. When high density polytetrafluorethylene [28] or silica beads [29] were used, a correct bead aspiration into the LOV was not possible due to lump

formation into the LOV microchannel. This problem was overcome by promoting a continuous recirculation of the suspension by a peristaltic pump. Another strategy proposed for ensuring the permanent homogeneity of the suspension was the continuous stirring of the suspension reservoir [30]. Although both strategies provided repeatable packaging of sorbent into the LOV microchannel, they are associated with the use of additional instrumentation that increases the complexity and the cost of the manifold. Therefore, the current state-of-art of BI-LOV excludes a diversity of materials with physical-chemical properties able to extend the application range of this technique, particularly for the SPE of samples with high matrix complexity. Hence, the objective of the present work was the development of a universal BI-LOV approach, able to cope with non-spherical and non-uniformly size distributed beads applied routinely to solid phase extraction protocols. For this, different strategies for in-line bead suspension handling and column packing will be assessed regarding their precision through evaluation of the sorbent mass packed. Moreover, the impact of the proposed strategies on the analytical performance will be evaluated after automatic extraction and elution of a model analyte (propranolol), further quantified by HPLC.

2. EXPERIMENTAL

2.1 Reagents and solutions

The chemicals used were from analytical grade and used with no further purification. All aqueous solutions were prepared in ultra pure water (resistivity $> 18 \text{ M}\Omega \text{ cm}^{-1}$) obtained from a MilliQ (Millipore, Bedford, MA, USA) system. Methanol (MeOH) and acetonitrile (ACN) HPLC grade, supplied by Merck (Darmstadt, Germany) were also used as solvents.

Four different sorbents (Table 1) were tested: Oasis HLB (Waters, Milford, MA, USA), Lichrolut EN (Merck), SupelMIP β -receptors (Supelco, Bellefonte, PA, USA) and Discovery DSC-MCAX (Supelco). Methanolic and glycerolic sorbent suspensions were prepared by adding 1000 μL of MeOH or 200 μL of MeOH followed by 800 μL of 87.5% (w/w) aqueous glycerol (Sigma-Aldrich, St. Louis, MO, USA) to 100 mg of sorbent, respectively. For Discovery-MCAX, 200 mg mL^{-1} methanolic suspensions were also prepared.

The stock solution (500 mg L^{-1}) of propranolol (Sigma-Aldrich) was daily prepared by dissolving the appropriate amount of solid in 10.00 mL of MeOH. Working standards for direct injection into liquid chromatograph were prepared by diluting the stock solution in MeOH. For the SPE extraction procedure, a 2.00 mg L^{-1} propranolol solution was prepared using 5.0 mmol L^{-1} ammonium acetate, pH 6.7 or 2% (v/v) CH_3COOH (Sigma-Aldrich) as solvents. These solvents were previously prepared by dissolving solid ammonium acetate (Sigma-Aldrich) or diluting the solution of CH_3COOH (Sigma-Aldrich) in ultra pure water. The SPE eluents used were 1% (v/v) HCOOH in ACN (MIP β -receptors) and 5% (v/v) NH_4OH in MeOH (Discovery DSC-MCAX). Both mixtures were prepared by dissolution of the commercial solutions, 25% NH_4OH (Sigma-Aldrich) and 98% HCOOH (Sigma-Aldrich), in the respective organic solvents. For the chromatographic determination of propranolol, a mobile phase containing MeOH and 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma-Aldrich) in the proportion 50:50 was used. This solution was filtered through a 0.22 μm membrane and degassed by ultra sound irradiation during 15 minutes before use.

2.2 Lab-on-valve manifold

The flow system used for the mesofluidic handling of bead suspension and automatic SPE of propranolol (Fig. 1) comprised a BU4S multisyringe module (MS) (Crison Instruments, Allela, Spain), as propulsion unit equipped with two 2500 μL glass syringes (Hamilton, Bonaduz, Switzerland), labelled as S2 and S3. The access to the solutions reservoirs (position off) or LOV (position on) was controlled by the three-way commutation valves (NResearch, Caldwell, NJ, USA) placed at the head of each syringe. The propulsion unit was connected to a customized mesofluidic platform (Ideia.M, Porto, Portugal) containing a central channel and eight peripheral ports with channels of 1.5 mm i.d. engraved in a polyetherimide block (Fig. 1). This monolithic device was mounted atop of eight-port multi-position selection valve (MPV, Crison Instruments). The access to the complete array of peripheral ports, one at a time, was provided by the central channel (CC), which was connected to S3 by the holding coil (HC). For the bead suspensions handling experiments only S3 was used and ports 3, 6 and 7 were closed. During automatic SPE of propranolol, all ports were used and S2 was connected to the dual port 3, facilitating the sample exchange. The beads were retained at the exit of channel 1 by a 1 mm thick polyethylene frit with a pore diameter of 20 μm (Supelco). The bead container was placed in the exit of the channel 4 of the LOV (45° rotation). Polytetrafluorethylene tubing (Omnifit, Cambrigde, UK) with 0.8 mm i.d. was used to connect the LOV ports to the solutions reservoirs. The connections between the syringes and respective solutions reservoirs and the HC (5000 μL capacity) were made of 1.5 mm i.d. tubing of the same material.

All the programmable flow sequences were executed by a personal computer running lab-made software written in Quick Basic 4.5 (Microsoft, Redmond, WA, USA). The parameters controlled by the software through a RS232 interface were the direction

(aspiration / propulsion) and speed (flow rate) of fluid handling unit (multisyringe module), the position of commutation valves, and also the selection (one at a time) of the different ports on the MPV.

Table 1, here please

Fig. 1, here please

2.3 Strategies employed for mesofluidic handling of beads

Procedures commonly used for SPE under BI-LOV format comprise four main operations that can be defined as (1) sorbent packing and conditioning, (2) sample loading and matrix removal, (3) elution and (4) bead discarding. In the present work customized protocols for operation (1) were evaluated (Tables 2 and S1), followed by application of operation (4), aiming bead recollection for mass evaluation. For propranolol extraction, operations (2) and (3) were added (Table S2), in order to perform the retention and elution of the analyte. For sake of simplicity, the steps respecting to the refilling of S3 were omitted in the following description of the operating procedures.

Microcolumn packing and conditioning. Four different protocols (I – IV) for performing the aspiration of solid suspension into the LOV and packing the solid-phase microcolumn, based on two different strategies, were evaluated (Table 2). They were characterized by the direct aspiration of a methanolic (I) or glycerolic (II) bead suspension or by introducing a resuspension step before the aspiration into the LOV of a methanolic suspension (III and IV) resulting in a fluidized suspension before bead aspiration. In the last case (IV), the sorbent bed was physically limited by the volume of microchannel 1, after discarding the excess of beads previously aspirated. For all cases,

the flow rates for bead aspiration and column packing were fixed at 0.5 mL and 2.0 mL min⁻¹, respectively.

I) Direct aspiration of methanolic suspensions: 500 µL of MeOH and 125 µL of bead suspension were sequentially aspirated into the HC. Syringe 3 was refilled, and after flow reversal, the column was packed and conditioned by sending 625 µL through port 1.

II) Direct aspiration of glycerolic suspensions: after aspiration of 750 µL of water, 125 µL of glycerolic suspension were inserted into the HC and the column was packed by flow reversal. Next, a plug of 625 µL of MeOH was aspirated into the HC and the syringe was refilled with carrier. Subsequently, the microcolumn was packed and conditioned with these two solvents. When a sorbent mass of 10 mg was targeted, the sorbent suspension aspiration step was adjusted to 86 and 72 µL for Oasis HLB and MIP β-receptors, respectively. For the propranolol extraction with MIP β-receptors, the volumes were adjusted and extra conditioning steps were added (Table S3).

III) Aspiration of methanolic suspensions after a resuspension step: in this case, after the aspiration of 525 µL of MeOH and the repositioning of the piston, a burst of 125 µL was sent to the bead suspension reservoir. For a period of approximately 10 s, S3 was refilled while the beads were settling in the container placed at microchannel 4. Thereafter, 125 µL of sorbent were aspirated. Then, the flow direction was reversed and the microcolumn was packed and conditioned. For experiments for which the sorbent mass targeted was 10 mg, the volumes of suspension aspirated were modified to 58, 95, 75 and 34 µL, when Oasis HLB, MIP β-receptors, Lichrolut EN and Discovery DSC-MCAX were used.

IV) Aspiration of methanolic suspensions after a resuspension step with control of channel 1 filling: for this strategy only two extra steps were added to the previously

described strategy (III). After the microcolumn package and conditioning, S3 was refilled and the exceeding beads (remaining stored in the MPV rotor and central channel) were discarded by sending 750 μL of carrier through port 8.

Beads disposal: the sorbent packed inside the microchannel 1 was wetted with 100 μL of MeOH, previously stored inside the HC. Next, the beads were aspirated into the HC and immediately disposed into waste channel by 1275 μL of carrier solution that was collected into a 2 mL capacity vial for subsequent sorbent mass determination. Finally, the column cavity was filled with 200 μL of carrier solution.

2.4 Microcolumn weighing procedure

As mentioned above, the microcolumns packed by the different fluidic protocols were collected (after discarding procedure, Table S1) by placing 2 mL capacity glass vials (Supelco) in port 2 during step e (Table S1). The vials were previously cleaned by a N_2 stream and weighed in an analytical balance AG285 (Mettler-Toledo, Columbus, OH, USA). The liquid content of the vials was evaporated overnight by oven drying with temperatures between 80-90 $^{\circ}\text{C}$. After cooling, the vials were weighed again. The sorbent mass present in the microcolumn was calculated as the difference between the mass obtained for the vial containing the dried sorbent and for the mass of the same empty vial. For quality control purposes, control charts of the mass of the empty vials were constructed ($n > 10$) and outliers ($\text{SD} > 5\%$) were eliminated from the experiment.

2.5 Automatic SPE and chromatographic determination of propranolol

Solutions containing 2.00 mg L^{-1} of propranolol prepared in 5 mmol L^{-1} ammonium acetate, pH 6.7 or 2% (v/v) CH_3COOH were extracted by MIP β -receptors and Discovery DSC-MCAX sorbents, respectively. The automatic SPE protocol (Table S2),

summarized in Table 3, was based on the information endorsed by the sorbent suppliers. The eluate was diluted 1:1 with 0.1 (v/v) TFA before liquid chromatographic analysis. The chromatographic analysis of propranolol was performed on a liquid chromatography setup Merck / LaChrom 7000 series (Hitachi, Tokyo, Japan). It was composed by a high-pressure pump (L-7455), a detector (L-7100) and an interface (D-7000). The system control and data acquisition were performed by D-7000 software. The system was used in the isocratic mode of elution using MeOH containing 0.1% v/v of TFA at a flow rate of 1.50 mL min^{-1} , using a Chromolith RP-18e ($100 \text{ mm} \times 4.6 \text{ mm i.d.}$) connected to a guard column ($5 \times 4.6 \text{ mm i.d.}$) of the same material as stationary phase. The samples were manually injected through a Rheodyne 7725i injector (Rohert Park, CA, USA) equipped with a $50 \mu\text{L}$ loop. Retention time and spectra were used for propranolol identification. Peak area at the maximum absorbance wavelength (288 nm) was used as analytical signal. A linear response ($r^2 \geq 0.998$) was reported for calibration curves established in the range $0.25 - 1.50 \text{ mg L}^{-1}$.

