CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF ANALYTICAL CHEMISTRY

APPLICATION OF NON-SEPARATION FLOW METHODS IN PHARMACEUTICAL ANALYSIS

Dissertation thesis

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UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

KATEDRA ANALYTICKÉ CHEMIE

VYUŽITÍ NESEPARAČNÍCH PRŮTOKOVÝCH METOD VE FARMACEUTICKÉ ANALÝZE

Dizertační práce

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STATEMENT OF ORIGINALITY

I declare that this thesis is my own work and all the information sources used here were properly cited. This work was not used to obtain any other title.

PROHLÁŠENÍ O PŮVODNOSTI

Prehlasujem, že táto práca je mojím pôvodným autorským dielom, ktoré som spracovala samostatne. Všetka literatúra a ďalšie zdroje, z ktorým som pri spracovaní čerpala, sú uvedené v zozname použitej literatúry a sú riadne citované.

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V Hradci Králové dne

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ABSTRACT

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Application of non-separation flow techniques in pharmaceutical analysis

Flow techniques are a branch of instrumental techniques in chemical analysis based on the handling of the sample in a tubing manifold. Since the introduction of what is recognized nowadays as modern flow techniques in the early seventies, this analytical approach gained popularity among scientist thanks to their main principles. Controlled dispersion and precise timing due to the programming enable full automation of a variety of analytical procedures.

Flow methods were employed in a multiple different applications: simple sample measurement, chemical reactions, kinetic studies, sample pretreatment, separation, and others in an automated way. They are especially valued for automation, decrease of chemicals consumption and thus lower cost and waste production, and shortening of the analysis time.

This dissertation presents new contributions to the field of flow techniques. It is divided into a theoretical part and an experimental part, the latter one listing five publications and one manuscript submitted for publication.

The theoretical part comprises three main chapters: sample pretreatment methods, flow methods, and selected sample handling methods in flow techniques. An overview is given on sample pretreatment and related procedures, and especially modern microextraction techniques are emphasised here. Both liquid and solid phase-based methods are discussed further.

The definition and basic instrumentation of flow techniques is described in the following section, highlighting the ones applied in the experimental works. The main features of each technique are briefly discussed including the technical differences and performance.

The third chapter is devoted to the applications of flow techniques in several sample handling procedures, applied in analysis of pharmaceuticals in their formulation or in a biological sample, or in analysis of other biologically active substances. It includes applications of enzymatic reactions and use of irradiation as well as the possibility of automation of selected microextraction techniques in a flow manifold.

The experimental part comprises five publications and one manuscript currently submitted for publication. Each of the attached publications is accompanied by a short comment clarifying the

most interesting features of the respective work, the method development, and a discussion about the novelty of the presented methods.

The first experimental work is focused on the execution of a reaction catalysed by an enzyme in a sequential injection system with spectrophotometric detection. The method is applied to the determination of an anaesthetic drug propofol in emulsion and compared to a simple flow method with fluorimetric detection.

The second experimental work studies the use of a Sequential Injection Analysis system for the automation of a liquid-liquid microextraction and a modified method for dispersive liquid-liquid microextraction of thiocyanates in human saliva samples.

In the third experimental work, a novel mode of head-space single drop microextraction performed in an In-syringe system applied to the determination ethanol as a model volatile analyte is described.

The fourth experimental work deals with solid phase based microextractions carried out on-line. A use of a flow-batch system for SPE, UV-photodegradation and a fluorimetric determination of the pesticide metsulfuron methyl is presented as an application from the field of environmental analysis that corresponds to the scope of cooperation with the Department of Chemistry at the University of the South in Bahía Blanca, Argentina.

The fifth experimental work was focused on the study of conditions influencing fluorescence of two model substances in a sequential injection system.

The last, sixth experimental work shows a novel mode of automation of microextraction by packed sorbent coupled directly to separation in a low pressure sequential injection chromatographic system. The method was applied to the determination of betaxolol in human urine samples and the work is presented as a manuscript submitted for a publication in a scientific journal with impact factor.

ABSTRAKT

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Název dizertační práce:

Využití neseparačních průtokových metod ve farmaceutické analýze

Prietoková analýza je jedným z odvetví inštrumentálnych techník v chemickej analýze. Je založená na manipulácii so vzorkou v prietokovom zariadení. Od uvedenia techník, považovaných dnes za moderné prietokové techniky, v sedemdesiatych rokoch minulého storočia, si tento analytický prístup vďaka svojim charakteristikám získal medzi vedcami popularitu. Riadená disperzia a presné časovanie vďaka programovaniu umožňuje plnú automatizáciu rôznych analytických procesov.

Prietokové metódy sú využívané v mnohých aplikáciách: jednoduché meranie vzoriek, chemické reakcie, kinetické štúdie, úprava vzorky, separácia a ďalšie, a to v automatickom móde.

Tieto techniky sú cenené hlavne kvôli automatizácii, zníženiu spotreby chemikálií, vyprodukovaného odpadu a nákladov na analýzu, skráteniu času analýzy.

Táto dizertácia predstavuje ďalší prínos v oblasti prietokovej analýzy. Je rozdelená na teoretickú a praktickú časť, pričom praktická časť obsahuje päť publikácií a jeden manuskript odoslaný k publikácii v odbornom časopise.

Teoretická časť je ďalej rozdelená na tri podkapitoly: metódy úpravy vzoriek, prietokové metódy a vybrané techniky manipulácie so vzorkou s využitím prietokových metód. Práca obsahuje prehľad metód úpravy vzoriek a príbuzných techník, a sústredí sa hlavne na moderné mikroextrakčné postupy. Sú zahrnuté ako extrakčné metódy z kvapaliny do kvapaliny, tak aj extrakcie tuhou fázou.

Ďalší oddiel teoretickej časti sa venuje definíciám a základnej inštrumentácii používanej v prietokových technikách, s dôrazom na tie, ktoré boli využité pri experimentálnej práci. Stručne sú predstavené hlavné charakteristiky každej techniky, vrátane technických rozdielov a prevedenia.

Tretí oddiel teoretickej časti popisuje využitie prietokových techník v rôznych metódach manipulácie so vzorkou. Tieto metódy boli použité na analýzu liečivých látok buď vo farmaceutickom prípravku alebo v biologickej vzorke, alebo v analýze ďalších biologicky aktívnych látok. Zahrnuté je využitie enzymatických reakcií a použitie žiarenia v prietokovom systéme ako aj možnosť automatizácie vybraných mikroextrakčných postupov s využitím princípov prietokových techník.

Praktická časť predloženej dizertačnej práce obsahuje päť publikácií a jeden rukopis v súčasnosti odoslaný k publikácii. Ku každej z predložených prác je priložený krátky komentár, v ktorom sú popísané najzaujímavejšie charakteristiky, vývoj metódy a novosť danej práce.

Prvá experimentálna práca je zameraná na prevedenie reakcie katalyzovanej enzýmom v sekvenčnom injekčnom systéme so spektrofotometrickou detekciou. Táto metóda je použitá na stanovenie anestetickej látky propofolu vo farmaceutickom prípravku. Zároveň je táto metóda porovnaná s jednoduchou prietokovou metódou s flourescenčnou detekciou.

Druhá experimentálna práca študuje použitie systému sekvenčnej injekčnej analýzy na automatizáciu mikroextrakcie z kvapaliny do kvapaliny a modifikovanú metódu na disperznú mikroextrakciu tiokyanatanov vo vzorkách ľudských slín.

Tretia práca popisuje nový spôsob mikroextrakcie do kvapky v priestore nad vzorkou, a to priamo v striekačke piestovej pumpy. Ako modelová analýza prchavého analytu bol pomocou vyvinutej metódy stanovený obsah etanolu vo víne.

Štvrtá experimentálna práca sa zaoberá on-line extrakciou tuhou fázou. Je popísané využitie „flow-batch“ systému na extrakciu, UV-fotodegradáciu analytu a fluorimetrické stanovenie pesticídu metsulfuron metylu. Práca teda spadá do oblasti kontroly životného prostredia, čo odpovedá oblasti zamerania spolupracujúceho zahraničného pracoviska (Department of Chemistry, University of the South, Bahía Blanca, Argentína).

Ďalšia, piata, experimentálna práca bola zameraná na štúdium podmienok ovplyvňujúcich fluorescenciu dvoch modelových látok v systéme sekvenčnej injekčnej analýzy.

Posledná, šiesta práca predstavuje novú metódu automatizácie mikroextrakcie na tuhom sorbente, s priamym spojením na separáciu v nízkotlakovom systéme sekvenčnej injekčnej chromatografie. Metóda bola použitá na stanovenie liečiva betaxololu ľudskom moči. Práca je priložená ako manuskript odoslaný na publikáciu v odbornom časopise s impakt faktorem.

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INDEX OF ABBREVIATIONS

AAS	atomic absorption spectrometry
AIA	all injection analysis
BA	batch analysis
BI, BIA	bead injection, bead injection analysis
CE	capillary electrophoresis
CFA	continuous flow analysis
CF-SDME	continuous flow single-drop microextraction
cFI	continuous flow injection
CPG	controlled pore glass
DI-SDME	direct immersion single-drop microextraction
DI-SPME	direct immersion solid phase microextraction
DLLME	dispersive liquid-liquid microextraction
DPX	disposable pipette tip extraction
dSPE	dispersive solid phase extraction
DV-SIA	dual-valve sequential injection analysis
EC	extraction cell
ETAAS	electrothermal atomic absorption spectrometry
EU	extraction unit
FAAS	flame atomic absorption spectrometry
FIA	flow injection analysis
FBA	flow-batch analysis
HF-LPME	hollow-fibre liquid-phase microextraction
HPLC	high performance liquid chromatography
HS-SDME	head-space single-drop microextraction
HS-SPME	head-space solid phase microextraction
i.d.	inner diameter
ICP-MS	inductively coupled plasma mass spectrometry
IS	immunosorbents
ISA	in-syringe analysis
LAV	lab-at-valve
LC	liquid chromatography
LLE	liquid-liquid extraction
LLLME	liquid-liquid-liquid microextraction
LIS	lab in a syringe
LOV	lab-on-valve

LPME	liquid-phase microextraction techniques
MEPS	microextraction by packed sorbent
MCFIA	multicommutated flow injection analysis
MIP	molecularly imprinted polymer
MPFS	multipumping flow system
MSFIA	multisyringe flow injection analysis
MSM	metsulfuron methyl
pFI	programmable flow injection
PDMS	polydimethylsiloxane
PEEK	polyether ether ketone
PP	protein precipitation
PTFE	polytetrafluorethylene
RAM	restricted access material
SBSE	stir bar sorptive extraction
SDME	single-drop microextraction
SFA	segmented flow analysis
SIA	sequential injection analysis
SIC	sequential injection chromatography
SPE	solid phase extraction
SPME	solid-phase microextraction
UV	ultraviolet
VIS	visible

1 INTRODUCTION

Flow analysis represents a well-established group of analytical techniques. Most well-known flow injection analysis and related techniques are based on the introduction and manipulation of a sample (including detection) in a stream of a carrier in a closed, low-pressure tubing manifold built up of a pump, valve(s), tubing and the detection cell. Since its introduction in the 1970´s [1] (taking the experience from previous similar techniques such as continuous flow analysis), it evolved into various new related techniques, branches, or generations. Although these may differ in the flow characteristics and handling in the course of one analytical run, for most of them the introduction of sample into a stream of carrier remains the mutual attribute.

The field of application of flow techniques is wide – from measurements and analysis in agriculture, environment, oceanography, and food analysis, through pharmaceutical and biomedical field to biosensors and basic research. The number of areas demonstrates that the features of flow techniques such as versatility, programmable flow, repeatability, and precise sample handling contribute benefits to these fields. Since coupling chromatographic columns to a flow manifold in 2003 [2], separation is also included in the scope of flow analysis, which then represents a complementary (not competitive!) tool to chromatography.

To obtain reliable results of any analysis, one must have a representative sample treated in such a way that it is possible to use the chosen detection technique. This can include extraction, clean-up, or in a broader context, change of the physicochemical (optical) properties of the sample. Therefore, sample pretreatment is of high importance and must be performed precisely and reproducibly. In this context, the features of flow techniques (comfortable way of handling solutions in a closed system, repeatability, precise sample handling, and programming) invite to use flow systems in such important and wide area of application as automated sample pretreatment.

Sample pretreatment is not a laboratory-specific field, but in contrary every analyst has to prepare samples for their analysis. Hence, the trends are decreasing the time of sample pretreatment, automation, and downscaling of the solvents and sample volumes. Flow analysis has its specific features to accomplish these requirements.

Precise timing is a key factor also in kinetic studies or e.g. enzymatic reactions. In time based studies, for instance light induced reactions, optimization and precise programming and thus timing is crucial.

In this thesis, exploitation of flow techniques for new modes of automation of different microextraction techniques as well as for the automation of an enzymatic reaction and photo-induction prior to a fluorimetric measurement is presented in form of five publications and one manuscript submitted for publication, including a theoretical discourse of flow methods, sample pretreatment techniques and examples of sample handling in a flow system.

2 OBJECTIVES

The general objective of this thesis was to study and verify the use of non-separation flow techniques in the analysis of biologically active substances (mainly in frames of pharmaceutical analysis) and to develop new methods based on these techniques, focusing on the automation of the analytical procedures.