Table 3, here please

3. RESULTS AND DISCUSSION

3.1 Mesofluidic-based strategies for handling bead suspensions

The sorbents used in the present work (Table 1) were chosen considering their physical-chemical properties and the potential application in reversed-phase SPE protocols under LOV format. Therefore, SupelMIP β -receptors, Lichrolut EN and Discovery DSC-MCAX were used. MIP β -receptors and Lichrolut EN are irregularly-shaped polymeric materials. The first is a molecularly-imprinted polymer for the selective extraction of

drugs with affinity for β -receptors and the second one is a PS-DVB sorbent with enhanced surface suitable for reversed-phase extraction of organic compounds at trace levels. Discovery DSC-MCAX, composed by functionalized silica-based non-spherical particles, is a mixed-mode solid-phase that retains molecules by hydrophobic interactions and cation-exchange mechanisms. For comparison purposes, Oasis HLB, a spherical polymeric sorbent previously used in BI-LOV protocols [22,25], was also applied. Considering the differences in the backbone structure, particle size and physical-chemical properties, these sorbents can be regarded as a relevant sample of commercially available products for reversed-phase SPE.

The sorbents studied were suspended using methanol or 75% (w/w) glycerol (glycerol/methanol/water) as solvent. These suspensions were handled by different flow programs that involved two different strategies (Table 2): *i*) the direct aspiration of the beads from the container or *ii*) the aspiration after a resuspension step, where a fluidized sorbent bed was generated. Concerning the amount of beads packed in each protocol sequence, two different approaches were tested. The first approach was the aspiration of a fixed volume of suspension from the container (125 μ L), while the second one was the aspiration of a variable volume corresponding to 10 mg of sorbent. By this way, it was possible to evaluate the precision of the mesofluidic column packing at different aspiration volumes.

3.1.1 Direct aspiration of the beads from methanolic and glycerolic suspensions

In BI-LOV methods described so far, the physical-chemical properties of bead materials were taken into consideration for choosing the suspension milieu used. Hence, the hydrophilic (polysaccharide-based materials) are generally suspended in aqueous [30,31] while hydrophobic (reversed-phased) materials are frequently suspended in alcoholic

solutions [21,25]. Considering the hydrophobic character of the sorbents used in this work, the first approach to the handling of the beads was the direct aspiration of suspensions prepared in 100% MeOH (Table S1). For the direct aspiration of 125 μ L of bead suspension, a lump formation was noticed when MIP β -receptors and Lichrolut EN were aspirated after 2 or 3 consecutive times from the container. This resulted in a poor precision in the sorbent packaging, with relative standard deviation values (RSD%) of 67.6 and 24.2%, respectively (Table 4). For the tested sorbents with more uniform size-distribution (Discovery DSC-MCAX and Oasis HLB), this strategy provided a better precision (7.9 and 3.8%).

Polysaccharide-based beads (Sephacrose and Sephadex), often used in BI-LOV protocols [4], are spherical and have a density similar to that of water. Thus, homogeneous suspensions could be prepared and high reproducible packaging into the LOV microchannels was achieved. In contrast, the reversed-phase sorbents used in this work, except for Oasis HLB, were non-spherical. Moreover, polysaccharide-based beads could have swelling properties and usually have higher density than the suspension solvent, settling at the bottom of the container. By this way, the introduction of the reversed-phase beads into the LOV manifold by a homogeneous suspension was tried. To this end, the viscosity of the suspension solvent was increased by the addition of glycerol. This alcohol was selected considering its high-viscosity and compatibility with both organic and aqueous solvents commonly used in solid-phase extraction procedures. Preliminary experiments were made preparing glycerol / water solutions in the range 50-75 % (w/w). Considering our previous knowledge on other MIP material [24], different suspensions containing 100 mg of MIP β -receptors per mL of solvent were prepared and its homogeneity was evaluated by visual inspection. The use of 75% (w/w) glycerol seemed to provide a homogeneous suspension. However, after processed by the

automatic system, the masses of sorbent obtained were not reproducible ($RSD\% > 20$), in disagreement with the visual result because there were still very small bead aggregates in the suspension. This source of impaired results was circumvented by wetting the beads with methanol before the addition of the glycerol solution, ensuring a subsequent homogenous solvation of the sorbent by the glycerol and water molecules. Thus, glycerolic suspensions were prepared by suspending 100 mg of sorbent in 200 μL of methanol followed by the addition of 800 μL of 87.5% (w/w) glycerol, resulting in the original 75% (w/w) glycerol in the final solvent composition. The four sorbents in test were suspended by this procedure and packed into LOV microchannel 1 by the fluidic protocol (II) described in Table S1. In this particular case, 125 μL of bead suspensions were collected and packed by the carrier solution. The volume for conditioning the beads was increased aiming a complete removal of the glycerol from the surface of the beads (Table S1). The sorbent mass weighed for Oasis HLB and MIP β -receptors (Table 4) deployed a precision suitable for the use in SPE protocols, with RSD values of 6.1 and 3.5%, respectively. On other hand, the Lichrolut EN and Discovery DSC-MCAX beads migrated along the time (< 10 min) either to the bottom or to the top of the suspension, respectively. This migration was observed by visual inspection and by the increase or decrease of the mass packed along the time (Fig. 2). Hence, for these two materials, 75 % (w/w) glycerol was not a suitable solvent to prepare homogenous suspensions.

For the aspiration of a lower volume of suspension containing c.a. 10 mg of sorbent (58 μL for Oasis HLB and 95 μL for MIP β -receptors), the RSD values obtained (4.7-6.9 %) were similar to those than obtained in the aspiration of 125 μL of glycerolic suspension (3.5-6.1 %) (Table 4), demonstrating the high precision of the propulsion unit and the robustness of programmed flow protocol.

Table 4, here please

Fig. 2, here please

3.1.2 Aspiration of beads in methanolic suspensions after a resuspension step

The second strategy tested in the present work was based in the introduction into the mesofluidic protocol sequence of a resuspension step of the bead suspension (Table 2) before collecting the beads. Taking into account that the beads were permanently settled at the bottom of the suspension, they were flushed by a burst of solvent (125 μ L) resulting in a dispersion of the particles along the solvent, followed by its resettlement for a period of 10 s. Since the collection of beads began before its complete settlement, the compactness associated with the mass of sorbent accumulated at the bottom of the container was prevented, and a homogeneous suspension could be aspirated through microchannel 4.

Thus, the four sorbents under evaluation were submitted to three different operating procedures that included a resuspension step with 125 μ L of methanol (Table S1, (III – IV)). The first two approaches included the aspiration of a fixed volume of 125 μ L for all sorbents or the aspiration of a variable volume, aiming a sorbent mass of 10 mg, as performed before for the direct aspiration of glycerolic suspensions. In the third approach, after aspirating 125 μ L of suspension, the beads that were over the volume capacity of microchannel 1 were discarded through channel 8, restricting the sorbent bed to the volume of the microchannel 1 cavity. In opposition to what happened in the direct aspiration of methanolic suspensions, the formation of lumps and clogging of the channels were not noticed in all resuspension approaches, which made feasible the packaging of all sorbents.

The precision of the packaging procedure, when fixed volume (125 μL) or fixed mass (10 mg, suspension volumes varying from 34 to 95 μL) experiments were carried out (Table 4), were similar in the range 6.0-10.4% and 3.1-13.8%, respectively. For the approach based on the restriction of the sorbent bed to the volume of microchannel 1, an enhanced precision for the sorbent packaging was observed, with RSD values comprised between 1.6 and 4.1%. These results were in agreement with our previous observations using a similar strategy [24], and provided the highest precision of all approaches evaluated. However, in this case, it is not possible to change the amount of beads loaded without a physical resizing of the LOV microchannel. A possible way of resizing the microchannel, without manufacturing a new device, would be the introduction of a portion of rigid polyetheretherketone (PEEK) tubing inside the channel, which may work simultaneously for decreasing the channel volume and also as a bead stopper. Notwithstanding, it should be considered that the use of PEEK tubing could also result in some restrictions upon the maximum flow rates allowed through the operation sequence as reported before [25].

3.2 Solid-phase extraction of propranolol

In order to assess the influence of the column packing protocol in the SPE procedure, standard solutions containing 2.00 mg L^{-1} of propranolol were extracted using either a polymeric (MIP β -receptors) or a silica-based (Discovery DSC-MCAX) sorbent, that were both able to extract this molecule from aqueous solutions. After extraction, the propranolol was quantified by liquid chromatography with diode-array detection. For both sorbents, the extractions were performed using either 10 mg of sorbent or the mass that occupies the volume of microchannel 1, which was aspirated from a methanolic suspension after a resuspension step. In addition, for MIP β -receptors the direct

aspiration of 10 mg of beads from a 75% (w/w) glycerol suspension was also assessed. The protocol sequences used (Tables S2 and S3) were summarized in Table 3. To prevent band broadening effect in the propranolol peak, 1500 μL of 0.1% (v/v) TFA were added to the eluate collected before injection into the chromatographic equipment. For the resuspension based approach using 10 mg of sorbent, the concentrations of propranolol found were 1.38 ± 0.06 and $1.46 \pm 0.08 \text{ mg L}^{-1}$ ($n \geq 6$) when MIP β -receptors and Discovery DSC-MCAX were used. These values changed to 1.48 ± 0.04 and $1.39 \pm 0.08 \text{ mg L}^{-1}$, respectively, when the sorbent bed was defined by microchannel 1 space, corresponding to 11 and 24 mg of sorbent, respectively. In addition, for the direct aspiration of 75% (w/w) glycerolic suspension of MIP β -receptors beads, the concentration achieved was $1.39 \pm 0.08 \text{ mg L}^{-1}$ while the mass of sorbent applied was 15 mg. In fact, there was no statistically significant difference for using either of the sorbents tested, evaluated by application of paired t -test to each pair of results [32]. The high precision ($\text{RSD} < 6\%$) and the high recovery levels (69-74%) demonstrated that the proposed approaches could be applied the determination of propranolol from real samples. Moreover, it should be emphasized that the optimization of the microSPE protocol, which was not aimed by the present work, could result in an improvement of the recovery levels.

4. CONCLUSIONS

In the present work, several strategies for the mesofluidic handling of irregularly shaped with non-uniform size distribution beads were evaluated. Despite its common application to spherical beads with homogenous size distribution, direct aspiration of the tested sorbents did not provide repeatable packing, even when the viscosity of the

media was changed by addition of glycerol. In fact, the change of viscosity worked only for one of the sorbents (MIP β -receptors beads), requiring further tailoring of glycerol concentration to maintain a homogenous suspension for other sorbents (Lichrolut EN and Discovery DSC-MCAX). Hence, this strategy may be effective after adequate selection of suspension milieu but it can not be regarded as a universal approach.

The resuspension of the sorbent prior to aspiration, providing a fluidized bed on the beads container, was indeed effective to foster a repeatable packing for all sorbents tested. Furthermore, the precision of sorbent packing was even enhanced when the protocol was designed to define the mass of sorbent by the physical dimension of the LOV microchannel. Nevertheless, considering the good precision (RSD < 6%) and similar recovery levels for the extraction of propranolol from aqueous solutions, the packing conditions offered by the resuspension approach are adequate for analytical applications. Future research in this area will be focused on the expansion of the materials and samples used, as well in new developments in LOV technology towards enhancement of its performance for renewable microSPE applications.

Acknowledgement

This work was financially supported by FCT through project PTDC/AAC-AMB/104882/2008, by Spanish Ministry of Education and Science through project CTQ2007-64331, and by Integrated Action n° E-48/09. Hugo M. Oliveira thanks Fundação para a Ciência e Tecnologia (FCT) and FSE (III Quadro Comunitário) for the PhD grant SFRH/BD/22494/2005.