The work aimed on the development of various automated sample pretreatment techniques, coupled on-line with the detection step and using the technique of sequential injection analysis. The goal was to develop methods for both solid phase- and liquid phase-based techniques: solid phase extraction, microextraction by packed sorbent, liquid-liquid microextraction and head-space single-drop microextraction. The automation of an enzymatic reaction in a flow system was also included.

The goal was to apply these methods to the treatment of different kinds of samples, ranging from pharmaceutical preparation (emulsion), biological (such as human saliva and urine) to food (wine) and environmental (surface water) samples.

The specific objectives were:

a) Development of an automated system to perform an enzymatic reaction for determination of an anaesthetic drug in its pharmaceutical formulation, to study the chemical conditions of the reaction and to test the applicability of the method to real sample measurement.
b) Comparison of the conditions for fluorescence detection in a flow system for different solvents in case of a naturally fluorescent substances and a substances with photo-induced fluorescence;
c) Development of method enabling a sample clean-up, light-induced decomposition, and detection in one fully automated system based on flow-batch principles;
d) Assembling of an analyser system for the automation of liquid-liquid microextraction and dispersive liquid-liquid microextraction and elimination of the problem of mixing two different phases in one flow system, optimization of all kinetic and dynamic conditions of the reaction and the flow system; on-line determination of biologically active substance in a complex matrix;
e) Evaluation of the possibility of automation of microextraction by packed sorbent by a sequential injection chromatographic system and optimization of the conditions for treatment of biological samples for the determination of betablockers;
f) Development and optimization of an automated system for head-space single-drop microextraction and testing the applicability of this system for determination of a model volatile substance.

3 THEORETICAL PART

3.1 SAMPLE PRETREATMENT TECHNIQUES

3.1.1 General characteristics and requirements on sample pretreatment

The development of a method enabling direct and fast measurement of an analyte in an untreated or only minimally treated sample is the aim of research of many analytical chemists. However, most analytical methods do not offer this possibility. Usually, at least a simple intervention is necessary to obtain a measurable sample even in non-complex matrices. The aim of the intervention is to obtain a sample, in which it is possible to detect or reliably quantify the analyte of interest.

Such intervention includes either the isolation of the analyte from the matrix or decreasing the quantity of interfering compounds from the matrix to a minimum (without losing the analyte) or modification of the analyte of interest into a measurable form, e.g. by a chemical reaction.

Figure 1 gives a chart of the time spent on the respective steps of an analysis in average for the most frequent analytical techniques such as chromatography. It shows that as much as 60% of the total analysis time is spent on sample pretreatment, 27% on data treatment, 6% on sampling, and only 6% on the actual analysis. The time devoted to sample treatment is justified as the performance of this step is crucial for the whole analytical procedure, since a mistake in this step can affect the result of the entire analysis without the possibility of corrections in later stages of the analysis.

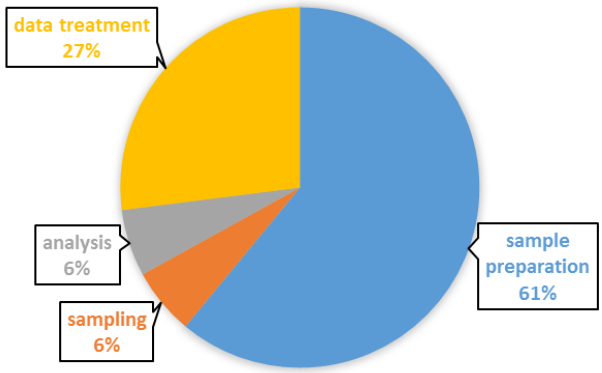

Figure 1: Time spent on an average chromatographic analysis[1].

[1]Adapted from [3].

General requirements on a sample pretreatment technique include:

- Selectivity/universality – depending on the purpose of the work, highly selective techniques or universal techniques suitable for a large range of substances might be preferred;
- Simplicity of operation and performance – the more complicated the technique, the more training of the operator is required, the higher the risk of errors in the procedure and the lower the sample throughput;
- High sample throughput – related to the previous point; relevant e.g. for laboratories carrying out routine analyses;
- Low sample consumption – as the sample quantity might be limited, e.g. biological samples;
- Low consumption of solvents – to copy with the principles of green chemistry, to reduce the exposition of the operator to vast toxic solvents vapours, and to reduce the quantity of produced waste together with the cost of analysis; especially important in laboratories with high sample throughput;
- Possibility of automation and compatibility with the used analytical manifold – automation accelerates and facilitates the analysis and significantly reduces the risk of errors in analysis;
- Low initial (e.g. instrument purchase, stuff training) and performance cost (e.g. maintenance);
- Prevention of analyte loss – a high recovery is crucial especially if the analyte concentration is low.

The selection of a sample pretreatment method is based on several considerations. The cost of analysis is always taken into account. Then, the available quantity of the sample volume plays an important role, as different laboratories work with different materials (e.g. clinical laboratories compared to waste waters analysis). Another factor is the need for preconcentration; this is more important for less sensitive detection techniques (e.g. spectrophotometry) than for very sensitive ones (e.g. mass spectrometry). The type of laboratory plays an important role; routine analyses with high sample throughput would require a sample pretreatment method, which is not significantly time consuming and costly in the sense of development and performance, on the other hand time might be of less importance e.g. for a research laboratory. Depending on the application, it should also be decided about usage of a more selective or a more universal methodology. The complexity of the method should be taken into account too, since the more steps the sample pretreatment has, the higher the chances of an error in the protocol.

The stability of the sample and its compatibility with the pretreatment technique must be considered such as the solubility in different solvents, buffer addition or thermic stability. For example, a sample heating cannot be used to accelerate the mass transfer in head-space extraction for substances, which decompose at higher temperatures.

The simplest sample preparation method is dilution. It does not require any instrumentation and the effect of matrix can be already significantly decreased, but the method´s sensitivity for the analyte can become a crucial factor. Other simple but not especially selective methods are centrifugation and/or filtration, which are feasible when sensitivity is not a limiting factor of the methodology and especially high selectivity is not required. However, one should bear in mind the possible loss of analyte on the filter or on the sedimented material.

Protein precipitation (PP) is a relatively old method (since 1950´s), but it is useful in laboratories designed for screening and with a very high sample throughput. The addition of an organic solvent, acid, or salt to the sample leads to protein denaturation and possible precipitation. Although some analyte can be lost by adsorption to the precipitate, this methodology remains, apart from simple sample dilution, one of the fastest and least costly. Nowadays, this method can be fully automatized by using a 96 well plate, while automation by flow techniques is not typical.

In the following chapters, the sample pretreatment techniques based on extractions are described. Both the conventional and modern micro-scale liquid- altogether with solid-phase extraction methodologies are included.

An emphasis is given to those techniques where automation by flow techniques has been extensively explored and which are of high relevance to the experimental works presented in this thesis in chapter 4. The aim of this part is not to give a comprehensive list of all developed techniques but to present an overview of them as a comparison to the methods presented in this thesis. Therefore, membrane-based sample pretreatment methods such as gas diffusion are not included in this work.

3.1.2 Liquid phase-based techniques

The liquid phase-based sample pretreatment techniques or the liquid phase microextractions (LPME) are based on a use of a liquid extraction agent while the sample can be either in solid or in liquid form.

3.1.2.1 *General principles of liquid phase-based techniques. Classical methods*

LPMEs are based on a different solubility of an analyte in two (or more) solvents, expressed as the partition coefficient (logP):

$$\log P = \log K_{1:2} = c_1 / c_2$$

where c_1 is the concentration of an analyte in phase 1, e.g. octanol, and c_2 is the concentration of the analyte in phase 2, e.g. water.

Ideally, the analyte is more soluble in the extraction solvent than in the sample solvent, usually water. In some cases, e.g. for analytes with appropriate acido-basic properties, the solubility of the analyte in the extraction solvent can be modified by the changing the pH (reduction of the analyte ionization), or ion coupling with a counter-charged reagent is performed to achieve a neutral complex soluble in an organic solvent (if the sample is aqueous and the analyte is ionic).

The conventional method, used for sample clean-up, extraction, and preconcentration of the analytes of interest, is denoted liquid-liquid extraction (LLE, Figure 2).

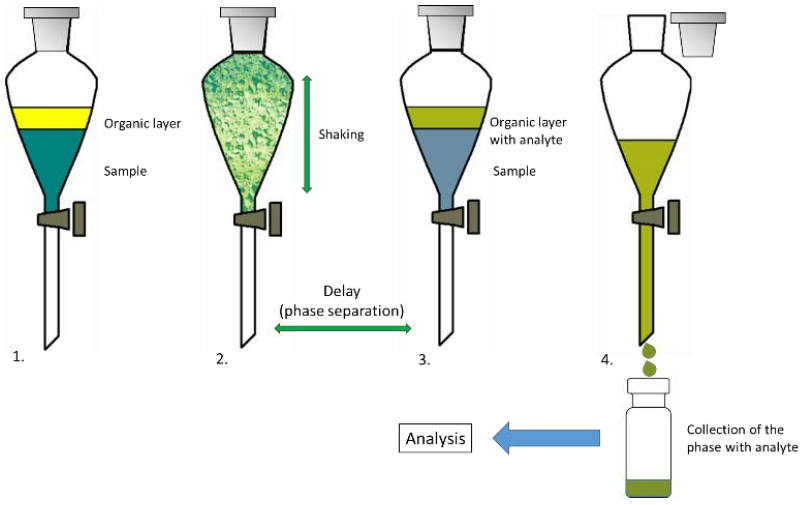

Figure 2: Classical liquid – liquid extraction in a separatory funnel.

It uses the sample and one extraction solvent. The traditional LLE is performed in a separatory funnel or a flask, which sizes range from 10 mL to several litres and the volume of the extractant solvent can reach hundreds of microliters to ten of millilitres [4]. The classical LLE is simple, method development and performance are typically not time consuming and no special instrumentation is required. LLE techniques are generally used for screening purposes or in

combination with very selective detection techniques. The selectivity can be improved by a derivatisation reaction performed in one step along with the extraction. However, large volumes of both the sample and the organic solvent consumed for the extraction, together with large waste production, low sensitivity or the need of evaporation of a large extraction solvent volume before further analysis are significant drawbacks of this method. Although the range of applications is wide and the performance is simple, downscaled formats are preferred nowadays.

3.1.2.2 *Liquid-liquid microextraction sample pretreatment techniques*

3.1.2.2.1 Generals remarks on liquid - liquid microextraction

Liquid phase microextraction techniques are based on classical LLE, however, the volumes of both the sample and extractant solvent are significantly reduced. The sample volumes can be downscaled from 40 - 50 mL in environmental analysis [4] to a few tens of microliters in case of biological samples. The volume of the extractant solvent in a miniaturized liquid extraction technique ranges typically between 1 - 20 μL [4].

Miniaturized versions of LLE were introduced in the lists of sample pretreatment methodologies in the 1990´s. The first experiments with a very low volume of extractant were published by Liu and Dasgupta in 1995 using a single drop for extraction of NH_3 and SO_2 from air [5]. This can be considered a pioneering work for all liquid-phase microextraction techniques. The modern LPME techniques overcome several of the above mentioned drawbacks of the conventional LLE, using lower volumes of sample as well as the organic solvent/acceptor phase per analysis and feasible automation. Also, higher preconcentration or enrichment factors are usually achieved.

Different criteria to classify LPME techniques can be found in the literature. One classification divides these techniques according to the mode of performance of the contact between the sample and the extractant solvent. Based on this criterion, the LPME mode can be divided into direct immersion, head-space, dispersive or hollow fibre protected (two or three phases) [4].

The large growth in the number of new, modified or improved liquid phase microextraction techniques documents the fact that these have a significant advantage over the traditional sample pretreatment techniques. Pena-Pereira et al. reported that more than 50 new methodologies were developed since 1995 [6]. Several other reviews on liquid-phase microextraction techniques were published: in the work of Pena-Pereira et al. the authors reviewed single drop microextraction, hollow-fibre microextraction and dispersive liquid - liquid microextraction [7]. In another work from Nováková and Vlčková from 2009, new trends in chromatography and modern sample preparation methods used in bioanalysis were discussed [8]. Recently (2014), the LPME techniques were overviewed by He [9]. Cost and time savings, ease of performance, and application to a wide range of samples [4] are several of the profits of liquid phase microextraction techniques.

3.1.2.2.2 Single-drop microextraction

The first use of a single drop as an acceptor medium for gaseous analytes from air was presented in 1995 by Liu and Dasgupta [5]. A drop of the sulphuric acid or hydrogen peroxide in manganese(II) sulphate was used to preconcentrate NH_3 and SO_2, respectively, from a gaseous sample. One year later, Jeannot and Cantwell used a single drop supported by a PTFE probe for extraction of a model compound analysed later by gas chromatography (GC) [10]. In 1997, the same authors described the idea of using single-drop microextraction (SDME) where a drop of an organic solvent was exposed to a sample by means of a syringe and the syringe´s needle served as the drop support [11]. After extraction and retraction of the needle, the syringe was used to inject an aliquot directly into the GC system. Since then, this technique gained a lot of attention and evolved into several modifications differing in the mode (static versus dynamic) or in the means of assuring the contact of the analyte with the acceptor phase (e.g. head-space, direct immersion, or continuous flow SDME) [12, 13]. The main advantages of this type of microextractions are high preconcentration factor, ease of performance, and no other special instrumentation requirement than a syringe.

Direct immersion single-drop microextraction

The origin of direct immersion single-drop microextraction (DI-SDME) dates back to 1996, when Liu and Dasgupta published a work about the so-called drop-in-drop system [14] and Jeannot and Cantwell described a system with a drop located at the end of a PTFE rod immersed in an aqueous sample [10]. Two modes of SDME can be applied. In a static mode, the drop of the extractant is held above or in the sample bulk. In the dynamic mode, the sample is continuously exchanged. This mode assures a constant analyte transfer into the drop.

In DI-SDME, a drop of the extractant is directly immersed into a sample. It can be performed in both the static and dynamic modes. Effective distribution of the analyte and its transfer into the acceptor drop is assured by stirring [4]. After a defined (optimised) period of time of enrichment, the drop is retracted back into the microsyringe and is subjected to further analysis. In this mode, the acceptor phase comes into direct contact with the sample, and therefore has to be water immiscible. The choice of the solvent has to be carefully considered, as solvents partly soluble in water are not suitable in this case. Attention must also be also paid to the drop stability – a higher stirring speed can dislodge the drop.

Head-space single-drop microextraction

Tankeviciute et al. and Theis et al. described the development of a head-space single-drop microextraction (HS-SDME) in 2001 [15, 16]. This technique is based on exposing a drop of an acceptor phase in the gas phase above the sample, so denoted head-space. Therefore, the sample can be solid, liquid, or even gaseous. The principle of the HS-SDME is depicted in Figure 3. The

analyte evaporates from the sample into the head-space and is adsorbed over time into the drop, hence the duration of the drop exposition must be studied.

The mass transfer from the sample into the head-space can be enhanced in several ways:

1: Sample stirring,

2: Increased temperature of the sample,

3: Ultrasound,

4: Decreased/depressed pressure [17].

In HS-SDME, the acceptor phase does not come into direct contact with the sample, therefore the use of organic solvents can be avoided. Instead, an aqueous phase can be used with advantage regarding the requirements of green analytical chemistry. Sensitivity and selectivity can be increased if a derivatisation reagent is used in the acceptor phase. On the other hand, this method is only suitable for volatile or semi-volatile analytes, which is a limiting factor for a wider use of HS-SDME. The duration of enrichment can be the limiting factor of the total analysis time and sample throughput. Also, the stability of the drop at the end of the needle can be problematic, especially at larger volumes (>10 µL). On the other hand, using a very small volumes of the drop might result in the drop evaporation and worsening the reproducibility. Generally, the drop volume is smaller than 5 µL, since larger volumes can also cause peak tailing in chromatographic analysis [18]. The principle of HS-SDME designates this method to be used conveniently in combination with chromatographic methods, especially GC [18].

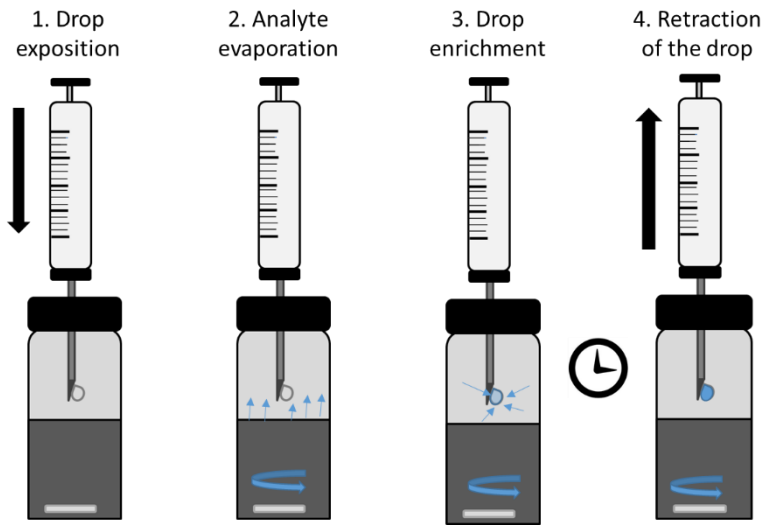

Figure 3: Head – space single drop microextraction.

Continuous flow single-drop microextraction

Continuous flow single-drop microextraction (CF-SDME) is a dynamic variant of SDME. It was introduced in 2000 [19] and the authors reported concentration factors of 260-1600 within an extraction time of 10 min. In this study, a glass chamber was used as an extraction vessel with a

septum-port for a syringe needle, the needle being located over a sample´s inlet. Then, the sample was pumped through the chamber and at the same time, a drop of 1-5 μL of the acceptor phase was generated at the needle´s tip. The drop was then exposed to the flowing sample through the opening in the glass chamber, so that it was always in contact with the same concentration of the analyte in the sample. As a consequence, a constantly high extraction rate can be achieved, while in static mode the concentration gradient and thus the extraction rate between sample and drop decreases over time.

In such a dynamic assembly, the drop is more prone to being dislodged, and therefore, a careful choice of the solvent must be done and flow rate optimization must be performed. Another disadvantage of this technique is a relatively large sample consumption taking into account its continuous flow. Another modification of this technique is called cycle-flow SDME [20], which solves this problem by pumping the sample back into the sample reservoir instead to the waste.

Liquid-liquid - liquid single-drop microextraction

This variation of the single-drop microextraction (LLLME) [21] is a static method based on a three phase system, where the final acceptor phase is an aqueous phase. Presented first in 1999, this method uses a layer of organic phase to extract an analyte from a sample, and from this layer simultaneously into an aqueous acceptor phase (back-extraction). This process is achieved by pH adjustment, so that the analyte is in non-polar/non-ionized state in the sample for ideal extraction into the organic phase and is transferred into its polar/ionized state by the aqueous acceptor phase, e.g. acidification of the sample for the extraction of phenols. Analytical systems requiring an aqueous sample can be coupled with this pretreatment technique, on the other hand, the operation is more complicated when compared to simple SDME [7].

3.1.2.2.3 Dispersive liquid - liquid microextraction

The development of dispersive liquid - liquid microextraction (DLLME) is dated back to 2006 and was first reported by Rezaee et al. [22]. A three-component system is employed in this technique. A very small quantity of the extraction solvent (in the range of μL) is mixed with a larger volume of another solvent, denoted as dispersive solvent. The mixture is injected rapidly into an aqueous sample and as a result, a cloudy solution is created (Figure 4). The function of the dispersive solvent is to increase the contact of the extraction solvent with the sample by dispersing it into small droplets in the sample volume. After this step, the layers are separated by their densities (by centrifugation or by a simple separation by gravitation) and the organic layer with the extracted analyte is submitted for further analysis.

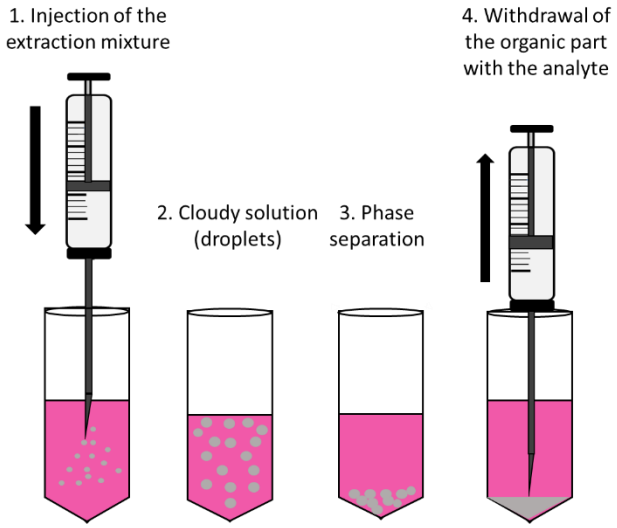

Figure 4: Dispersive liquid - liquid microextraction with extraction solvent of denser than water.

The advantage of DLLME are in the reduced volume of the extraction solvent and increased enrichment factor due to the larger surface, and in the speed of further manipulation in cases when evaporation of the solvent is necessary prior to the next processing. The following advantages are no need for any special instrumentation or devices and the speed of the process due to the direct and significantly enlarged contact area between the sample and the extraction solvent, especially in comparison to SDME. Also, addition of a derivatisation agent to the extraction solvent is possible to increase the sensitivity and/or selectivity of the analytical methodology [23]. In the contrary, the combination of solvents intended for use has to be tested, since several conditions must be fulfilled: the extraction solvent must be water immiscible and the analyte must be very well soluble in it.

Originally, solvents with density higher than water were preferred due to easier retraction of the solvent with the extracted analyte from the bottom of the extraction vessel. Nowadays, also alternative methodologies were suggested, corresponding more to the demands of the green chemistry concept. For the extraction, solvents lighter than water are also used. Difficulties with withdrawing the organic part after the phase separation were overcome e.g. using modified vessels or by solidification of the organic drop by cooling. A review of the DLLME modifications using solvents lighter than water and corresponding instrumentation is given elsewhere [24]. The traditionally used chlorinated organic solvents can be replaced by more environment-friendly ionic liquids. These kinds of solvents have been gaining popularity recently and a review on their employment in DLLME was published lately [25].

The dispersive solvent must be soluble in both organic and aqueous phases, and not be a good solvent for the analyte, so that the analyte migrates preferably into the extraction solvent. To achieve dispersion, the action of the dispersive solvent can be either supported or replaced by

application of ultrasound [26], increased temperature [27], decreased temperature [28], addition of salts [29], stirring [30], vortexing [31] and bubbling [32].

3.1.2.2.4 Hollow-fibre liquid phase microextraction

A three-phase system (similarly as in LLLME) denoted as hollow-fibre LPME (HF-LPME) was presented in 1999 [33]. In this case, a hollow fibre (usually polypropylene) is used as a support for a microliter volume of the acceptor phase, so that it is more stable than in the case of SDME [9]. The fibre is attached to a needle of a syringe and initially immersed into an organic solvent, so that the pores of the fibre wall are impregnated with the solvent, as depicted in Figure 5. The lumen of the fibre is filled with the acceptor solution. The acceptor can be identical with the solvent in the walls and the system is then two-phased, which is convenient as a sample pretreatment for a GC analysis. Alternatively, it can be filled with an aqueous solution and is then compatible with HPLC or capillary electrophoretic (CE) systems [8]. When compared to the previously mentioned techniques, the manipulation with the fragile fibre is less convenient but on the other hand, the organic phase is better protected from the particles contained in the sample matrix. The sample to acceptor phase ratios are large, so high enrichment factors can be achieved using this technique [8].

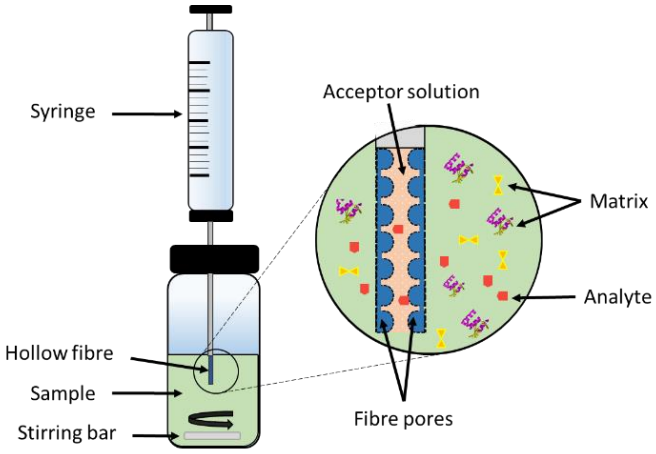

Figure 5: Hollow-fibre liquid - liquid microextraction

3.1.2.2.5 Salting out liquid - liquid extraction and QuEChERS

Salting-out is based on decreasing the solubility of an organic solvent in aqueous media by increasing the salt concentration. The range of solvents potentially used for extraction is enlarged by the water-miscible ones such as acetonitrile, acetone, or short-chain alcohols, which are suitable for extraction of the neutral polar substances. The idea of salting-out originated in 1989 [34]. Usually, a large amount of salts must be added into the water sample to achieve phase separation, usually chlorides or sulphates.

Moreover, addition of salts to the sample helps to decrease the analyte solubility in the sample and thus promotes its transfer to the acceptor phase – non-polar substances into water-immiscible solvents or volatile analytes into the head space over the sample in case of the head-space microextraction variation [35].

A similar concept was presented in 2003 for the determination of pesticides [36], which gave base for the name QuEChERS – an acronym, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe. It is also based on the addition of solvent, salt, and buffer to an aqueous sample solution with posterior clean-up of the organic phase by a solid phase extraction (SPE). The method gained popularity and commercial kits with the chemicals needed are now available.

3.1.3 Solid phase-based methods

Solid phase-based methods are sample pretreatment techniques based on using a solid phase (sorbent), on which the analytes are adsorbed. Afterwards, the analyte is released either by a chemical way, e.g. using a solvent or changing the pH of the analyte, or by a physical way, e.g. by heat.

3.1.3.1 *General principles of solid phase-based sample pretreatment techniques. Classical methods*

Solid phase extraction (SPE) is still the most common technique used for sample pretreatment [37]. It is based on a partition of the analyte between a sorbent and the sample solvent. SPE is usually performed in a cartridge filled with the sorbent.