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Fig. 1. Illustration of the manifold used for handling bead suspensions and performing microSPE of propranolol. MS, multisyringe; S_i, syringes (2500 µL capacity); LOV, lab-on-valve; HC, holding coil; CC, central channel; W, waste; Ca, carrier solution; EC, eluent collection; S, sample; B, bead suspension; C, conditioning solvent (methanol), A, air; E, eluent; BR, beads exceeding removal. For sake of simplicity the connection between S2 and the LOV dual-channel (3) was omitted. For the extraction of aqueous propranolol by MIP β-receptors, the channel 8 corresponded to water and the exceeding beads were discarded by the waste channel (2).

Fig. 2. Mass of sorbent weighed for consecutive packing procedures of beads suspended in 75% (w/w) glycerol + 10% (v/v) MeOH. □, Lichrolut EN; ♦, Discovery DSC-MCAX; ●, SupelMIP β-receptors.

Table 1. Properties of the sorbents used in the present work.

Sorbent	shape	size / μm	specific surface / m^2g^{-1}
Discovery DSC-MCAX	irregular	50	480
Oasis HLB	spherical	30	n. a.
Lichrolut EN	irregular	40 – 120	1200
SupelMIP β -receptors	irregular	56*	n. a.

* average value

n. a., not available

Table 2. Summary of the protocol sequences for the two strategies proposed for microcolumn packaging of bead suspensions under LOV format.

Step description	Strategy	
	Direct aspiration ^a	In-line resuspension ^b
Aspiration of MeOH / water	✓	✓
Resuspension of beads	×	✓
Beads settling	×	✓
Aspiration of bead suspension	✓	✓
Packing and conditioning of the microcolumn	✓	✓

✓, step included; ×, step not performed in this strategy

^a This strategy was applied to methanolic and glycerolic suspensions

^b This strategy was applied to methanolic suspensions, with and without control of channel filling

Table 3. Steps taken for automatic in-line SPE of propranolol using SupelMIP β -receptors and Discovery DSC-MCAX in BI-LOV format.

Operation	MIP β -receptors	Discovery DSC-MCAX
Conditioning	500 μ L of 1% (v/v) HCOOH in ACN 500 μ L H ₂ O 1400 μ L of 5 mmol L ⁻¹ NH ₄ COO	500 μ L of MeOH 500 μ L of 2% (v/v) CH ₃ COOH
Sample loading	1000 μ L of 2.00 mg L ⁻¹ propranolol standard	1000 μ L of 2.00 mg L ⁻¹ propranolol standard
Washing	1000 μ L of 5 mmol L ⁻¹ NH ₄ COO	1000 μ L of 2% (v/v) CH ₃ COOH
Analyte elution	1500 μ L of 1% (v/v) HCOOH in ACN	1500 μ L of 5% (v/v) NH ₄ OH in MeOH

Table 4. Average sorbent mass (mg) obtained for microcolumn packing by application of different mesofluidic protocols^a

Sorbent	Microcolumn packing protocol					
	Direct aspiration			Fluidized suspension		
	Methanol (I)	Glycerol (II)		(III)		(IV)
	Fixed volume	Fixed volume	Variable volume ^b	Fixed volume	Variable volume ^b	Filled channel
Oasis HLB	28.8 ± 1.1 (3.8%, <i>n</i> = 8)	13.2 ± 0.8 (6.1%, <i>n</i> = 8)	10.2 ± 0.7 (6.9%, <i>n</i> = 9)	23.6 ± 2.2 (9.3%, <i>n</i> = 8)	10.0 ± 1.3 (13.0%, <i>n</i> = 8)	17.2 ± 0.7 (4.1%, <i>n</i> = 8)
MIP β-receptors	6.8 ± 4.6 (67.6%, <i>n</i> = 5)	14.5 ± 0.5 (3.5%, <i>n</i> = 10)	8.6 ± 0.4 (4.7%, <i>n</i> = 7)	15.1 ± 0.9 (6.0%, <i>n</i> = 5)	9.7 ± 0.3 (3.1%, <i>n</i> = 7)	11.1 ± 0.2 (1.8%, <i>n</i> = 10)
Lichrolut EN	18.2 ± 4.4 (24.2%, <i>n</i> = 10)	n. a.	n. a.	20.1 ± 2.1 (10.4%, <i>n</i> = 10)	10.9 ± 1.5 (13.8%, <i>n</i> = 7)	16.7 ± 0.4 (2.4%, <i>n</i> = 8)
Discovery	32.9 ± 2.6	n. a.	n. a.	23.2 ± 1.7	9.3 ± 1.0	24.5 ± 0.4
DSC-MCX	(7.9%, <i>n</i> = 8)			(7.3%, <i>n</i> = 7)	(10.7%, <i>n</i> = 8)	(1.6%, <i>n</i> = 8)

^aValues in parenthesis correspond to relative standard deviation and number of replicates.^bIn this case different volumes were used to attain a sorbent mass of c.a. 10 mg.

n. a., not available, please see text for more information

Fig. 1.

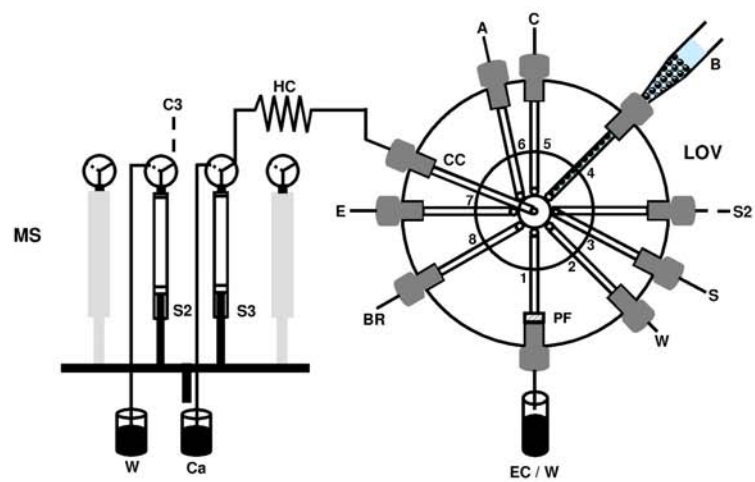
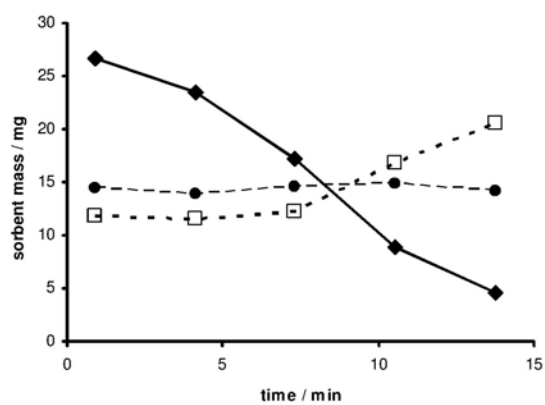


Fig. 2.



Supplementary information

Universal approach for mesofluidic handling of bead suspensions in lab-on-valve format

Hugo M. Oliveira¹, Manuel Miró², Marcela A. Segundo^{1,*}, José L. F. C. Lima¹

¹ *REQUIMTE, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal*

² *Department of Chemistry, Faculty of Sciences, University of the Balearic Islands, Carretera de Valldemossa km 7.5, E-07122 Palma de Mallorca, Illes Balears, Spain*

*Corresponding author. Tel: +351 222078994. Fax: +351 222078961. E-mail: msegundo@ff.up.pt

Table S1. Operating procedure for the different strategies used for column packing and conditioning and beads disposal.

Description	S3	vol / μL	Flow rate / mL min^{-1}	LOV (position)
(I) Direct aspiration of methanolic suspension				
a) aspiration of methanol	on / aspirate	500	2.5	5
b) aspiration of beads	on / aspirate	125	0.5	4
c) refilling of syringes	off / aspirate	262.5	3.75	8
d) column packing and conditioning	on / dispense	887.5	2.0	1
(II) Direct aspiration of glycerolic suspension				
a) filling of syringes	off / aspirate	750	5.0	5
b) aspiration of beads	on / aspirate	x^a	0.5	4
c) column packing	on / dispense	875	2.0	1
d) aspiration of methanol	on / aspirate	625	2.5	5
e) refilling of syringes	off / aspirate	1000	5.0	4
f) column conditioning	on / dispense	$1500 + x^a$	2.5	1
(III – IV) Resuspension (methanolic suspension)				
a) aspiration of methanol	on / aspirate	525	2.5	5
b) repositioning of the piston (dummy step)	off / dispense	100	2.5	5
c) resuspension of beads	on / dispense	125	5	4
d) refilling of syringes, settling of beads	off / aspirate	450	3.75	4
e) aspiration of beads	on / aspirate	y^b	0.5	4
f) column packing and conditioning	on / dispense	$750 + y^b$	2	1
g) refilling of syringes ^c	off / aspirate	750	3.75	8
h) discarding of exceeding beads ^c	on / dispense	750	5.0	8
Beads disposal (common for both strategies)				
a) aspiration of methanol	on / aspirate	250	2.5	5
b) refilling of syringes	off / aspirate	950	3.75	8
c) wetting beads	on / dispense	100	1.5	1
d) aspiration of beads	on / aspirate	375	3.75	1
e) discarding of beads and vial collection	on / dispense	1275	6.25	2
f) cleaning column cavity	on / dispense	200	1.5	1

^a $x = 125\mu\text{L}$ for fixed volume strategy. For variable volume (target mass of 10 mg), $x = 86$ and $72\mu\text{L}$ for Oasis HLB and SupelMIP β -receptors, respectively.

^b $y = 125\mu\text{L}$ for fixed volume strategy. For variable volume (target mass of 10 mg), $y = 58, 95, 75$ and $34\mu\text{L}$ for Oasis HLB, SupelMIP β -receptors, Lichrolut EN and Discovery DSC-MCAX, respectively.

^c Steps performed only when the sorbent was restricted to the volume of microchannel 1 (IV).

Table S2. Operating procedure for the propranolol extraction using the resuspension strategy.

Description	S3	vol / μL	Flow rate / mL min^{-1}	LOV (position)
<i>1- Microcolumn packing and conditioning</i>				
a) aspiration of methanol	on / aspirate	525	2.5	5
b) repositioning of the piston (dummy step)	off / dispense	100	2.5	5
c) resuspension of beads	on / dispense	125	5.0	4
d) refilling of syringes, settling of beads	off / aspirate	450	3.75	4
e) aspiration of beads	on / aspirate	y ^a	0.5	4
f) column packing	on / dispense	750 + y ^a	2.0	1
g) aspiration of water ^b	on / aspirate	750	3.75	8
h) discarding of exceeding beads ^b	on / dispense	750	5.0	2
i) aspiration of water ^c	on / aspirate	500	3.75	8
j) aspiration of eluent	on / aspirate	500	3.0	7
k) refilling of syringes	off / aspirate	1400	5.0	1
l) sorbent conditioning	on / dispense	2400	2.5	1
<i>2- Sample loading and matrix removal</i>				
a) aspiration of air	on / aspirate	150	3.0	6
b) aspiration of sample	on / aspirate	1350	2.5	3
c) repositioning of the piston	off / dispense	75	2.5	8
d) sample loading	on / dispense	1000	1.0	1
e) refilling of syringes	off / aspirate	1000	3.75	2
f) washing of HC	on / dispense	1425	7.5	2
g) refilling of syringes	off / aspirate	1000	3.75	1
h) sample cleanup	on / dispense	1000	2.0	1

^a y = 125 μL when the sorbent bed was restricted to the volume of microchannel 1. For a target mass of 10 mg y = 95 and 34 μL for MIP β -receptors and Discovery DSC-MCAX, respectively.

^b Steps only performed when filling channel was performed.

^c Steps performed for conditioning MIP β -receptors.