The procedure is carried out in several steps. First, the sorbent is conditioned – wetted typically with solvent such as acetonitrile or methanol. Then, an equilibration step with a solvent similar to the sample composition is carried out. After the sample loading, the matrix is washed out with an aqueous solution, sometimes with some addition of organic solvent (depending on the matrix). The final step is the elution of the retained analyte. Further, solvent evaporation and reconstitution with a mobile phase can be done prior to chromatographic analysis.

Over the years, a wide range of different sorbents were introduced to the market. Nowadays, different materials and chemistries are available, corresponding to the requirements of selectivity, recovery, and chemical stability. SPE sorbents are available for both polar and non-polar, as well as for ionic substances. An overview of the new sorbents for SPE is given elsewhere [38, 39].

Many years of use and wide-spread of the technique prove its numerous benefits: high recovery, ease of operation, and feasibility of automation [8, 37]. Both off-line (sample pretreatment performed manually or using an instrument separated from the analyser) and on-line (directly coupled to the posterior analyser) modes are possible. The extraction is typically performed using a manifold for multiple SPE cartridges comprising a vacuum pump to speed up the percolation.

There are many similarities with chromatographic columns. This fact also led to the idea of a full SPE automation and column switching approach in liquid chromatography (LC). In column switching LC, the first column is used for the analyte extraction, which is transferred to the second column for separation using a switching valve. The time of analysis and sample manipulation is significantly reduced [40].

Although SPE is superior to LLE regarding the organic solvent consumption, modern liquid-based microextraction techniques are surely improved in this regard because only microliters of solvents are typically used, whereas larger volumes are typically used in SPE. Also, automation of these techniques is feasible nowadays, using either robotic systems or flow analysis based systems. Another pitfalls of typical SPE sorbents are: the flow rates cannot be controlled easily, drying of

the solvent must be prevented [8], equilibration of the sorbent must be performed before each loading of the sample, which increases the analysis time, and the cartridges are prone to clogging, which is omitted when using liquid phase-based technique [4]. Despite some disadvantages, this method still remains the method of choice in bio-analytical laboratories [8].

3.1.3.2 *Miniaturized solid phase-based sample pretreatment techniques*

3.1.3.2.1 General remarks on miniaturized solid phase-based sample pretreatment techniques

Although SPE has still a leading position among sample pretreatment methods, it does not fulfil all requirements stated previously in chapter 3.1.1. Its relatively large format in which correspondingly large volumes of sample, solvents and time of extraction are used, what motivated the scientists to the development of downscaled and faster methodologies. Classification can be done according to e.g. the number of phases of the extraction procedure, onto which kind of sorbent the analyte is retained, or divided into head-space and direct sample contact [41] as well as into static and dynamic mode. The following chapters present a short overview of the modern techniques. More comprehensive publications on miniaturized solid phase-based sample pretreatment procedures can be found in the literature [42, 43].

3.1.3.2.2 Microextraction by packed sorbent

Microextraction by packed sorbent, denoted by some authors also as a microextraction by packed syringe (MEPS), represents a miniaturized version of SPE. In MEPS, a barrel is filled with 1 – 4 mg of a sorbent, and it is attached to a microsyringe of 100 or 250 μL (Figure 6). The procedure is equal to classical SPE: conditioning of the sorbent usually with acetonitrile or methanol, equilibration with a solvent identical or similar to the sample medium, sample loading, matrix washing usually with water or water with small addition of organic solvent, followed by elution with a specific solvent compatible with the used detection system (Figure 7). The solutions pass the sorbent bed twice, as they are aspirated and dispensed by the syringe plunger movement. The sample volumes typically range in tens of microliters, but larger volumes up to 1000 μL can also be handled by repeated aspiration. The extraction efficiency can be thus increased by repeating the sample loading step.

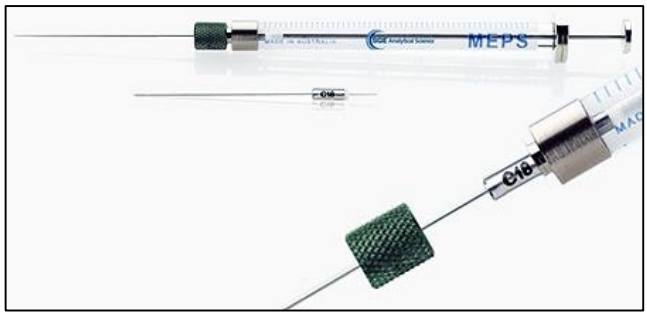

Figure 6: MEPS cartridge with a microsyringe[2].

Unlike the SPE, MEPS can be used repeatedly (up to 100 times for plasma and urine samples, [45]), which dramatically decreases the cost of sample pretreatment. Full blood pretreatment with MEPS was also demonstrated [46]. Aditionally, MEPS is compatible with LC, GC and capillary electrophoresis (CE).

The performance can be quite tedious and time consuming when performed manually, and an experienced operator is required. The flow rate is the key factor for high extraction efficiency and analyte recovery. Too fast aspiration and discarding do not allow complete adsorption of the analyte on the sorbent [8] and fluctuation in the flow rate impairs the repeatability.

MEPS extraction can be performed either manually, semi-automatically using a special motor-driven pipette, or fully automatically using the autosampler of a used analytical instrument.

Apart from the tedious manual performance, small variety of sorbents can be considered to be another disadvantage. However, smaller dimensions, shorter time, possibility of full automation and repeatable use make MEPS a useful tool for sample pretreatment.

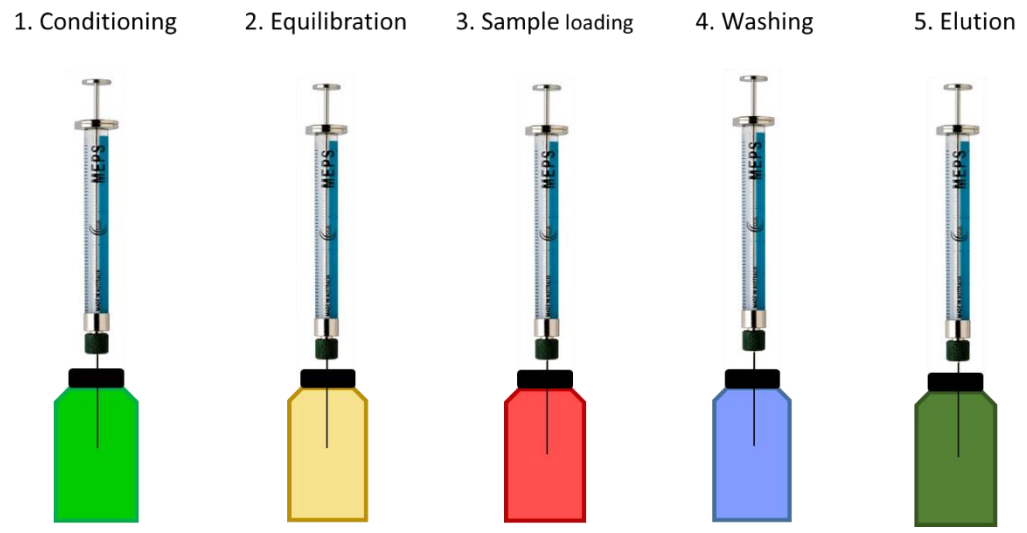

Figure 7: Microextraction by packed sorbent.

[2] Adapted from [44].

3.1.3.2.3 Solid phase microextraction

Solid phase microextraction (SPME) is a solvent-less microextraction technique. It was firstly presented in 1990 [47]. SPME is based on distribution of an analyte between the matrix and the sorbtive material. This is typically a fused-silica fibre, which is usually coated with polydimethylsiloxane (PDMS) or polyacrylate [48]. The fibre is attached onto a needle of a special syringe. It is exposed to the sample during the extracting process and held inside the syringe during the perforation of the septum of the sample void and during transportation. Then, the analyte is released from the stationary phase by means of thermic desorption in the injector of a GC system instead of elution with a solvent. There are two possible modes of SPME. The fibre is either immersed in the sample (direct immersion, DI-SPME) or held above it (head-space, HS-SPME). Derivatisation is feasible in SPME, either with a reagent bound onto the fibre coating or added into the sample [48].

This technique is surely superior to other sample pretreatment techniques in the terms of solvent consumption. It is also a relatively simple system consisting of one modified syringe. On the other hand, some drawbacks such as possible long desorption time, limited capacity, and fragility of the fibre prevent the SPME to become widely used. Carry-over might be a serious problem with some substances even at elevated desorption temperatures [48]. The risk of irreversible binding of substances with high molecular weight (e.g. proteins) by direct immersion must be taken into consideration. Moreover, its application is restricted to the analytes with high vapour pressure and thermic stability to enable thermic desorption [8].

3.1.3.2.4 Stir bar sorptive extraction

The principle of stir bar sorptive extraction (SBSE) is the extraction of the analyte based on the partition coefficient. It was introduced in 1999 as a solvent-less sample pretreatment technique [49]. A stir bar is coated with the acceptor phase, commonly PDMS, and placed in a vial with the sample. During stirring, the analyte is adsorbed onto the bar coating. Then, the bar is removed, washed and wiped gently in order to remove salts, proteins, or polymers from the sample matrix. Afterwards, the analyte of interest is submitted to further analysis either upon thermic desorption or liquid desorption in case of thermolabile substances [50]. A representation of SBSE is in Figure 8.

A good sensitivity can be obtained with this technique, since the whole extract can be submitted to analysis and simultaneous derivatisation is also possible, e.g. by binding the reagent to the stir bar coating, its addition to the sample before extraction or addition of the derivatisation agent into the desorption chamber [50].

Stirring time, volume of the sample, and size of the bar as well as temperature and salt addition can influence the extraction efficiency. Although the technique is quite simple to be carried out, limited extraction of polar substances (due to the non-polar character of PDMS) and especially

long extraction times (usually not less than 30 min, [51, 52]) are the main drawbacks of SBSE. The applications, potentials, and limitations of this technique were summarized in a recent review [53].

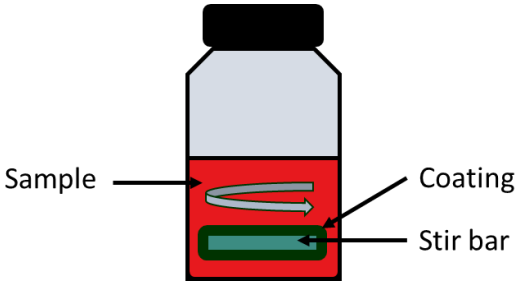

Figure 8: Stir bar sorptive extraction.

3.1.3.2.5 Other solid phase-based microextraction techniques

Dispersive solid phase extraction

The dispersive solid phase extraction (dSPE) usually follows the QuEChERS procedure. It was firstly proposed by Anastassiades for pesticides analysis [36]. The acetonitrile fraction from the QuEChERS procedure is submitted to dSPE. A specified quantity of sorbent is added to the sample with the addition of salt and shaken or vortexed and then centrifuged for separation of the sorbent from the sample. The sorbent is used to remove the sample matrix. The solvent is then submitted for further analytical treatment, i.e. solvent evaporation and sample reconstitution in a mobile phase [36].

The advantage of this method is a simpler performance than the SPE in a cartridge, since no vacuum pumps and preconditioning steps are required. Also, the problems with the cartridge blockage are omitted and a combination of different solvents is feasible.

Disposable pipette tip extraction

In disposable pipette tips extraction**,** or DPX, the sorbent is loosely placed, e.g. not fixed, in a pipette tip (Figure 9). It is dispersed in the sample by air bubble mixing at sample aspiration. The advantages over the classical SPE are apparent: bidirectional flow enabling faster aspiration and removal of a solvent, smaller sorbent quantity and therefore also lower volumes of sample and solvents. Both, batch-like and semiautomated performances, are possible [54].

SPE can be miniaturized using a 96-well plate, too. Each well accommodates a 1-2 mL individual cartridge with a 10-100 mg of the sorbent. Although it is similar to SPE, the transfer of an optimized SPE method to a 96-well format is not straightforward [55], since the physical characteristics (mass, volume, flow rate control) of the sorbent beds are different. The method development can be therefore time consuming and costly.

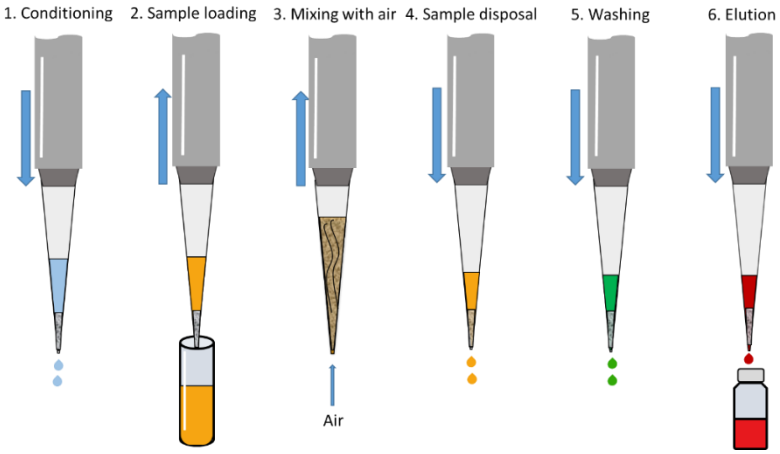

Figure 9: Disposable pipette tips extraction.