Table S2. Operating procedure for the propranolol extraction using the resuspension strategy (continued).

Description	S3	vol / μL	Flow rate / mL min^{-1}	LOV (position)
<i>3- Elution</i>				
a) aspiration of air	on / aspirate	250	3.0	6
b) aspiration of eluent	on / aspirate	1625	2.5	7
c) refilling of syringes	off / aspirate	500	3.75	1
d) repositioning of the piston	off / dispense	125	2.5	1
e) elution	on / dispense	1500	0.5	1
f) washing of HC	on / dispense	750	750	2
<i>4- Beads disposal</i>				
a) aspiration of methanol	on / aspirate	250	2.5	5
b) refilling of syringes	off / aspirate	950	3.75	8
c) wetting of beads	on / dispense	100	0.75	1
d) aspiration of beads	on / aspirate	375	3.75	1
e) discarding of beads	on / dispense	1275	6.25	2
f) cleaning of column cavity	on / dispense	200	1.5	1

Table S3. Protocol sequence for microcolumn packing of MIP- β -receptors suspended in 75% (w/w) glycerol during the extraction of propranolol.

Description	S3	vol / μL	Flow rate / mL min^{-1}	LOV (position)
<i>I- Microcolumn packing and conditioning</i>				
a) filling of syringes	off / aspirate	750	5.0	5
b) aspiration of beads	on / aspirate	72	0.5	4
c) column packing	on / dispense	875	2.0	1
d) aspiration of methanol	on / aspirate	625	2.5	5
e) refilling of syringes	off / aspirate	1000	5.0	4
f) column conditioning	on / dispense	1572	2.5	1
i) aspiration of water	on / aspirate	500	3.75	8
j) aspiration of eluent	on / aspirate	500	3.0	7
k) refilling of syringes	off / aspirate	1400	5.0	1
l) sorbent conditioning	on / dispense	2400	2.5	1

**Exploiting automatic on-line renewable
molecularly imprinted solid-phase extraction
in lab-on-valve format as front end to liquid
chromatography: application to the
determination of riboflavin in foodstuffs**

Exploiting automatic on-line renewable molecularly imprinted solid-phase extraction in lab-on-valve format as front end to liquid chromatography: application to the determination of riboflavin in foodstuffs

Hugo M. Oliveira · Marcela A. Segundo ·
José L. F. C. Lima · Manuel Miró · Victor Cerdà

Received: 25 October 2009 / Revised: 30 January 2010 / Accepted: 1 February 2010 / Published online: 27 February 2010
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Abstract In the present work, it is proposed, for the first time, an on-line automatic renewable molecularly imprinted solid-phase extraction (MISPE) protocol for sample preparation prior to liquid chromatographic analysis. The automatic microscale procedure was based on the bead injection (BI) concept under the lab-on-valve (LOV) format, using a multisyringe burette as propulsion unit for handling solutions and suspensions. A high precision on handling the suspensions containing irregularly shaped molecularly imprinted polymer (MIP) particles was attained, enabling the use of commercial MIP as renewable sorbent. The features of the proposed BI-LOV manifold also allowed a strict control of the different steps within the extraction protocol, which are essential for promoting selective interactions in the cavities of the MIP. By using this on-line method, it was possible to extract and quantify riboflavin from different foodstuff samples in the range between 0.450 and 5.00 mg L⁻¹ after processing 1,000 µL

of sample (infant milk, pig liver extract, and energy drink) without any prior treatment. For milk samples, LOD and LOQ values were 0.05 and 0.17 mg L⁻¹, respectively. The method was successfully applied to the analysis of two certified reference materials (NIST 1846 and BCR 487) with high precision (RSD<5.5%). Considering the down-scale and simplification of the sample preparation protocol and the simultaneous performance of extraction and chromatographic assays, a cost-effective and enhanced throughput (six determinations per hour) methodology for determination of riboflavin in foodstuff samples is deployed here.

Keywords Solid-phase extraction · Molecularly imprinted polymer · Flow analysis · Lab-on-valve · Riboflavin · Foodstuff

Introduction

Sample preparation prior to chromatographic analysis is a critical step to ensure a correct isolation and quantification of the target analytes [1]. Moreover, the pre-treatment protocol is the most time-consuming, labor-intensive and error-prone part of the analytical procedure [2]. Among a wide range of extraction techniques applied for isolating analytes from the sample matrix [1], solid-phase extraction (SPE) has undeniably an important role as sample preparation tool due to simplicity of use and automation, low cost and a large variety of commercially available sorbents from different suppliers. Nevertheless, the classical SPE sorbents retain the analytes by non-selective interactions (hydrophobic or hydrophilic) that result in a partial co-

Electronic supplementary material The online version of this article (doi:10.1007/s00216-010-3522-1) contains supplementary material, which is available to authorized users.

H. M. Oliveira · M. A. Segundo (✉) · J. L. F. C. Lima
REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia,
Universidade do Porto,
Rua Aníbal Cunha, 164,
4099-030 Porto, Portugal
e-mail: msegundo@ff.up.pt

M. Miró · V. Cerdà
Department of Chemistry, Faculty of Sciences,
University of the Balearic Islands,
Carretera de Valldemossa km 7.5,
07122 Palma, Illes Balears, Spain

extraction of interfering substances [3], especially when complex matrices are present. Therefore, the development of tailor-made sorbents with molecular recognition properties for analytical purposes has been a topic of growing interest [4, 5].

Since its introduction by Sellergren [6], molecularly imprinted solid-phase extraction (MISPE) became a powerful tool for sample preparation owing to the possibility of developing selective extraction procedures, which are enhanced by combining MISPE with separative techniques [7]. Despite their attractive features, the poor compatibility of some polymers with aqueous samples and the difficulty on having a strict control of the extraction procedure were the major drawbacks during the development of MISPE protocol [8]. Hence, the design of new strategies for synthesizing molecularly imprinted polymers (MIP) [9] and the introduction of automatic extraction protocols could have a key role not only in the enhancement of analytical results [2, 10] but also in the implementation of MISPE as a sample preparation tool for large-scale routine analysis.

In this context, MISPE automation based on flow analysis and related techniques [11] can offer straightforward and cost-effective methodologies for the hyphenation between sample preparation and chromatographic analysis [12]. Furthermore, the progress on miniaturization enabled the introduction of the third generation of flow injection analysis, so-called lab-on-valve (LOV) [13]. The configuration of the LOV module, which comprises a monolithic structure with microchannels engraved on it, allows the accurate insertion and transport of bead suspensions in a fully automatic fashion, defined as bead injection (BI). BI has been applied for implementing a wide range of on-line SPE protocols that included renovation of the sorptive surface between each determination [14]. Nonetheless, the use of BI-LOV as SPE automation tool for sample preparation technique coupled to chromatographic analysis has been underexploited, with only two on-line [15, 16] and one off-line [17] methodologies reported in the literature.

Therefore, the objective of the present work was the implementation of a hyphenated MISPE-BI-LOV methodology for performing MISPE as front end to liquid chromatography for the determination of riboflavin in foodstuff samples. Riboflavin is a water-soluble vitamin that can only be obtained through dietary intake [18] and it is commonly added during the manufacturing of fortified foods, particularly milk and milk-based formulas. Moreover, due to its fast photodegradation during preparation, preservation and storage [19], riboflavin content is a key parameter in the quality control of fortified foods, requiring suitable methods for routine control.

Experimental

Reagents and solutions

All reagents used were of analytical grade. Ultra-pure water (resistivity $>1.8 \times 10^5 \Omega \text{ cm}$) was obtained using a MilliQ system (Millipore, Bedford, MA, USA). A 2.4% (v/v) acetic acid solution prepared by dilution of glacial acetic acid (Sigma-Aldrich, St. Louis, MO, USA), was used as solvent for preparation of standard solutions. For sample digestion, 2.5 mol L⁻¹ sodium acetate (Sigma-Aldrich) and 50% (w/v) trichloroacetic acid (Sigma-Aldrich) solutions were prepared by dissolving the appropriate amount of the respective compound in water.

The stock solution of riboflavin (Sigma-Aldrich) was prepared by accurately weighing 5.00 mg of compound and dissolving it in 100 mL of solvent. In order to ensure that complete dissolution had been attained, the solution was heated in a water bath for 10 min at 37 °C. After cooling, working standard solutions were obtained by dilution of the stock solution. All riboflavin solutions were stored in the dark at 4 °C.

The MIP for riboflavin selective extraction was obtained from Supelco (Bellefonte, PA, USA). The beads (100 mg) were suspended in 1,000 μL of a hydro-alcoholic mixture containing 50% (v/v) MeOH/H₂O. Suspensions containing 2-propanol (Sigma-Aldrich) and ethanol (Sigma-Aldrich) in the same proportions were also prepared. The MISPE eluent was a mixture of 50% (v/v) MeOH/H₂O containing 1% (v/v) of acetic acid.

The mobile phase containing 5 mmol L⁻¹ of octanesulfonic acid (Sigma-Aldrich), 0.5% (v/v) triethylamine (Sigma-Aldrich), 2.4% (v/v) acetic acid, and 15% (v/v) of methanol HPLC-grade (Merck; pH 5.25 ± 0.01) was prepared according to the procedure proposed by Albalá-Hurtado et al. [20]. The final solution was filtered by a 0.22- μm cellulose membrane and degassed by ultrasound irradiation for 15 min before use.

Samples

Certified reference samples NIST-1846 (infant formula—milk-based) and BCR-487 (pig liver) were purchased from LGC Standards (Teddington, UK). Infant formula sample was prepared by dissolving 25.00 g of sample in 100 mL of hot water (approximately 90 °C). After cooling, this solution was transferred to a 250-mL volumetric flask and the remaining volume was completed with water. Pig liver sample (0.500 g) was subjected to an acidic (0.1 mol L⁻¹ HCl)/enzymatic (acid phosphatase, P3627, Sigma-Aldrich) digestion following the procedure described by Tang et al. [21]. The energy drink sample was degassed by ultrasound irradiation and filtered by a 0.45- μm polyvinylidene

fluoride filter before processing through the automatic system.

Liquid chromatography analysis

The quantification of riboflavin content in the samples was performed in a liquid chromatography (LC) equipment Merck/Hitachi LaChrom 7000 series (Hitachi Ltd., Tokyo, Japan). It was constituted by an interface (D-7000), a high-pressure pump (L-7455), and a diode array detector (L-7100) controlled by D-7000 software. This software was also used to record all the chromatographic data. A reversed-phase monolithic column Chromolith RP-18e (100×4.6 mm i.d.; Merck) connected to a guard column of the same material (5×4.6 mm i.d.) was used as stationary phase. Separation was conducted by isocratic mode (mobile phase containing 5 mmol L⁻¹ of octanesulfonic acid, 0.5% (v/v) of triethylamine, 2.4% (v/v) of acetic acid and 15% (v/v) of methanol in water; flow rate=1.0 mL min⁻¹) and the detection wavelength was set at 268 nm. A 200-μL loop made of 0.75 mm i.d. polyetheretherketone tubing was assembled to a Rheodyne (Rohnert Park, CA, USA) 7725i six-port manual injector (IV), working as interface between the automatic flow system and the chromatograph. A column heating system (CH-500, Eppendorf, Westbury, NY, USA) was also employed.

LOV-MSFIA manifold

The different components of the proposed manifold were arranged as schematically illustrated in Fig. 1. The propulsion unit was a multisyringe burette (Crison Instruments, Allela, Spain) equipped with two syringes (Hamilton, Bonaduz, Switzerland) of 2,500 μL (syringes S1 and S2) and one of 5,000 μL (syringe S3). The insertion of liquids into the flow network and the access to the solutions reservoir was controlled by the three-way commutation valve (NResearch, Caldwell, NJ, USA) placed at the head of each syringe. The LOV was constituted by a group of microchannels (1.5 mm i.d.) engraved on a polyetherimide (PEI) block. This device was mounted atop of an eight-port multiposition valve (MPV; Crison Instruments). All the microchannels communicated to a central channel (CC) which was connected to S2 through the holding coil (HC) allowing access to all peripheral ports, one at a time. The bead suspension container (channel 4) was made by attaching a pipette tip (1,000 μL) to a fitting. The beads were stopped at the exit of channel 2 by a polyethylene frit with a pore diameter of 20 μm (Supelco). A PEI T-junction was placed between the exit of channel 2 and the injection loop in order to allow the mixing of eluate stream (propelled by S2) with water (propelled by S3) during the elution step. All connections were made

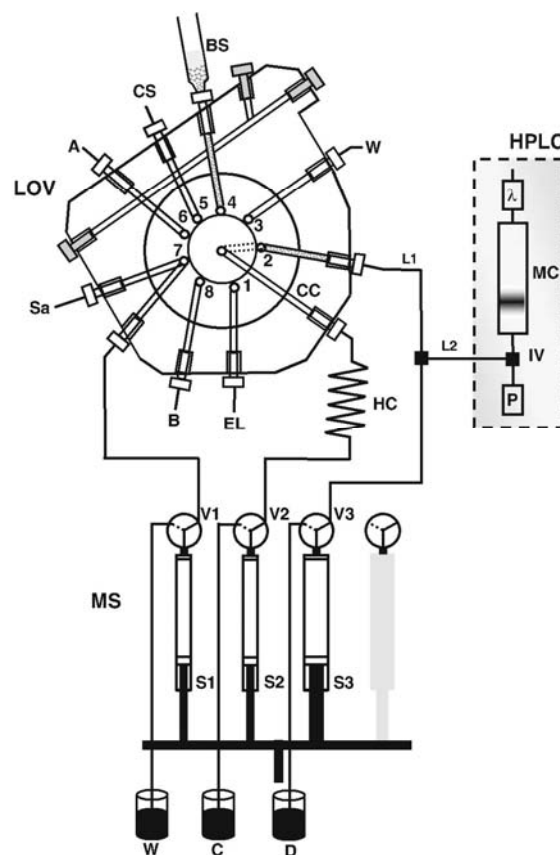


Fig. 1 Schematic representation of the manifold for determination of riboflavin in foodstuff. *LOV* lab-on-valve, *MS* multisyringe, *HPLC* high-performance liquid chromatography, *S_i* syringe, *V_i* three-way commutation valve (dashed line position off, solid line position on), *A* air, *CS* conditioning solvent (50% (v/v) MeOH/H₂O), *BS* bead suspension in conditioning solvent, *C* carrier solution (H₂O), *D* diluent (H₂O), *W* waste, *CC* central channel, *EL* eluent (50% (v/v) MeOH/H₂O+1% (v/v) CH₃COOH), *B* channel for bead discarding, *Sa* sample/standard solution, *HC* holding coil, *L1* connection tubing (8 cm), *L2* connection tubing (44 cm), *P* chromatographic pump, *IV* injection valve, *MC* monolithic chromatographic column, *λ* diode array detector

by 0.8 mm i.d. polytetrafluorethylene (PTFE) tubing (Omnifit, Cambridge, UK), except the HC (with 5 mL capacity) and connections between solution reservoirs and syringes, where 1.5 mm i.d. tubing of the same material was used.

The bi-directional movement of syringes and respective operating speed, the position of commutation valves and the port selection on the MPV were controlled by a personal computer running a lab-made software written in Quick Basic 4.5 (Microsoft, Redmond, WA, USA) through RS232 interface.

Operating procedure

The operational sequence for extracting riboflavin from different food matrices by the proposed method (Electronic Supplementary Material, Table S1) comprised four main steps defined as (1) sorbent loading and conditioning, (2) sample loading and matrix removal, (3) elution and LC injection and (4) bead disposal. For the sake of simplicity, the steps concerning the refilling of syringes were omitted from the following description.

1. *Sorbent loading and conditioning.* After aspiration of 975 μL of conditioning/suspension solvent (50% (v/v) MeOH/H₂O), the flow direction was reversed and the beads in the pipette tip were resuspended with 87.5 μL of this solution. Thereafter, the flow on this channel was stopped for 15 s for beads settlement in the container while S2 was refilled with 500 μL of water. A volume of 62.5 μL of beads suspension was subsequently collected and, after flow reversal, beads were loaded into channel 2 and immediately conditioned with 50% (v/v) MeOH and water carrier that were previously stored in the HC. Simultaneously, S3 was activated in order to clean the tubing connecting to the injection valve. Finally, the exceeding beads that remained in the HC were discarded by sending 500 μL of carrier through channel 8.
2. *Sample loading and matrix removal.* For sample exchange, S1 was activated, providing sample aspiration through the auxiliary channel of port 7 (Fig. 1), resulting in washing of the connecting tubing to sample reservoir. Next, a plug of 125 μL of air and 1,350 μL of sample were sequentially aspirated into the HC. After flow reversal, 1,000 μL of sample was percolated through the sorbent. The sample and air remaining in the HC were subsequently eliminated to waste (channel 3) by propulsion of 2,000 μL of carrier, guaranteeing also the removal of remains of sample from the HC. Subsequently, matrix components were eliminated from the packed MIP microcolumn by pumping 2,000 μL of water through the sorbent. During the last part of this sequence (Table S1, step 2k), S3 was also activated in order to circumvent the dispersion of the sample in the tubing connecting S3 to the confluence point at the T-junction.
3. *Elution and LC injection.* Two hundred fifty microliters of air and 437.5 μL of eluent (50% (v/v) MeOH/H₂O + 1% CH₃COOH) were consecutively aspirated into the HC. Thereafter, the direction of flow was reversed and, simultaneously, S2 and S3 were activated, propelling 312.5 μL of eluent through the sorbent bed that were merged with water at 1:2 proportion at the confluence point. This mixture was fed into the IV loop (200 μL),

where it was partially injected into the chromatograph by a heart-cut approach and the chromatographic run was started. Next, the HC coil was washed by propelling 2,000 μL of carrier to waste and the chromatograph IV was returned to load position.

4. *Bead disposal.* The beads packed into channel 2 were wetted with 200 μL of 50% (v/v) MeOH, previously aspirated into the HC. After flow reversal, the packed sorbent was moved into the HC and subsequently disposed for waste (channel 3) by pumping carrier (1,037.5 μL). Finally, the tubing connections to IV and respective loop were cleaned with 1,500 μL of water propelled by S2 and S3.

Results and discussion

Chromatographic method

The chromatographic method used for the determination of riboflavin was adopted from the reversed-phase method for the determination of water-soluble vitamins reported by Albalá-Hurtado et al. [20]. All chromatographic conditions (mobile phase, flow rate, temperature, and detection wavelength) were maintained, except the chromatographic column. The original packed column (octadecylsilane, 5 μm , 250 $\times$ 4.6 mm) was replaced by a monolithic column (100 $\times$ 4.6 mm) of the same material. This originated a reduction of run time from 19 min in the original method to 7 min in the present work. The analytical performance of the monolithic column was evaluated by performing consecutive calibration curves (intra- and inter-day) with standard solutions of riboflavin in the range of 0.100–5.00 mg L^{-1} (injection volume of 20 μL). Linearity was attained ($r^2 > 0.998$) and deviations on sensitivity were $< 2\%$ in both situations ($n=3$, intra-day comparison; and $n=10$, inter-day comparison).

Design of the MISPE-BI-LOV system

The manifold proposed for the selective extraction of riboflavin in food samples was designed taking into account the different steps necessary to automate the complete extraction protocol. For application of the present manifold as front end to liquid chromatographic analysis, chemical resistance of the LOV unit and flexibility of the propulsion unit were considered. The use of organic solvents during SPE protocols, especially acetonitrile and methanol, is crucial to achieve good recoveries. Nevertheless, the commonly available LOV modules [15, 22] are made of polymethylmethacrylate, which has a satisfactory resistance to methanol but not to acetonitrile. Hence, LOV and T-

junction (Fig. 1), made of polyetherimide, were herein selected to enable the use of this organic solvent in the assays. On other hand, the use of a multichannel propulsion unit (multisyringe burette) was important to provide alternative flow paths, that could be accessed simultaneously and/or intermittently [23], resulting in a flow network that had the LOV central channel as its main processing pathway. In this particular manifold, these features were exploited through the additional channels provided by S1 and S3 for sample exchange and eluate composition adjustment, respectively.

Mesofluidic handling of molecularly imprinted polymer bead suspension

Previous work reported that in order to guarantee a repeatable manipulation of beads in LOV, the sorbent should fulfill some requirements, such as spherical shape, uniform size distribution and, in the case of hydrophobic beads, water-wettability [14], along with homogeneity of bead suspension. The suspension milieu was generally dependent of the hydrophilic/hydrophobic nature of the particles and it was usually an aqueous, hydro-alcoholic or alcoholic solution [24].

In the present work, the beads were suspended in methanol and handled by an operational protocol similar to that described by Quintana et al. [15]. The results were not satisfactory because a fast deposition of the beads was observed, resulting in a lump that interrupted the aspiration into the HC. In fact, the molecularly imprinted beads used in this work were neither spherical nor uniform in size distribution (average particle size $66.5\ \mu\text{m}$ with predominant distribution in the range $30\text{--}90\ \mu\text{m}$ (75%)). This clogging problem has been reported before for the use of PTFE beads in LOV that had high density and irregular shape. It was then circumvented by suspending the beads in an ethanolic media and promoting the continuous recirculation of the suspension by an auxiliary propulsion unit as recommended by Miró et al. [22]. Nonetheless, in order to avoid the use of additional instrumentation, the extension of the time necessary for bead settling inside the microchannel 4 (Fig. 1) was targeted here by using two complementary strategies: increasing the viscosity of suspension solvent and introducing a resuspension step before the aspiration of beads into the HC.

The viscosity was modified by suspending the beads in binary mixtures (50% (v/v)) of water with either methanol, ethanol, or propanol, which corresponded, approximately, to the maximum viscosity of these water-alcohol binary systems [25]. Using a batch approach, when 25 mg of sorbent were suspended in $1,000\ \mu\text{L}$ of hydro-alcoholic mixture, the time taken for bead settling followed the order: propanol (50% (v/v)) > ethanol (50% (v/v)) > methanol (50%

(v/v)) >> methanol. To test the solvents in the MISPE automatic system, the hydro-alcoholic suspensions were then transferred to the bead container (Fig. 1). After settling the beads, the supernatant was reduced to one third of its initial volume and the suspension was processed according to the operation protocol (Table S1, sections 1 and 4), where the bead suspension is agitated by a burst of solvent (Table S1, section 1, step d) before bead aspiration. For all tested binary mixtures, the time necessary for settling the beads was suitable for repeatable sorbent aspiration, enabling the precise packaging and disposal of the MIP microcolumn. A 50% (v/v) MeOH/H₂O solution was chosen for further experiments because it could also be applied for sorbent conditioning before sample loading. However, this strategy caused the progressive decrease of bead concentration in the suspension at the container as distribution of beads in the suspension was not homogeneous. Hence, a lower mass of sorbent was aspirated along consecutive injections, lowering the amount of beads packed into channel 2. To overcome this problem, a larger amount of beads was drawn into the HC, larger than the amount necessary to fill completely the microchannel 2. Hence, after assembling the SPE microcolumn, the extra beads were collected through channel 8 (Table S1, step 1j) and reused after drying. For each analysis, a mass of $11.2 \pm 0.2\ \text{mg}$ ($n=10$) of sorbent was used.