SPE in a disc format

This type of SPE brings the problem of the sorbent structure non-uniformity as well as the processing time are significantly reduced. The discs are made of a fibre support (PTFE, glass) loaded with the sorbent. They are available in different sizes, as a 0.5 mm membranes or loaded in a cartridge and about 1 mm thick. More detailed review on SPE in a disc format was done e.g. by Poole [56].

3.1.3.2.6 Modern sorbents for solid phase-based microextraction

Sorbents used in miniaturized solid phase extractions show many similarities to the chromatographic sorbent. From the classical materials (silica, polymer, cellulose), the ones based on silica still keep their leading position in use. Silica gels modified with C18, C8, CN, NH_2 groups are typically used in SPE as well as in the miniaturized formats. However, modern materials were developed in order to increase the selectivity and performance of sample clean-up, some enabling also direct on-line use. Although they were not used in the experimental work, they are included in this thesis in order to give an up-to-date report on sample pretreatment possibilities. More comprehensive reviews including nanoparticles, carbon nanotubes, and metallic particles with magnetic properties can be found elsewhere [57, 58].

Restricted access material

Restricted access material (RAM) was firstly used in 1991 by Desilets et al. [59]. Authors bound polyoxyethylene groups on the surface of the stationary phase for chromatography. Direct injection of a sample into a chromatographic system was possible, since the polyoxyethylene groups acted as a sieve, thus the size exclusion principle was applied. Biomolecules are washed away and the smaller molecules penetrates inside the stationary phase and are retained inside the stationary phase according to their affinity to it. RAM can be therefore considered as an enhanced form of on-line sample pretreatment technique [60]. Nowadays, RAM can be tailor-made to fit the desired

application – non-retentive surface and functionalized pores for retention can be improved by "adding" a semipermeable polymer or protein coating or using molecularly imprinted polymers (MIP) as the inner retentive phase [60].

Application of RAM enables direct injection of a sample into a chromatographic system. The contamination of the sample due to manipulation between the sample preparation and actual analysis is therefore prevented and the sample throughput is increased [60]. RAM cartridges were also coupled into a sequential injection system for sample preparation [61, 62].

Molecularly imprinted polymers

MIPs are special polymers developed to increase the selectivity of solid phase-based extraction. They are synthetized by polymerization of functional and cross-linking monomers around a template molecule – the analyte of interest – in order to achieve a polymer, which possesses a recognition property for the template or similar molecules [63]. The aim of the synthesis is to achieve a polymer with chemical memory, which resembles the template by size, shape, and binding sites and is therefore able to recognize the target molecule or chemically similar molecules.

Depending on the synthesis method, so-called template bleeding can occur, which makes the quantification of the target analytes more complicated [64]. However, methods solving this problem were also proposed, such as using radioactive labelling [65].

There have been several reviews devoted to the synthesis, characterization, and applications of MIP [66, 67]. The use of MIP as a sorbent for SPE or SBSE or other miniaturized solid phase-based extraction techniques in order to hyphenate the advantages of these techniques with the selectivity of MIP was also revised [63].

Immunosorbents and aptamers

Immunosorbents (IS) and aptamers represent groups of highly selective solid phase sorbents. IS are sorbents with bonded antibodies, employing the antibody-antigen reaction ensuring high selectivity towards the target substance. IS can be used both in off-line or on-line modes and the extraction procedure is similar to the SPE with C18 sorbents [68]. Cross-reactivity can be also advantageous in extraction of a group of substances from a complex matrix. Wider exploitation of IS is restrained by complicated preparation of antibodies for small molecules [68].

Aptamers are oligonucleotides (DNA or RNA), which can bind to a wide variety of substances, from small molecules to supramolecular structures. They are applied especially due to their high specificity, affinity, and good stability [69]. Their advantage over the IS is faster re-conditioning (within minutes) for application after the denaturation, better batch-to-batch repeatability and in-vitro preparation [69].

3.2 FLOW TECHNIQUES

"The ideal flow analyser can be identified by three things: There must be careful design, it must be practical, it can be assembled by anyone". (Zagatto et al., [73])

3.2.1 Definition and general remarks on flow techniques

There are several definitions of flow techniques, established in their early years: "a simple and versatile analytical technology for automating wet chemical analysis, based on the physical and chemical manipulation of a dispersed sample zone formed from the injection of the sample into a flowing carrier stream and detection downstream" [70], or "information gathering from a concentration gradient formed from an injected, well-defined zone of a fluid, dispersed into a continuous unsegmented stream of a carrier" [71], and also "a flow analysis technique performed by reproducibly manipulating sample and reagent zones in a flow stream under thermodynamically non-equilibrated conditions" [72]. However, they mostly apply only to flow injection analysis (FIA) and do not cover the whole spectra of different flow methodologies.

Flow techniques can be described as an analytical approach where the chemical analysis is accomplished inside a manifold, typically assembled using narrow tubing. A discrete aliquot of a liquid sample is precisely measured and inserted into this manifold and pushed towards a detector by means of a propelling device (pump). The sample undergoes a physicochemical modification inside the tubing manifold on the way to the detector [73]. The modification (such as reagent addition or extraction) is performed so that the detector is able to detect the presence of the analyte in the detection cell. The simplest modification, dilution by the carrier, can be however undesirable, depending on the application. For most of the flow techniques, it is valid that the equilibrium of the modification steps taking place inside a flow manifold is not reached, yet high reproducibility of these steps allows to perform them before the steady-state [73].

The main purpose of invention of flow techniques is automation of analytical procedures. The call for an automated system emerged in 1950 together with the growing number of clinical studies [74]. Seven years later, a first analyser was built by Skeggs in 1957 [75]. This was the so-called segmented flow analysis (SFA) system (in Figure 10), the predecessor of the later coming flow techniques, which exploit a carrier solution.

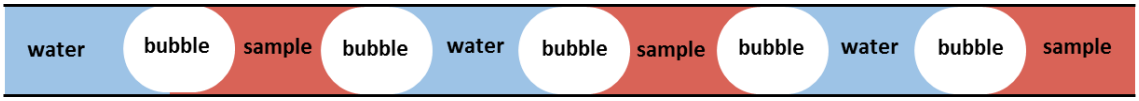

Figure 10: Segmented flow analysis – sequence of aspiration[3].

[3] Adapted from [75].

This system differs from the other flow methodologies by reaching the equilibrium of the sample modifying step [76]. Although not fully automated due to the missing computer-control at that time, SFA offered a relatively high sample throughput [76] and is still applied in certain fields [73].

The pioneering work of what is called modern flow methods was done by Jaromír Růžička and Elo Hansen in the seventies, and their first work on the topic was published in 1975 [1]. In the next chapters the key features and components of the most flow techniques will be discussed.

3.2.2 Basic principles of flow techniques

3.2.2.1 *Sample introduction*

The sample can be introduced into the flow manifold by different modes. Generally, several microliters to several hundreds of microliters of sample are used [77], with the exception of flow-batch approaches, where the sample volume can vary from one to more than ten millilitres [78, 79].

Generally the small volumes of samples contribute to small waste production [73]. An important feature is the closed environment of the flow manifold where the sample is introduced. This is advantageous for both the sample and the operator: sample loss or contamination is prevented in this manner, which decreases the risk of bias [73] and the operator is protected from the harmful effect of chemicals being used.

3.2.2.2 *Controlled dispersion*

A significant part of a flow manifold is made of narrow tubes, usually 0.3 – 1.0 mm i.d. In flow techniques, the flow is typically laminar within the common range of flow rates. A zone of sample introduced into such a tube will obtain a parabolic flow profile (due to friction) on the way downstream, with the faster flow in the centre and slower movement next to the tubing walls [77]. The front and end boundaries of the sample zone therefore overlap and mix with the carrier solution, decreasing the concentration of the sample in the overlapped part. Dispersion (D) is mathematically expressed as

$$D = c_0/c_{max}$$

where c_0 is the concentration of the sample at the moment of injection and c_{max} is the maximum concentration of the sample recorded by the detector, i.e. the concentration of the sample, which underwent all dispersive processes on the way to the detector [71]. The minimum value of D is 1.

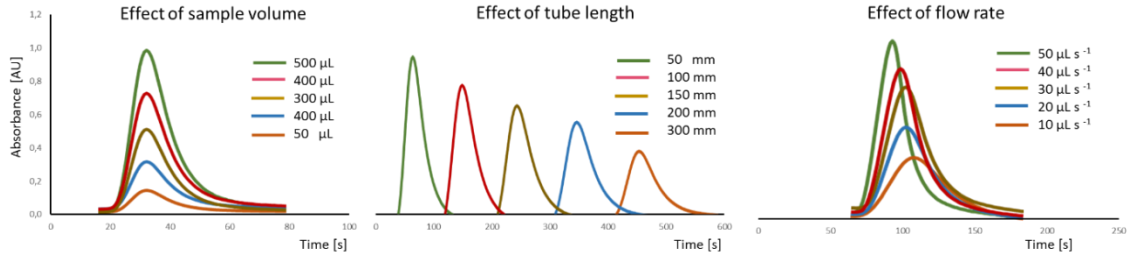

Figure 11: Effect of different flow conditions on dispersion[4].

Sample volume, flow rate and tubing dimensions are the factors mostly influencing sample dispersion as shown in Figure 11. High dispersion can affect negatively the limits of detection. On the other hand, overlapping with the adjacent zones of the carrier containing a reagent is advantageous when formation of a reaction product is aimed [80].

Dispersion contributes to mixing of solutions inside a flow manifold. Unlike in batch mode, where the mixing is homogeneous, a concentration gradient is formed in a flow system as the solutions pass downstream. This is reflected by a signal in shape of asymmetric peak in most measurements [80].

3.2.2.3 *Reproducible timing*

Reproducible timing is the third and key feature of flow techniques. Precise and reproducible manipulation with solutions in a flow system allows performing measurements before reaching equilibrium of the sample modifying processes, thus the analysis time in comparison to standard batch-wise methods can be significantly shortened. Suitability of signal data collection before reaching the equilibrium should be evaluated for each application [73]. Improvement in reproducibility is related to the expansion and accessibility of computers and their inclusion in laboratory instrumentation.

[4] Adapted from [74].

3.2.3 Common components of a flow system

3.2.3.1 *Propelling device*

"There is no ideal pump." Jaromír Růžička

Propelling devices induce flow in the flow manifold. Taking into account the small volumes, which are handled in a flow system, precision is an important characteristic of such a device. Several principles have been used over time to propel liquids. The following text describes briefly the most common ones, although other principles were used to drive a liquid inside a flow system, such as electroosmosis [81], gas pressure [82], or gravity [83] and an overview on the propelling principles can be found elsewhere [76, 77].

3.2.3.1.1 Peristaltic pumps

Peristaltic pumps are driven by a motor, which spins a module with rollers placed around. These rollers then press flexible tubes arranged in one or more (depending on the type) channels around them and the liquid inside the tubes is then pushed towards the output. Reconstitution of the original state of the tube causes then the liquid suction from the inlet side and the flow is induced [84]. The flow rate is adjusted by the tube i.d. and rotation rate [80].

Peristaltic pumps are typically used in flow injection analysis (see chapter 3.2.4), where a continuous flow is characteristic. Flow programming is less convenient in this case, since delay of the pump action can occur, which affects undesirably the time precision and is impermissible in stop-flow measurements [77]. However, flow programming using a suitable interface for computer connection and use in sequential injection analysis (see chapter 3.2.5) is possible. The disadvantage of peristaltic pumps is the flow pulsation caused by pushing and releasing the tubes and the need for relatively frequent tube exchange due to abrasion.

3.2.3.1.2 Piston pumps

Piston or syringe pumps are constructed from a stepper-motor, which drives a plunger inside a syringe. The syringe is fabricated from inert materials such as glass and Teflon®. The pump movement is bidirectional and produces a pulseless flow [85]. Introduction of the first cam-driven piston pump to low-pressure flow systems was related to the introduction of sequential injection analysis, where flow programming is inherent. Piston or syringe pumps are able to induce a variety of flow rates depending on the application [77]. Although syringe pumps require time for refilling from the reservoir and the sampling frequency is therefore lower compared to the peristaltic pumps, the advantages outweigh these drawbacks [85]. Some of the pump types are shown in Figure 12.

3.2.3.1.3 Solenoid pumps

Solenoid pumps generate a pulsed flow using an electromagnet built inside. A diaphragm separates the operating mechanism from the flow channel. When the valve is on (solenoid is energized), the diaphragm is retracted, creating a partial vacuum inside the pump. This pulls liquid through a valve at the inlet and simultaneously closes a valve at the outlet. When the solenoid is de-energized a spring pushes the diaphragm down, the vacuum is released and expelling a discrete volume of liquid is propelled through the outlet valve. Simultaneously, the inlet valve is closed [86].

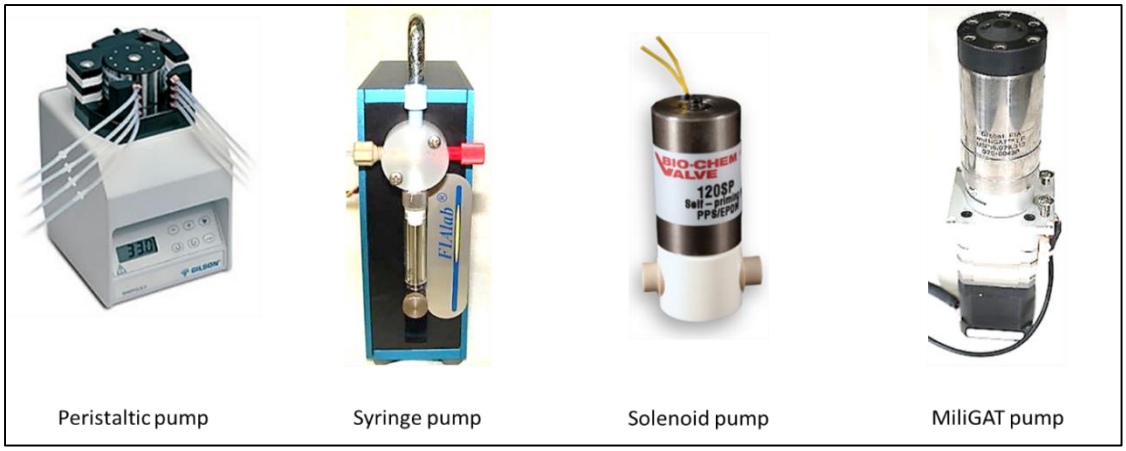

Figure 12: Propelling devices used in flow techniques.

The advantage of solenoid pumps over the peristaltic pumps is the smaller size and weight, which can be of significant advantage in some applications [87]. Also, individual control of each channel, unlike with a multichannel peristaltic pump, is convenient.

The disadvantage is, similarly as with peristaltic pumps, generating a pulsed flow [87]. Moreover, the flow is unidirectional and liquids only can be pumped (air is not desirable). Overheating, which can stop the pump, can be caused by fast flow rates over a long time.

3.2.3.1.4 MilliGAT™ pump

The MilliGAT™ pump, developed by GlobalFIA®, is the latest type of pump, which is supposed to overcome the drawbacks of the peristaltic pumps (pulsation and frequent tubing exchange) and the syringe pumps (necessity of barrel refilling). These two conditions were fulfilled and the MilliGAT™ can pump bi-directionally both liquids and gasses at flow rates ranging from nL min^{-1} to mL min^{-1} [88]. However, not even this pump has a universal usage since it contains metal components and is therefore not applicable in trace metal analysis, it is not resistant to strong bases and acids and to high pressures, the action inside cannot be visually controlled (not transparent), and it requires a professional service [80].

The pulseless and continuous pumping is here achieved by employing four pistons inside a cylinder, which are moved by means of a motor. As they turn, they run a circular path. The pistons

change their vertical positions by turning as they are attached to an inclined support [80]. While one is aspirating, the opposite piston is discharging and the two remaining pistons are in the middle of the action of aspiration or discharging [77] from the ports in the cylinder.

3.2.3.2 *Tubing and mixing coil*

The typical tubing used in flow methods has an i.d. of 0.3 – 1.0 mm [77]. In flow methods, the purpose of the tubing is often different than just delivering liquids; thorough mixing of e.g. sample and reagents is required [74]. To improve the mixing, coiled or knitted reactors are often employed in flow systems. Pumping the sample and reagents in such reactor (mixing coil) promotes radial flow and thus better mixing of the liquids is achieved [74]. Reversed flow also serves for this purposes [89].

The materials of tubing and connectors in a flow system should be inert and resistant against different chemicals. The mechanical characteristics should be also considered, according to desired purpose. Teflon® is nowadays the prevailing material for tubing, although other, such as PEEK™ or Tefzel® are used for some applications due to improved characteristics over Teflon® such as mechanical stability [74].

3.2.3.3 *Sampling device*

Metering the exact volume of the sample and passing it into the manifold is accomplished together by a pump and the sampling device. Introduction of the sample or "injection" into the stream of carrier solution can be achieved by different modes. The most typical devices are described below and shown in Figure 13.

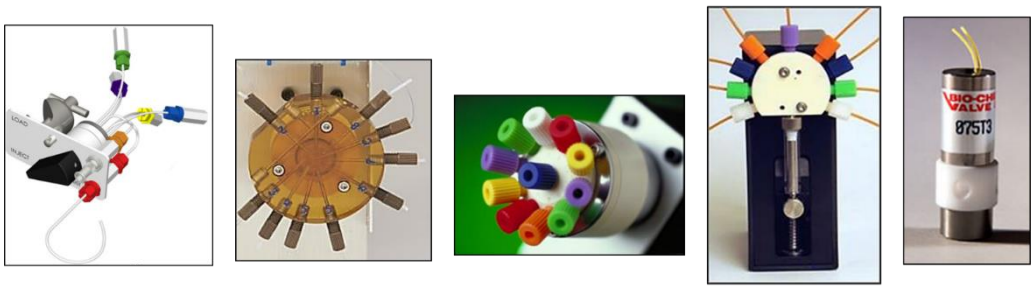

Figure 13: Selected injection devices used in flow techniques.

At the inception of FIA, the sample was literally injected into the system by means of a syringe needle through a septum, having given also the name to the technique [77]. Injecting the desired volume of sample and reagents by means of several burettes [74] is typical for multi-syringe FIA (MSFIA) [90], using peristaltic pumps and solenoid valves is characteristic for multicommutated

FIA (MCFIA) [91] and solenoid piston pumps are used in multipumping flow systems (MPFS) [92]. Description of other sample introducing modes and devices can be found elsewhere [77].

3.2.3.3.1 Injection valve

Injection valves are designed as two (or three) attached disks, from which one is rotating (rotor). For a six-port valve, six channels are drilled into the top disk (stator): 1. loop inlet, 2. sample inlet 3. waste outlet, 4. loop outlet, 5. channel leading to the manifold, 6. carrier inlet. The dimensions of the tube for loop determine the sample volume. Connection of the channels at loading and injection positions is depicted in Figure 14. At the loading step, pure carrier flows into the manifold. At the injection step, the rotor turns and the loop connects with the channels opposite to those at the loading step. They are linked in such way that the carrier washes the content of the loop into the system [74]. The working principle of a proportional injection valve is similar, but instead of discs, blocks are used and junction of channels is achieved by moving the middle block [93].

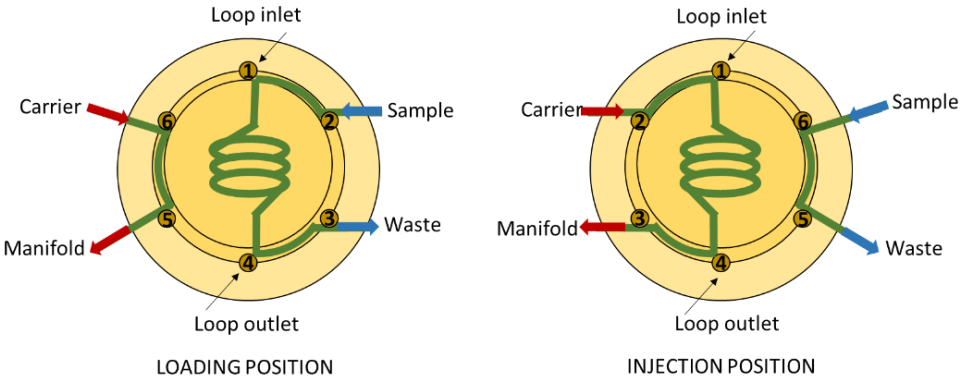

Figure 14: Scheme of an injection valve.

3.2.3.3.2 Multiposition valve

This type of valve (multiposition or selection valve) consists also of a rotating disc and other disc with drilled lateral channels – ports (6, 8, 10, or more ports) and a central port. The central port connects always the mixing reactor/holding coil with one of the ports, which is chosen by movement of the turning disc. Tubes from the sample and reagents reservoirs and waste are mounted to the respective ports. The selected volumes of solutions (controlled by a computer) are aspirated (or discarded) by a pump. This type of valve and its mode of operation (possibility of reversed flow) is more typical for sequential injection analysis [94].

3.2.3.3.3 Solenoid valve

It can be constructed as either two- or a three-channel valve. The two-channel valve is used to facilitate or prevent fluid stream [74, 91]. In a three-channel valve, the solution is pumped through one common channel and one of two optional channels. The solution flows constantly through the

channel in OFF position and is directed to the other channel (ON) for the chosen period of time [74]. The volume of the sample is determined by the time of the valve opening [77]. Solenoid valves are typically used in multicommutated systems [95].

3.2.3.4 *Detection*

Flow techniques are compatible with a large scale of detection systems. The detector is either specifically designed to be used in a flow system or a conventional laboratory detector is adjusted to be used in flow instead of batch conditions.

The most common detection techniques in flow techniques are spectroscopic methods such as UV and VIS spectrophotometry, fluorescence and chemiluminescence, atomic absorption spectrometry (AAS) [73, 96-98]. The priority in applications is given by the easiness of operation, robustness and high reproducibility of measurements [74].

In flow methods, detection cells are constructed with regards to the characteristics of flow. The detection cell shape should reduce the dead volume; inner volume should be large enough to prevent light beams collimation, but small enough to prevent retaining and merging of fluids zones, sensitivity loss and peak broadening as a consequence [73, 77]. Typically, the flow cells volume range from several units to tens of microliters [80, 98].

Adjusting of a conventional laboratory instrument for a flow technique can be done by mounting a cuvette-like flow-cell, comprising inlet and outlet. Flow cells designed to be used in flow systems have the inner flow-through channel typically Z-shaped facilitating the potentially occurring bubbles to escape and not to collide with spectrophotometric measurements [73]. They are conventionally used with optical fibres, using appropriate holders [80]. In that case, external light source and data collector are coupled into the flow system.

The flow cells are made of inert material, with glass or quartz windows positioned either in-line (UV, VIS) or at a right angle (fluorimetric measurements). Alternative flow cells (long light path, Figure 15) were developed in order to increase the sensitivity of spectrophotometric measurements. They are gaining popularity, since increasing the path length leads to sensitivity increase according to the Lambert-Beer law:

$$A = c \cdot l \cdot \varepsilon$$

A - absorbance, l – path length, ε – molar absorption coefficient (l $mol^{-1}cm^{-1}$).

Spiral flow cells (Figure 15) are used e.g. in chemiluminescence measurements [99].

Use of electrochemical techniques such as potentiometry, voltammetry or coulometry [100-103], mass spectrometry [104-106] and others, and their hyphenation with flow techniques was described in several monographs [77, 98].

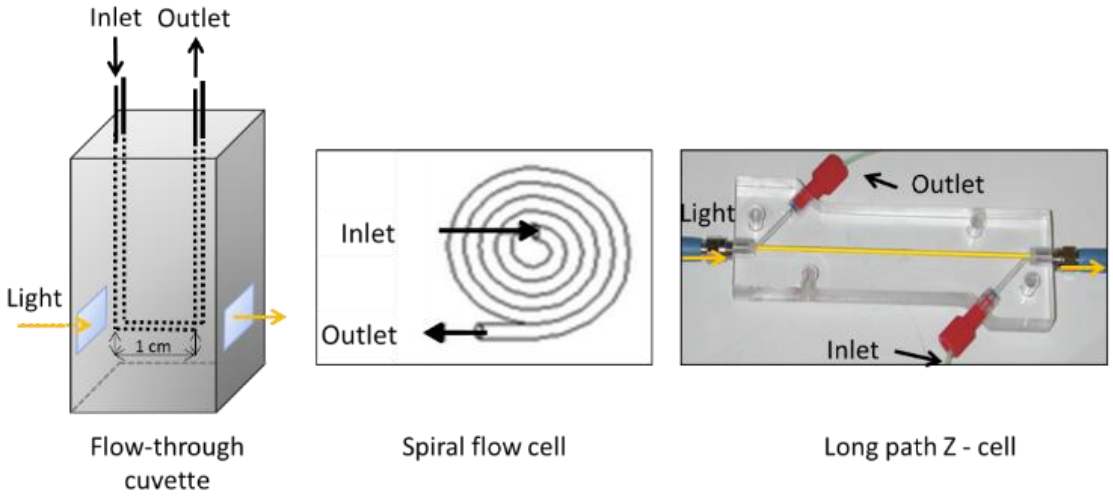

Figure 15: Examples of flow through detection cells.[5]

3.2.3.5 *Computer and software*

Computer control of the whole flow analyser is one of the premise for automation done by flow, sequential injection, or related techniques. Nowadays, several control softwares are available and supplied for the users by the developers of flow systems. FIAlab for Windows [107], FloZF [88] and AutoAnalysis [108] are available to control the action of each component of a flow manifold, to collect data, and to perform data evaluation and to enable the flow system user to write an operation protocol [109].

The development in the field of control programmes is continuous, due to the versatility of flow systems and hence to the possibility to connect - and consequently to control - additional devices. Automatic optimization and communication with other softwares are also the aims of further progress [109].

[5] Adapted from [77, 80].