On-line coupling of MISPE to chromatographic separation

On-line coupling of MISPE to chromatography have been described using essentially column switching schemes [4]. Here, for the target application, this strategy would not be suitable as direct introduction of sample rich in protein, such as milk, would precipitate in the presence of mobile phases containing either acid or acetonitrile. To overcome this limitation, we propose here the assembling of a MISPE column inside the LOV piece, where sample loading, analyte retention, and elution can take place without pumping the chromatographic mobile phase through the sorbent material.

The MIP selectivity towards riboflavin is due to tailored polymer composition by selection of template (riboflavin tetra-acetate) and functional monomer (2,6-bis (acrylamido)pyridine), which controls the binding site, and also selection of cross-linking monomer (pentaerythritol triacrylate), which influences the polarity and functionality of the polymer matrix per se [9]. In this case, selective retention is attained due to electrostatic forces from the three-point hydrogen-bond array established between the functional monomer and the imide motif present in the riboflavin molecule while the hydrophilic cross-linking monomer allowed the analyte retention in aqueous media [26].

Hence, the protocol proposed for determination of riboflavin in milk [27, 28] consisted on MIP conditioning with methanol followed by water; after sample application (and drying of MISPE column in reference [28]), the elution took place by addition of methanol (10% acetic acid) [28] or 70% acetonitrile [27]. To implement this protocol in the LOV format, the several solutions/solvents required were placed in the different lateral ports, except for water that was used as carrier. These liquids were aspirated into the holding coil (Fig. 1) whenever required and percolated through the MISPE column by flow reversal. Thus, MIP conditioning was easily attained by stacking a methanol plug in the holding coil, selecting the MISPE column port, reversing the flow direction and propelling methanol and water sequentially through the column. This procedure was advantageous when compared to the manual counterpart as the MISPE column never become dry before sample passage, as recommended by MIP supplier [27]. A similar strategy was adopted for sample feeding, followed by washing with water for matrix removal.

The elution step in on-line SPE-LC systems is usually a difficult task due to the need of matching the eluate with the mobile phase composition, otherwise peak broadening is often observed [10]. This is the case in our work because the elution of the target analyte should be performed with a high content of organic solvent (acetonitrile or methanol) while the mobile phase was 85% (v/v) in water. Therefore, it was necessary to perform a detailed evaluation of all variables involved in the desorption of the target compound and subsequent adjustment of eluate composition.

Preliminary experiments were performed in order to evaluate the influence of the organic solvent content on the peak broadening by diluting the analyte in the organic solvent (acetonitrile or methanol) and in different mixtures of organic solvent/water. When 20 μL of solution containing pure acetonitrile or methanol as organic solvent were injected, a wide band was recorded and no peak was detected (Fig. 2). However, a progressive narrowing of the riboflavin band, reflected on higher peak heights, was observed when the water content increased (Fig. 2), especially for methanol, which was selected for further experiments. Peak sharpness was also considered when defining the maximum volume of eluate to be injected. Hence, for MeOH percentages close to that applied in the mobile phase (20%), it was possible to inject up to 400 μL of sample whereas for 33% (v/v) MeOH the sample volume was reduced to 100 μL .

These results demonstrated that the matching between eluate and mobile phase composition was crucial for obtaining a narrow peak in the chromatogram. Thus, two aspects were considered simultaneously in order to tailor the elution conditions: the concentration of organic solvent in the MISPE eluent and the subsequent dilution necessary

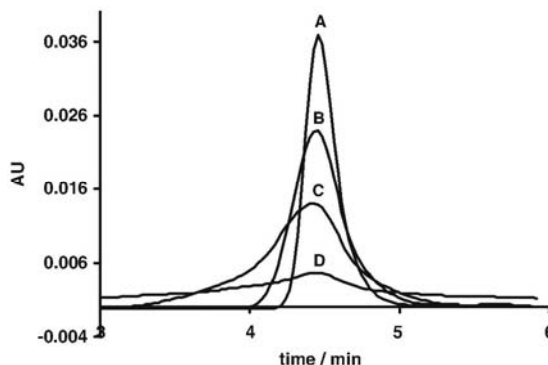


Fig. 2 Analytical signals from direct injection of 20 μL of a riboflavin standard (1.00 mg L^{-1}) prepared in different methanol percentages (v/v). **a** 33%, **b** 50%, **c** 67%, **d** 100%

to attain the organic/aqueous content of the mobile phase (ca. 15:85 (v/v)). In the proposed methodology, this dilution was performed in-line, in a fully automatic fashion. By taking advantage of the multichannel propulsion provided by the multisyringe burette, the eluent was merged with water at a T-junction placed after the MIP microcolumn and before the injection loop (Fig. 1). The dilution factor (Table 1) was obtained through the ratio between the volumes of syringes S2 and S3 (Fig. 1). Hence, different MISPE eluent composition/dilution factors were evaluated (Table 1) in order to attain similar composition of the injected eluate (about 15% MeOH). The experimental set consisted of merging MISPE eluent containing 30, 50, and 70% (v/v) of MeOH (propelled by S2 (Fig. 1) after aspiration from port 1 through the HC) with water dispensed by S3 (Fig. 1), of which volumes were 2.5, 5.0, and 10.0 mL, respectively (Table 1). A solution containing 15% of MeOH without any subsequent dilution by S3 was also used.

For each condition, the elution profile was evaluated by propelling volumes of MISPE eluent in the range of 187.5–875 μL and by representing it as peak area as a function of eluent volume (see Electronic Supplementary Material, Figure S1). As depicted in Fig. S1, the elution profile presented a sharp increase, followed by a smoother decrease after the maximum value was reached. The volume of MISPE eluent necessary to attain the maximum analytical signal varied from 250 to 625 μL (Table 1), and it decreased upon eluent dilution after the T-junction. This difference in eluent volumes is not related to the elution process itself but it arises from the different diluent volumes added after the T-junction and the fact that the connection between the T-junction and the IV loop (Fig. 1, L2) has a fixed volume (about 220 μL). Hence, at least 420 μL (220 μL from L2+200 μL from IV loop) of 15% MeOH eluent should be propelled to fill the IV loop while for 70%

Table 1 Influence of MISPE eluent composition and in-line dilution factor on the analytical signal recorded for a standard solution of riboflavin (1.00 mg L⁻¹; sample volume=1,000 µL; LC injection volume=200 µL)

Composition of eluent from S2 (%MeOH)	Dilution factor by addition of water	Composition of injected eluate (% MeOH)	Maximum peak area	Nominal eluent volume corresponding to maximum peak area (µL)	Volume of eluate injected (real value; µL)	Ratio (maximum peak area/real volume of eluate)
15	—	15	185595	625	200	928
30	1:1	15	92931	500	100	929
50	1:2	16.7	99089	312.5	67	1479
70	1:4	14	65090	250	40	1627

MeOH eluent, only 84 µL would be necessary as the remaining 336 µL would be diluent from syringe S3.

When absolute values of maximum peak areas were compared directly, it seemed that elution would be favored by a low MeOH percentage. However, the real eluate volume introduced into the 200-µL loop of the injection valve varied upon dilution at T-junction, as also presented in Table 1. Hence, when the ratio between the maximum peak area and the real injected volume of eluate was examined, the best results concerning elution efficiency were attained for higher MeOH percentages, which provided an eluent strength suitable for the desorption of the analyte from the MIP cavities. It should be emphasized here that the objective was a suitable recovery of the analyte present in a complex matrix (clean-up) and not its enrichment or pre-concentration. Thus, 50% (v/v) MeOH was chosen as MISPE eluent for heart-cut injection into LC, considering its high desorptive capacity (only 10 % lower than that attained for 70% (v/v) MeOH regarding the maximum peak area to real eluent volume ratio, see Table 1) combined with a dilution (1:2) that guaranteed a narrow peak and a suitable sensitivity for the quantification of the analyte.

Besides the organic solvent content in the eluent, the presence of acids or bases has also been reported to facilitate the elution step in MIP by disrupting hydrogen bonds and residual electrostatic interactions between the sorbent and analyte [3]. Considering the basic character of riboflavin and the interactions described in this MIP-based selective extraction [9], 1% (v/v) acetic acid was added to the four MISPE eluent compositions tested (Table 1). In all cases, an increase in peak area between 10% and 20% was observed. Thus, 50% (v/v) MeOH with 1% acetic acid (v/v) was chosen as MISPE eluent.

Using these conditions, analyte recovery was estimated by determination of the riboflavin mass injected in the chromatograph after processing 1.00 µg of riboflavin (1.00 mL of 1.00 mg L⁻¹ solution) through the MISPE system using the protocol established in Table S1. For this, a calibration curve (injection volume=200 µL) was also

established by direct introduction of standards. The value attained was 3.3%, indicating the fraction of analyte present in the original sample that is introduced in the chromatograph. It should be emphasized here that this value is acceptable in flow injection conditions, where mass-transfer processes with low yields (<10%), which includes dialysis, gas diffusion, or analyte retention/desorption in a solid phase, are commonly applied for sample treatment [29]. Despite the low recovery, accurate results are attained as long as the system is correctly calibrated, in this case by submitting standard solutions to the same processing steps as samples.

Analytical performance of the method

The proposed methodology was evaluated in terms of linear application range, breakthrough volume, repeatability, and sample throughput.

Calibration curves were established by extracting 1,000 µL of five standard solutions and plotting the peak area against the concentration of riboflavin. Linearity ($r^2 \geq 0.998$) and good precision (RSD<4%) were found for concentrations in the range of 0.450–5.00 mg L⁻¹, which are quite acceptable considering that the main goal of the MISPE procedure was sample clean-up and not analyte enrichment.

Breakthrough volume was determined by loading a mass of 2.50 µg of riboflavin contained in variable volumes (1.00–5.00 mL) through the MISPE microcolumn. No breakthrough occurred for sample volumes up to 2.00 mL. For volumes above 2.00 mL, a decrease in the analytical signal from 8% (3.00 mL) to 36% (5.00 mL) was observed.

Precision was evaluated as the RSD calculated after five consecutive injections of a standard containing 1.00 mg L⁻¹ of riboflavin using either a new sorbent portion for each determination or a permanent MISPE microcolumn. The repeatability (first case) and reproducibility (second case) were 3.8%, and 6.3%, respectively. Despite the improved RSD whenever the packed column is reused, a carryover effect was observed even after cleaning the microcolumn

with 2 mL of MISPE eluent and conditioning with 1 mL of water. After five consecutive injections of riboflavin standard, this solution was replaced by a blank (MilliQ water), and a peak area equivalent to 10% of the original analytical signal was recorded. Thus, sorbent renewal is essential to attaining accurate results.

The time required for the analytical protocol should include the time necessary for performing the MISPE (9.6 min) and the chromatographic determination (7 min). These two steps were carried out in parallel, as one sample was processed through the chromatograph while the target analyte from the next sample was extracted in the LOV system. Thus, it was possible to perform more than six determinations per hour, comprising both sample treatment and chromatographic run.

Retention time of riboflavin peak was typically 4.85 min. Repeatability ($n=10$, intra-day) and reproducibility ($n=8$, inter-day) of this value were calculated as 0.5% and 3.0%, respectively. The effect of temperature was also evaluated, accounting for a decrease on retention time of 5, 8, 12, and 15% when temperature was raised from 20 °C to 25, 30, 35, and 40 °C, respectively.