3.2.4 Flow Injection Analysis

Flow injection analysis (FIA) was patented in 1974 and one year later published by Růžička and Hansen in 1975 [1] as a descendent of SFA or continuous flow analysis (CFA). The mutual feature with the previous technique is the use of a peristaltic pump and a tubing manifold for sample handling. However, there are several differences: unlike SFA, in FIA, air bubbles are not employed to separate sample zones and reagents. Laminar flow is here predominant. Zone merging by overlapping and diffusion occurs. Furthermore, while SFA works at a reaction equilibrium, this does not have to be reached in FIA due to the precise timing, sample introduction and controlled dispersion, and the sample throughput is therefore significantly higher [73, 74].

In FIA, unlike in SFA, the sample zone is injected into a stream of carrier or reagent. The reaction product is formed at the boundaries of the zones and the presence of this product is recorded by the detector as a change in signal, reflecting change of the parameter measured by the detector. For some applications, such as environmental analysis, so called reverse flow injection can be more convenient, when the sample is abundant or the cost of reagent requires this configuration. In this case, a relatively small volume or reagent is injected into the stream of sample [110].

A FIA system can be either single-lined or consists of multiple channels. The early FIA systems worked solely at continuous flow conditions, using peristaltic pumps and tubing [80]. An injection valve was used to introduce sample into the system. Operation of flow injection system was improved and simplified by the implementation of computers into the control of flow system components in the eighties [73].

The three basic features: sample injection, controlled dispersion and reproducible timing are followed here. A precise volume of sample is metered by the loop of the injection valve and injected into a flowing stream of the carrier or reagent. Due to dispersion, a concentration gradient is formed. This is controlled by the flow rate and channel´s i.d. and length. Due to precise handling and programming, the concentration, which is read by the detector, is always reached at the same time, thus the timing is reproducible in terms of sample transport or chemical reaction duration.

Although the continuous flow was characteristic for FIA, development in the field brought flow programming also into this method [80]. The typical characteristics of a FIA manifold are listed in Table 1.

Table 1: Characteristics of FIA[6].

Sample volume	Flow rate	Channel i.d.	Channel length	Sampling frequency
50 – 300 µL	1 – 2 mL min^{-1}	0.2 – 0.8 mm	Up to 2 m	60–120 h^{-1}

[6] Adapted from [80].

3.2.5 Sequential Injection Analysis

3.2.5.1 *Classical Sequential Injection Analysis*

Sequential injection analysis (SIA), also called the second generation of flow techniques, was presented in 1990 by Růžička and Marshall. The new generation is based on computer control of all system components, and this hindered the wide spread of the technique shortly after its introduction [74].

The components differ from that of FIA; piston or syringe pumps are used to aspirate and dispense carrier, sample, and reagents. A multiposition valve is the centre of the system. It is connected with the pump via the central port and a holding coil, in which the aspirated solutions are stored and mixed (Figure 16). The lateral ports are employed to connect the central channel with the sample and reagents reservoirs via tubing, or are connected to the detector or other peripheral devices, respectively. Another difference in comparison to FIA is the implementation of a holding coil, which prevents the aspirated solutions from entering the syringe barrel [74]. This is not the case of In-syringe analysis (chapter 3.2.6.2).

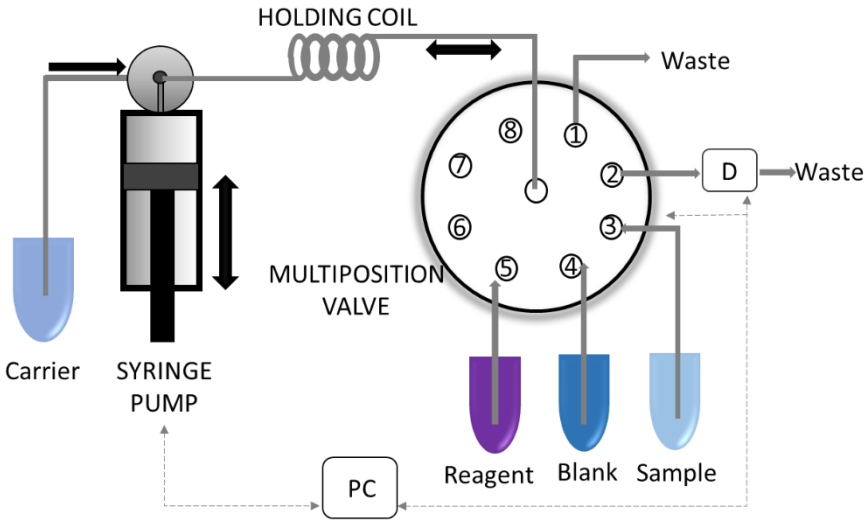

Figure 16: Sequential injection system comprising the basic components.

Two principles are added to the three corner stones of flow techniques: sequential aspiration and flow reversal [73]. Sample and reagent(s) are sequentially aspirated into the holding coil, upstream from the detector. By changing the flow direction, the solutions are propelled towards the detector. The adjacent zones overlap and their mixing and formation of the reaction product is improved in comparison to FIA with unidirectional flow [94].

Aspirated volumes and times, during which the sample stays in the system, are selected by means of a software unlike in FIA where the choice of tubing dimensions determines these parameters [74]. SIA enables selecting the flow rates according to the performed action: if a long

time is necessary due to a slow reaction, slow flow rate is programmed. For actions such as system washing, high flow rates are chosen. Stopped-flow measurements (see chapter 3.3.1) are amenable with SIA system [111].

The discontinuous mode of operation brings significant advantage in comparison to the previous technique: savings in solution consumption and waste production since they are aspirated sequentially and not pumped constantly. The versatility is supported by the possibility of connecting external devices to the basic assembly through the lateral ports of the multiposition valve (reactors, extractors, secondary pump and valve).

On the other hand, the limitation is in the volume of the syringe barrel, which must be refilled periodically. This gives SIA a disadvantage in the sample throughput compared to FIA. However, precise programming and/or use of a MilliGAT™ pump enables to reach similar sampling frequencies (Table 2).

The requirements of miniaturization led to the development of micro-SIA [112] and further formats, such as described in the following chapter. Since its inception, SIA was employed in different analytical fields, ranging from pharmaceutical [113] environmental [114, 115], agricultural [116], industrial [117], and others.

Table 2: Characteristics of SIA[7].

Sample volume	Flow rate	Channel i.d.	Holding coil length	Sampling frequency
20 – 150 μL	0.5 – 200 μL s^{-1}	0.3 – 1.5 mm	Up to 1 m	Up to 100 h^{-1}

3.2.5.2 *Bead Injection Analysis. Lab-on-Valve*

The versatility of flow techniques enables use of beads (regular spherical particles) in the system, without a significant hardware change. The first experiments, giving rise to bead injection analysis (BI, BIA), were performed by Růžička in 1993 [118]. In BI, beads of a typical diameter of 20 - 150 μm are aspirated into a system, which comprises a component (chamber) to entrap them [80]. Beads can carry an immobilized reagent. After the beads are entrapped in the system, the sample is aspirated and pumped through the bead bed. In this step, the reaction product is formed. Then, the product measurement can be performed in several ways:

1. An eluent passes the beads and washes the captured analyte(s) or reaction product(s) into a detection cell;
2. The measurement is performed directly on the beads – this is possible when transparent Sephadex® beads are used and the change of signal is given solely by the reaction product or the analyte captured on the beads [119, 120];

[7] Adapted from [74, 80].

3. The product is washed into the furnace for AAS, where the analytes are subsequently detected [121-123].

The critical moment in BI is the aspiration of a uniform amount of beads for each analysis and their correct positioning in a detection cell for direct measurements. Nowadays, BI is carried out in a Lab-on-Valve (LOV) format, which offer reliable and reproducible BI performance [80].

The concept of LOV as another descendant of flow techniques was presented in 2000 [124]. The LOV platform is a monolithic unit with carefully drilled channels placed atop of a multiposition valve, which fuses the function of a valve, flow channels, and detection cell [123]. It is therefore more compact and one step further in downscaling flow systems.

LOV is a versatile system, enabling UV/VIS or fluorescence detections simply by connecting optical fibres [125]. Hyphenation with electrochemical detection [126], inductively coupled plasma mass spectrometry (ICP-MS) [127], or CE is also feasible. The LOV concept is suitable to perform biochemical, biotechnological, or biological assays, including assays with cells [128, 129]. An example of a system with the LOV platform is in Figure 17.

The remarkable advantage of this approach is that a new microcolumn is formed for each analysis, so that the risk of carry-over is minimized. LOV gives an improved platform for BI thanks to the solid straight channels, thus minimizing the risk of accumulation of beads in non-desired sites along their pathway.

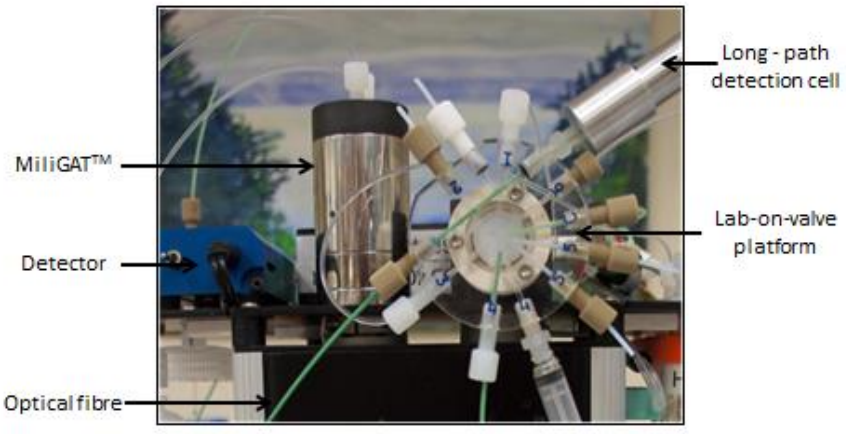

Figure 17: LOV system with MilliGAT™ pump[8].

[8] Adapted from [80].

3.2.6 Flow-Batch approaches

In flow techniques, the solutions are typically processed in a tubing manifold of typically < 2 mm i.d. In following chapters, the approaches are denominated batch if a chamber-like device is used in addition to handle (injection, mixing, reaction, extraction) the sample.

3.2.6.1 *Flow-Batch systems with external vessels*

The **batch analyser** (BA) was a system proposed and patented by Wang and Taha at the beginning of 90´ [130]. It was based on the injection of a small volume of sample very close to the detector surface. The detector (electrode) was placed in a large volume tank. The signal shape was similar to that one obtained in FIA and reflected the passage of the sample over the detector surface [130]. Reproducible fashion of sample delivery towards the detector is the common feature of BA and FIA. As the system concept is especially convenient for electrochemical detection, BA found use in determination of species with redox properties [131, 132]. Further potential of BI was described in 2004 [133].

Flow-batch analysis (FBA) was presented by Honorato in 1999 and used for titrations [134]. In this system, sampling and signal readout are carried out similarly as in flow methods, but the reaction takes place in a chamber belonging to the system. The chamber and valves for multicommutation are additional components to a propelling device, tubing, and detector, as used also in FIA or SIA. The solutions can be added to or removed from the chamber by a control system. Although the flow is typically continuous since peristaltic pumps are mostly used in FBA, the commutation system (use of three-way solenoid valves, Figure 18) enables recycling of the solutions to their reservoirs when the valves are switched off. The chamber is usually equipped with a stirring device [135].

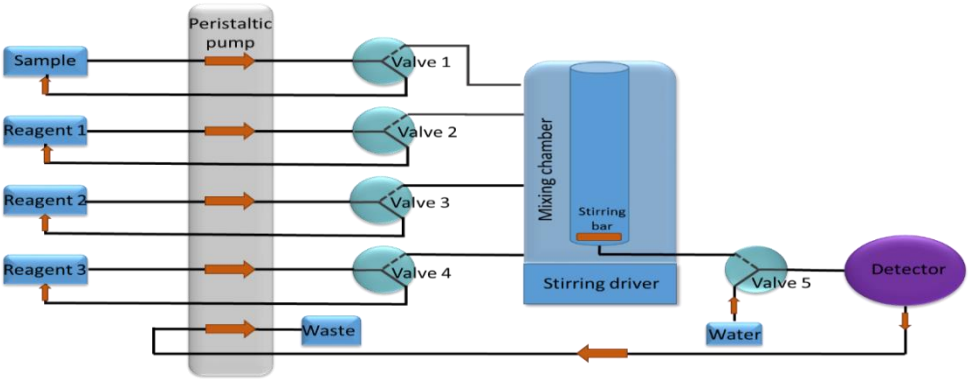

Figure 18: FBA system[9].

[9] Adapted from [135].

The advantage of these systems is higher sensitivity, since more efficient mixing can be achieved in the chamber and thus a same amount of a reaction product is formed faster in comparison to the simple zone overlapping in a tube [135]. Signal readout can be performed directly in the chamber, in case it comprises glass or quartz windows for spectrophotometric measurements [78]. The disadvantages are basically related to the use of peristaltic pumps, which generate pulsed flow and require relatively frequent tubing exchange due to mechanical and chemical changes.

Applications of FBA, such as titration, sample, standard addition and other were reviewed by Diniz et al. [135]. Miniaturized FBA systems were also proposed [136].