Application to the determination of riboflavin in foodstuff samples

The new methodology was applied to the determination of the riboflavin content in milk-based infant formula (NIST 1846), which was processed without any prior treatment, providing a clean chromatogram (Fig. 3). The concentration found ($16.6 \pm 0.7 \text{ mg kg}^{-1}$) was in agreement with the certified value ($17.4 \pm 1.0 \text{ mg kg}^{-1}$), for which $t_{\text{calculated}}$ (2.44) was $< t_{\text{tabulated}}$ (3.18) at $\alpha=0.05$ and $n=4$ (Table 2). Limits of detection (LOD) and quantification (LOQ) were expressed as the concentration equivalent to a signal to noise ratio of 3 and 10, respectively [30]. For this type of

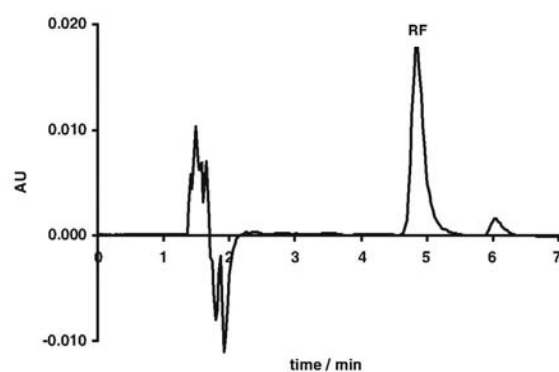


Fig. 3 Chromatogram obtained for milk-based infant formula (NIST 1846) processed by the MISPE-BI-LOV system (RF riboflavin)

sample, LOD and LOQ were 0.05 and 0.17 mg L^{-1} , corresponding to 0.50 and 1.66 mg kg^{-1} on the dry product.

Previous applications of the MIP sorbent were restricted to milk sample analysis. The method developed here was however also applied to the analysis of other foodstuff, namely pig liver (BCR 487) and an energy drink with high sugar (107 g L^{-1}) and caffeine (320 mg L^{-1}) content. These samples represent matrices with different, complex composition for which the imprinted polymer was not initially intended (Table 2), and where the use of chromatographic separation is mandatory for reliable quantification of the target vitamin.

For the pig liver sample, a previous digestion was necessary to release the protein bound riboflavin. The non-existence of significant differences between the obtained concentration ($99.5 \pm 4.3 \text{ mg kg}^{-1}$) and the reference concentration ($106.8 \pm 8.8 \text{ mg kg}^{-1}$) at a 95% confidence level was corroborated by the statistical t -test of comparison of means ($t_{\text{tabulated}}$ (2.13) $> t_{\text{calculated}}$ (1.75)). Nevertheless, the chromatogram obtained (Fig. 4) was not so clean as that obtained for the milk-based (Fig. 3) sample. This fact reflected the high vitamin content present on the digest and its non-selective interaction with the MIP, which has been reported for folic acid in particular [28]. Although this fact did not compromise the determination of the target analyte, an extra run time (5 min) to guarantee a complete clean-up of the chromatographic column before the subsequent analysis was necessary. Hence, for future applications of the method with these kinds of samples, the washing procedure protocol would have to be adjusted in order to minimize these non-selective interactions and the flow rate of the chromatographic run could be increased to speed up the analysis. The values of LOD and LOQ for this type of sample were 0.12 and 0.39 mg L^{-1} , which corresponded to 23.2 and 77.4 mg kg^{-1} in the original sample. The higher values found reflected the increased baseline noise observed in the chromatogram (Fig. 4).

An energy drink was also processed by the present method. In this case, the accuracy was assessed by analyzing samples fortified at two different levels (0.50 and 1.00 mg L^{-1}). The recoveries obtained (Table 2) were above 90% in both cases, demonstrating the potential of the system in handling matrices with high sugar content. LOD and LOQ found for this type of sample were also appropriated for current determination, with values of 0.10 and 0.35 mg L^{-1} , respectively.

Comparison to previously described sample preparation methods

Despite the large variety of analytical procedures available for the quantification of riboflavin in milk or milk-based samples, comprising the use of biosensors [31] or dispos-

Table 2 Application of MISPE-BI-LOV protocol to determination of riboflavin in foodstuff

Sample	Concentration found	Certified value
Infant formula (CRM NIST 1846)	16.6±0.7 mg kg ⁻¹	17.4±1.0 mg kg ⁻¹
Pig liver (CRM BCR 487)	99.5±4.3 mg kg ⁻¹	106.8±8.8 mg kg ⁻¹
Energy drink	0.93±0.05 mg L ⁻¹	—
Energy drink (+0.50 mg L ⁻¹)	1.30±0.09 mg L ⁻¹ (90.9%) ^a	—
Energy drink (+1.00 mg L ⁻¹)	1.80±0.09 mg L ⁻¹ (92.8%) ^a	—

^a Recovery percentage

able electrochemical sensors [19], fluorimetric titration [32], liquid chromatography [33], and capillary electrophoresis [34], all these recently reported methods included an extraction step prior to the determination of the analyte. Extraction techniques including protein precipitation with acetonitrile [33] or ammonium sulfate [19] and supercritical fluid extraction [34] were used. Although these extraction techniques allowed the determination at the target levels, extraction is a time-consuming and labor-intensive step, taking about 25–30 min, which did not include additional manual operations, such as sample metering, dilution or filtration. Hence, these methodologies are not suitable for fast screening analysis of foodstuffs.

On other hand, the proposed MISPE-BI-LOV methodology was able to carry out these tasks in less than 10 min in a fully automatic fashion with a similar analytical performance and within the same time frame of the chromatographic assay. Further, the microscale operation provided by the MISPE-BI-LOV system also allowed a drastic reduction in organic solvent and sorbent consumption. Only 856 µL of methanol and 11.2 mg of MIP were used per determination. Compared with the manual batch protocol endorsed by the MIP supplier [27], the proposed automatic procedure entails a reduction of 72% in organic solvent required and 55% in sorbent consumption.

Furthermore, the LOV system is commercially available and an integrated system comprising the LOV module,

multisyringe burette and controlling software would cost about one fifth of a conventional robotic system. Concerning run costs, the major consumable item is the solid-phase sorbent. Compared to Prospekt single-use cartridges for robotic systems (300–500€ per 100 analysis) or to on-line reusable pre-columns (150–400€ per 100 analysis), each MISPE-BI-LOV analysis would require 11.2 mg of sorbent, costing about 2.70€ per analysis. This value is comparable with the cost of reusable on-line pre-columns (which are not suitable for direct milk analysis) and it is lower than the cost of single-use cartridges applied in robotic systems.

Conclusions

A high-throughput automatic method for performing renewable MISPE in foodstuffs followed by LC analysis is here described for the first time. This was achieved by a hyphenated methodology, based on the flexibility of the propulsion unit (multisyringe burette), the microscale operation of LOV and the enhanced throughput provided by chromatographic monolithic columns. The analytical protocol enabled the handling of irregularly shaped beads suspension with a high precision (RSD<3%), and a simplification of the overall extraction process by suppression of some analytical steps in the batch MISPE procedure, including sorbent drying and eluate evaporation, without deterioration of the riboflavin assay. The applicability of this system was demonstrated by the matrix clean-up and determination of riboflavin in different food matrices, with direct injection of milk and energy drink without any prior treatment. Since riboflavin is a relevant parameter in food quality control, the present method is undeniably a suitable tool for fast determination of this analyte in foodstuff samples. Finally, it contributes towards “Green Analytical Chemistry” as a sustainable analytical procedure, where significant reduction in analysis time, reagent consumption, and thus, costs, was attained.

Acknowledgments This work was financially supported by FCT through project PTDC/AAC-AMB/104882/2008, by Spanish Ministry of Education and Science through project CTQ2007-64331, and by Integrated Action no E-48/09. Hugo M. Oliveira thanks Fundação para a Ciência e Tecnologia (FCT) and FSE (III Quadro Comunitário) for the PhD grant SFRH/BD/22494/2005. The authors thank Dr

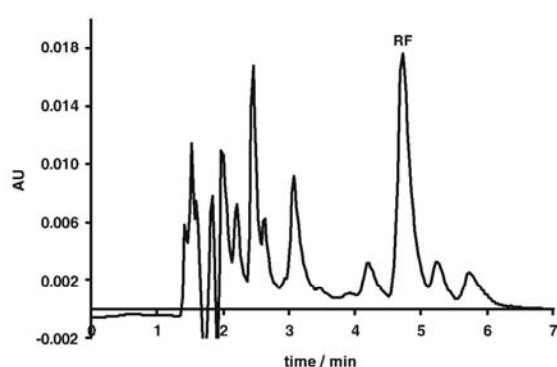


Fig. 4 Chromatogram recorded for the pig liver (BCR 487) digest analyzed by the MISPE-BI-LOV system (RF riboflavin)

Ferran Hierro from the Microscopy Laboratory at the University of the Balearic Islands for providing the electron microscopy picture of the MIP material and Mr. Marcelo Vaz Osório for technical assistance.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

**Exploiting automatic on-line renewable molecularly-imprinted
solid-phase extraction in lab-on-valve format as front end to liquid
chromatography: application to the determination of riboflavin in
foodstuffs**

Hugo M. Oliveira, Marcela A. Segundo, José L. F. C. Lima, Manuel Miró and Victor Cerdà

Table S1. Operating procedure for the automatic separation and determination of riboflavin in foodstuff samples

Description	S1	S2	S3	vol ^a (μL)	Flow rate ^a (mL min ⁻¹)	LOV (position)	HPLC IV (position)
1. sorbent loading and conditioning							
a) aspiration of conditioning solvent	*	on/aspirate	*	975	2.5	5	load
b) filling of syringes	*	off/aspirate	*	217.5	3.75	--	load
c) change of piston direction (dummy step)	*	off /dispense	*	200	3.0	--	load
d) resuspension of beads	*	on/dispense	*	87.5	7.5	4	load
e) filling of syringes; settling of beads	*	off/aspirate	*	500	2.0	--	load
f) collection of beads	*	on/aspirate	*	62.5	1.5	4	load
g) filling of syringes	*	off/aspirate	*	547.5	2.5	--	load
h) preparation of SPE column	*	on/dispense	*	567.5	3.0	2	load
i) conditioning of SPE column	*	on/dispense	on/dispense	947.5 (S2) 1895 (S3)	2.5 (S2) 5.0 (S3)	2	load
j) discarding of exceeding beads	*	on/dispense	*	500	3.75	8	load

Table S1. Operating procedure for the automatic separation and determination of riboflavin in foodstuff samples (continued)

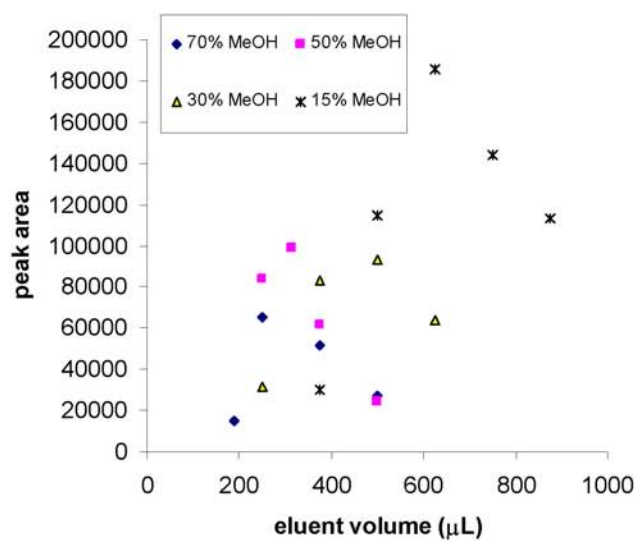
Description	S1	S2	S3	vol ^a (μL)	Flow rate ^a (mL min ⁻¹)	LOV (position)	HPLC IV (position)
2. sample loading and matrix removal							
a) washing of sample tubing and channel	on/aspirate	*	*	1000 (S1)	3.0 (S1)	7	load
b) repositioning of syringes	off/dispense	*	*	1000 (S1)	5.0 (S1)	--	
c) aspiration of air into HC	*	on/aspirate	*	125	3.0	6	load
d) aspiration of sample	*	on/aspirate	*	1350	2.0	7	load
e) change of piston direction (dummy step)	*	off/dispense	*	250	7.5	--	load
f) sample loading	*	on/dispense	*	1000	1.0	2	load
g) filling of syringes	*	off/aspirate	*	1775	3.75	--	load
h) washing of HC with elimination of sample and air	*	on/dispense	*	2000	7.5	3	load
i) filling of syringes	*	off/aspirate	*	2000	3.75	--	load
j) matrix removal from SPE column	*	on/dispense	*	1250	1.5	2	load
k) matrix removal and washing of tubing connecting to IV	on/dispense	on/dispense	*	750 (S2) 1500 (S3)	1.5 (S2) 3.0 (S3)	2	load

Table S1. Operating procedure for the automatic separation and determination of riboflavin in foodstuff samples (continued)

Description	S1	S2	S3	vol ^a (μL)	Flow rate ^a (mL min ⁻¹)	LOV (position)	HPLC IV (position)
3. elution and LC injection							
a) aspiration of air into HC	*	on/aspirate	*	250	3.0	6	load
b) aspiration of eluent	*	on/aspirate	*	437.5	2.5	1	load
c) filling of syringes	*	off/aspirate	*	1750	5	--	load
d) change of piston direction (dummy step)	*	off/dispense	*	125	2.5	--	load
e) elution of analytes and filling of the injection loop	*	on/dispense	on/dispense	312.5 (S2) 625 (S3)	0.50 (S2) 1.0 (S3)	2	load
f) eluate injection and starting of chromatographic run	--	--	--	--	--	--	inject
g) elimination of air and washing of HC	*	on/dispense	*	2000	2	3	inject
h) rotation of IV to load position	--	--	--	--	--	--	load
4. bead disposal							
a) aspiration of conditioning solvent	*	on/aspirate	*	300	2.5	5	load
b) filling of syringes	*	off/aspirate	*	937.5	3.75	--	load
c) wetting beads with conditioning solvent	*	on/dispense	*	200	1.5	2	load
d) aspiration of beads into HC	*	on/aspirate	*	500	3.75	2	load
e) withdrawal of used beads	*	on/dispense	*	1037.5	6.25	3	load
f) washing of tubing connecting to IV	*	on/dispense	on/dispense	500 (S2) 1000 (S3)	2.5 (S2) 5 (S3)	2	load

^a Volume and flow rates refer to syringe S2, excepted when indicated in parenthesis.

Figure S1. Elution profile attained for eluents with different composition^a



^aEluent volume refer to the volume propelled by syringe S2.

8

Conclusions

8.1 Contributions to the development of automatic sample preparation protocols

The automatic systems based on multisyringe flow injection analysis described in the present thesis, which carried out automatic SPE procedures, could be included in two different groups. In the first one (chapters 3 and 6), the manifolds were used as a research tool in order to obtain relevant information about the sorption (chapter 3) or the ability to be suspended and handled by the system. The second group, including chapters 4, 5 and 7, include new methodologies for the automatic extraction and determination of phenolic compounds, UV filters and riboflavin, respectively.

Flow injection analysis and derived techniques are valuable tools for research due to the repeatable working conditions attained by the automatic manifolds. Taking advantage of these features, three polystyrene-divinylbenzene sorbents with different characteristics, and that were able to trap phenolic compounds by a reversed-phase mechanism, were evaluated under exactly the same experimental conditions (chapter 3). The information gathered was important to understand the major interactions between the target analytes and the sorbents. Thus, for polymers with a low cross-linking degree (Amberlite XAD-4), hydrophobic interactions were prevalent, resulting in enrichment factors directly related with the hydrophobicity of the molecules. On other hand, for polymers with high-cross linking degree (Macronet-MN 200 and Lichrolut EN) a negative correlation between enrichment factor and molecular weight was found, indicating the existence of molecular exclusion mechanism besides the primary hydrophobic interactions. Therefore, the highest analytical performance was achieved for Lichrolut EN, which was selected for the further development of an analytical methodology aiming the determination of phenolic compounds in environmental samples (chapter 4).

Lab-on-valve was used as research tool in order to study the potential of different silica and polymer-based sorbents to be handled in the bead injection mode. The current state-of-art of bead injection excluded the use of heterogeneous size distribution materials due to the lack of precision during the microcolumn packing [1]. Therefore, new strategies for packing the microcolumns in a fully automatic fashion were achieved, by adjusting the fluidic protocols and / or the suspension milieu in a customized LOV design. Hence, LOV is now able to handle heterogeneous sorbent materials with high precision, enlarging the BI-LOV application scope as a front end to liquid chromatography.

Table 8.1. Flow based analytical methodologies developed for sample preparation coupled to liquid chromatography determination.

Analyte	Sample	Sample volume	Linear range	LOD	Repeatability (RSD %)	Determination frequency (det h ⁻¹)
Phenolic compounds ^a	Water and soil extracts	Up to 100 mL	150 – 3500 ng	< 1 ng mL ⁻¹ ^c	< 8%	4 – 10
UV filters ^b	Bathing waters	Up to 12.0 mL	60 – 1920 ng	0.45 – 3.3 ng mL ⁻¹ ^d	< 13%	6.7
Riboflavin	Foodstuff	1.00 mL	0.45 – 5.00 mg L ⁻¹	0.05 and 0.12 mg L ⁻¹ ^e	< 5.5	6

Note: ^a, including 2,4-Dinitrophenol; 2-Methyl-4,6-dinitrophenol; Phenol; 4-Nitrophenol; 2-Chlorophenol; 2-Nitrophenol; 2,4-Dimethylphenol; 4-Chloro-3-methylphenol; 2,4-Dichlorophenol; Pentachlorophenol; 2,4,6-Trichlorophenol; ^b, including benzophenone-3, butylmethoxydibenzoyl methane, ethylhexylmethoxycinnamate, homosalate; ^c, for a sample volume of 100 mL; ^d, for a sample volume of 12.0 mL; ^e, for infant milk formulas and pig liver, respectively.

Multisyringe flow injection systems, including a flow network (chapter 4) or a LOV device (chapters 5 and 7), were hyphenated to LC, aiming the automatic extraction by SPE and subsequent determination of different analytes in environmental and food matrices. The implementation of mass calibration [2] (Table 8.1) for the determination of phenolic compounds or UV filters created an enhanced dynamic linear range, extremely useful for environmental samples, which can be contaminated in broad range of concentrations. Furthermore, good precision (RSDs < 13%) and enhanced capacity of analyte detection, with LODs in the ng mL⁻¹ levels for the environmental applications, were also achieved.

In-line dilution of the eluate in order to match the organic / aqueous composition of the LC mobile phase using a single propulsion device can be only performed by MSFIA systems. Taking advantage of the multichannel propulsion, eluate and diluent were merged after a T junction, and the resulting mixture filled the loop of the high-pressure injection valve. This strategy allowed the introduction of unusual volumes of sample into the chromatographic system (up 400 µL) without any noticeable broadening or overloading effects in the chromatographic peaks.

By performing the sample preparation procedure and the chromatographic analysis in the same time frame, the time necessary to complete the whole analytical procedure was always below fifteen minutes (Table 8.1) in all cases. This drastic reduction of the analysis time in the developed methodologies resulted from two complementary factors. The first one is the adjustment of SPE protocols to the flow injection format, where protocols can be simplified and the solutions are easier and faster changed than in manual batch procedures (chapter 4). Furthermore, the whole SPE protocol was downscaled (chapter 5 and 7). The second reason was the use of monolithic columns as stationary reversed-phases [3], which contributed for the reduction of the time necessary for the chromatographic run. Analysis time was reduced, at least, in a factor of 14, 5 and 2 for the determinations of phenolic compounds, UV filters and riboflavin, respectively, when compared to other methodologies for the analysis of the same target analytes [4-17]. Therefore, the reported methods are also suitable tools for screening analysis.

As mentioned above, monolithic columns were chosen as stationary phase. These columns showed a high-robustness during long-term usage and allowed a drastic reduction not only in the analysis time but also in the working pressures of the chromatographic system. Furthermore, the separative performance attained was adequate for the samples or analytes under analysis in all situations.

8.2 New contributions from the developed methodologies under the scope of green analytical chemistry

The new automatic methodologies for sample preparation introduced in the present dissertation demonstrated to be “greener” alternatives to its predecessors. Hence, the organic solvent consumption was minimized and did not exceed 4.1 mL per determination of phenolic compounds (Table 8.2), corresponding to an improved environmental performance when compared to other automatic approaches [4, 8-11, 14]. For the miniaturized SPE procedures, reported in the chapters 5 and 7, this value was even low, corresponding to 1.55 and 0.86 mL, respectively. For the determination of UV filters in bathing waters this represented a reduction higher than 90% compared to earlier reported SPE methods [6, 12, 16], whereas for the quantification of riboflavin in foodstuff samples, a reduction of 72% in the organic solvent consumption was attained compared to the batch procedure endorsed by the MIP supplier [18].

A reduction in effluent generation during the SPE procedure was also noticed in the proposed methodologies (Table 8.2) when compared to other automatic (chapter 4) or batch methodologies (chapter 5 and 7). By this way, regardless the sample volume, waste generated per determination was comprised between 9.1 and 15.2 mL. Another important point is the composition of the effluent. Considering that organic solvent consumption was also reduced, the major components of the effluent were, besides the sample matrix, diluted acids ($\text{HCl } 10^{-2} \text{ mol L}^{-1}$ or acetic acid 2.4% (v/v)) or water, which are non-persistent pollutants.

Table 8.2. Reagents (organic solvent and sorbent) consumption and effluent generation of the proposed methodologies^a.

Methodology	Organic solvent (mL)	Effluent (mL)	Sorbent (mg)
Phenolic compounds	4.2	9.1	n. a.
UV filters	1.55	11.1	6
Riboflavin	0.86	15.2	11.2

Note: ^a, values obtained for sample preparation not including sample volume; n. a., not applicable.

8.3 Future trends and perspectives

Considering the latest advances in the liquid chromatography area, essentially related with the introduction of new instrumentation and stationary phases, it is clear that sample preparation will be still the limiting factor for the development of sensitive and high-throughput LC methodologies.

In this context, automation based on flow injection and derived techniques represent a valuable asset due to its low cost and flexibility compared to the present state-of-art based on robotic stations for sample preparation. As demonstrated by the present work, these techniques are quite amenable to perform automatic sample preparation and coupling to conventional LC equipment, resulting in a reduction of the analysis time for a scale where flow injection-LC methodologies could be easily used as screening tools.

On other hand, mass spectrometry detectors, which require very complex sample treatment procedures, are now emerging and assuming an important role in the next generation of analytical methods. In this particular case, flow injection analysis can represent an alternative to the current manual or robotic procedures available for sample preparation. In fact, there are a few applications emerging in this area [19], showing this field as a promising and fruitful area of research.

Finally, the subject of the present dissertation is a undeniably, an open-ended topic with proved growing interest about it, considering the rising number of publications reported in last years. On-line renewable SPE in non-robotic format is a unique feature of flow injection analysis and new methodologies relying on this strategy are expected in the next years. Furthermore, with new sorbents available and improved technology, flow injection based manifolds will definitely be able to process more complex samples with an enhanced performance, towards meeting green analytical chemistry requirements.

8.4 References

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