3.2.6.2 *Lab-In-Syringe technique*

In 2012, Maya et al. presented a work where he aspirated a mixture of octanol and acetonitrile for DLLME and a sample of benzo(a)pyren into a syringe of a MSFIA system [137]. Later on, he performed a similar method in a SIA system [138] and used the syringe even as a detection cell and established by this a novel *Lab-In-Syringe* (LIS) technique, also called in-syringe analysis (ISA).

This technique altered one of the rules in SIA, that any other solution than the carrier can enter the syringe. In LIS, the solutions are handled (by means of mixing, reaction, extraction) inside the syringe. This technique omits the classical use of a carrier as in FIA or SIA, and differs from FBA, where the respective channels are filled with solutions and have to travel relatively long distances (at least the length of the peristaltic tube) to the mixing chamber. In LIS, the distances from the solution reservoir to the syringe barrel are kept as short as possible. This can be facilitated by the 9-port selection valve [139], which is positioned directly atop of a syringe instead of a two-way valve (Figure 19). So far, this technique was mainly used for liquid phase-based microextractions, including DLLME [140, 141] and HS-SDME, as described in the article in chapter 5.3.

The use of the syringe as a mixing chamber has several advantages: the volume of the chamber can be adjusted (the upper limit being the syringe volume), enabling to increase the volume of the sample in order to achieve higher sensitivity. The system, using the SIA components, is compact – it can omit some of the tubing and mixing coil and the chamber itself can be used as a detection cell [138], minimizing also the dead volume.

The disadvantage is that additional syringe cleaning steps must be included in a control program before measurement of every individual sample, unlike in SIA, where the system is self-cleaning thanks to the carrier.

To achieve higher efficiency not only of the syringe cleaning step, but especially higher efficiency of dispersion (in DLLME) and extraction, stirring devices were proposed in LIS by Horstkotte et al., such as simple motor with an attached magnet positioned right next to the syringe,

as described in chapters 4.4 and 5.3. A novel and more sophisticated way of stirring was also proposed by the same author, using adapters creating magnetic field as driver for the stirring bar inside the syringe [141, 142]. This applies that a small-size stirring bar is placed inside a syringe, increasing the dead volume by disabling the piston to reach the very top position. However, adequate washing, which is more efficient using the stirring, and precise calibration eliminate this concern.

The use of solvents with density higher or lower than that of water and their dispensing into the detection cell is convenient in LIS, since the position of the syringe can be arranged either with the head up or alternatively, in head-down position [143].

Although relatively new, this technique was used not only in connection with spectrophotometric detectors, but also with AAS [144].

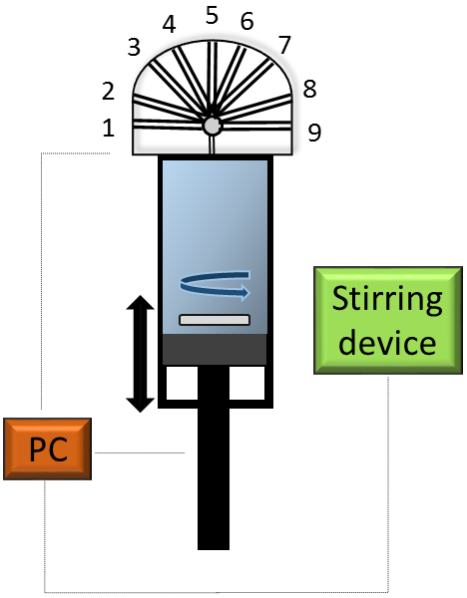

Figure 19: LIS system with 9-port selection valve.

3.2.7 Sequential Injection Chromatography

Šatínský et al. [2] tested to couple a monolithic columns into a sequential injection system and to separate four active substances of a topical pharmaceutical formulation in 2003 and established a technique denominated as sequential injection chromatography (SIC). SIC combines the advantages of both high performance liquid chromatography (HPLC) (determination of multiple analytes in one sample) and flow techniques (programmable flow) by coupling a separation column generally to one of the ports of a multiposition valve.

There are some clear advantages of SIC over HPLC: programmable instead of continuous flow and thus generating significantly lower volumes of waste, significantly lower cost of instrument and analysis, convenient pre- or post-column derivatisation, variability in flow manipulation and in flow path (use of Z-cell, long-path flow cell) for detection [2, 145].

In the early years of SIC, one of the limitations was the low working pressure of the SIA system (50 psi, [80]), allowing to use only monolithic columns for separation. This was partly solved by development of a SIChrom™ system with the working pressure of more than 500 psi. Increased working pressure of the system enabled to broaden the scale of possible columns to be used to longer monolithic and core-shell columns such as presented in the work of Chocholouš et al. [146], where three core-shell particle columns (30 mm × 4.6 mm, core–shell particle size of 2.7 μm) were tested. Ion-exchange column of 150 mg of strong anion exchanger sorbent filled into a 20 mm long × 3 mm i.d. cartridge was used in a SIC system for separation of UV filters [147]. Coupling of short monolithic columns was successfully applied even in flow injection-based systems [148] and MSFIA systems [149, 150].

The feasibility of liquid handling in flow systems enables also to generate gradient flows in the SIC format [151]. Sample pretreatment and subsequent separation and detection performed in one SIC system is also advantageous [152, 153].

Although the benefit brought to the family of flow techniques by SIC is evident, the comparison with HPLC shows some shortcomings of SIC; the pumps used in SIChrom™ are not comparable to the ones used in HPLC and thus the use of longer particle columns and separation of a larger number of analytes in one sample is cumbersome. Also, the existing data evaluation with the software provided with SIChrom™ system is rather simplistic in comparison with those used in HPLC [145]. However, comparison of SIC with HPLC in the meaning of performance is not fully adequate since SIC still belongs to the family of low-pressure flow techniques and it does not aim to be a competitor to HPLC.

SIC was applied mainly to pharmaceutical analysis of different formulations to determine the active substances and excipients [153-155] but process monitoring assays were also performed [156]. Environmental samples were analysed by SIC with UV detection using a long-path flow cell [157] or with ICP-MS detection [158].

3.2.8 Other flow techniques

The versatility of flow techniques offers the possibility for different system assemblies. Different ways of fluid propelling, merging, and delivering to the signal read-out system, i.e. different instrumentation gives raise to different flow techniques, and their categorization can be troublesome. Linden suggested classification of flow-based methods in 1994 [159]. MCFIA (techniques based on the use of three-ways solenoid valves), MSFIA (using multiple syringes, which can be moved simultaneously, for the aspiration and propelling of different solutions; each syringe coupled to a three-way valve), multi-pumping flow systems (MPSF; based on the use of solenoid valves), all injection analysis (AIA) (aspiration of all solutions into a holding coil and their circulating for a pre-defined time), chip-on-valve (incorporating the reaction coil into a monolith flow circuit mounted on a selection valve) and others are recognized by scientists among flow techniques. The reader is referred to literature devoted to these techniques, since their description exceeds the scope of this work [90-92, 160, 161].

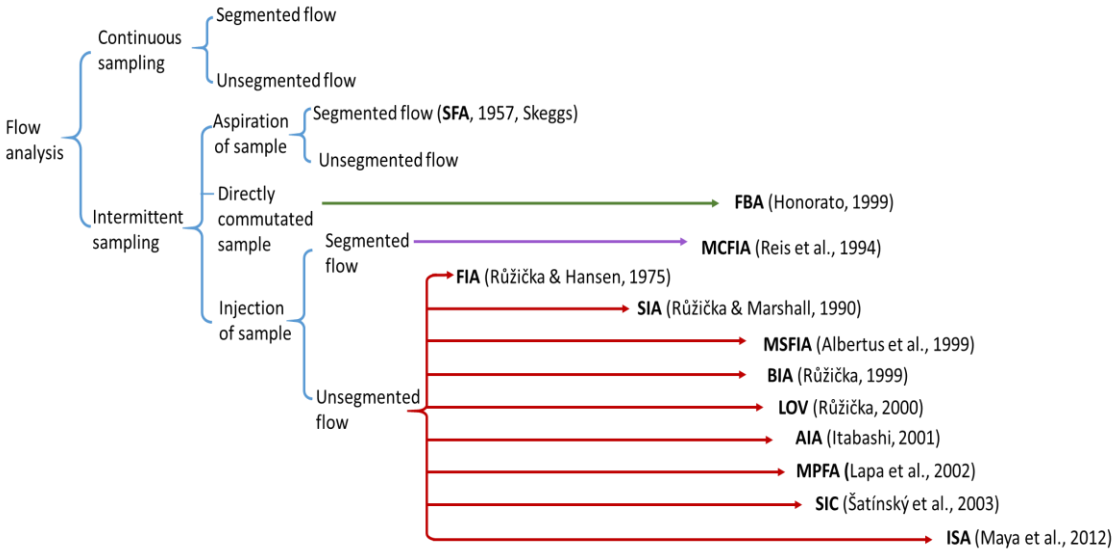

Figure 20: Classification of selected flow techniques[10].

[10] Adapted from [135, 159, 162].

3.3 SELECTED SAMPLE HANDLING METHODS IN FLOW TECHNIQUES

3.3.1 General remarks on sample handling in flow techniques

The versatility of flow analysers is given by the possibility of physical reconfiguration of the system and implementation of a large choice of components for various applications. Flow programming, which does not require any physical changes, contributes equally to the versatility of flow techniques such as SIA, SIC or LIS. Since different actions in a flow system (sample aspiration, mixing, detection etc.) require different conditions (flow rates, volumes, etc.) to achieve their most efficient performance, flow programming can be very useful for this purpose [80]. As an example, stopped-flow can be exploited in situations where a longer sample incubation time is necessary, e.g. in enzymatic reactions, when higher sensitivity of the method is aimed (steady-state can be achieved), or in case of phase separation. The flow rate is set to zero (the pump is stopped) for a defined (optimized) period of time. During this time, there is almost no dispersion of the sample zone but the chemical reaction or physical separation can continue [73]. As another example, more efficient mixing of the sample and reagents can be achieved applying the sandwich technique [163]. The sample is aspirated between two zones of the same reagent. Also, repeated reverse flow (typical for SIA) can be applied to improve mixing. Different flow programming and sample manipulation strategies as well as multiple applications were described in the monographs dedicated to flow techniques [73, 77, 80].

Adjusting the flow for different purposes helps to decrease waste generation (continuous versus programmable flow), to comply with the action carried out in a flow system, e.g. incubation, addition of a reagent, phase separation, continuous monitoring etc.), and to achieve satisfactory analytical performance in the meaning of the time of analysis, sampling rate, sensitivity etc.

In the following chapters, several applications of flow techniques exploiting different flow strategies for sample pretreatment will be described and the main features of the methods will be discussed. The methods described were employed in the works included in this dissertation. Liquid phase-based extractions were automated in works described in details in chapters 5.2 (LLME) and 5.3 (HS-SDME). Solid phase-based extraction was explored in works presented in chapters 5.4 (SPE) and 5.6 (MEPS). Enzymatic reactions were performed in a SIA system, as demonstrated in chapter 5.1 and irradiation for analyte alteration was applied in works described in chapter 5.4.

3.3.2 Enzymatic reactions in flow systems

3.3.2.1 *General introduction to enzymatic reactions*

Enzymes are proteins with a three-dimensional structure, which act as biocatalysts, i.e. they increase the reaction rate. The substrate molecule binds to an active site of the enzyme and is converted into a product. Binding to the active site is highly specific due to the three-dimensional structure. The high specificity of the enzymatic reaction, together with high turnover number, biodegradability and natural origin [164] are some of the advantages of the use of enzymes in analytical reactions in comparison with some other chemical reagents. The selectivity enables the determination of a substance of interest in complex matrices with rather universal (less specific) detection techniques [165]. A specific enzyme can be therefore used in pharmaceutical analysis to determine the active substance, decomposition products, an excipient, inpurities or metabolites in the pharmaceutical formulation or in a biological sample. These substances can be present at low concentration in relation to the matrix components, and a specific reaction might reduce the sample pretreatment or interference masking. On the other hand, some drawbacks, such as molecular complexity, high cost of production and easiness of irreversible changes in structure and activity [164] hinder broader applications of enzymatic reactions.

Analytical applications of enzymes are based on the Michaelis-Menten kinetics model and on the following equation:

$$V = V_{max} \cdot [S] \cdot (K_M + [S])^{-1}$$

where V is the reaction rate, V_M maximum reaction rate (depends on the enzyme activity), K_M the substrate concentration, at which the reaction rate is half the maximum reaction rate, also called Michaelis-Menten constant, and S is the substrate concentration.

Performing a time-dependent enzymatic reaction in a flow system has the advantage of precise timing and the possibility of stopped-flow measurements. Also, utilization of small quantities of an enzyme, measured accurately in the flow system, decreases the cost of analysis [77].

Flow techniques can be either used to perform the determination of an enzyme activity [111, 166], or the enzyme is used within a flow manifold as a catalyst of a specific reaction for determination of the substance of interest, which can act either as a substrate, inhibitor or activator [167].

There are several strategies for enzymatic determinations; enzymes can be handled either in a form of a solution, or immobilized [77, 165, 168], both of them having advantages and disadvantages, as discussed in the following chapters. The graphic representing the frequency of use of enzymes in SIA in different forms and having a different role is shown in Figure 21.

3.3.2.2 *Use of reactors with immobilized enzymes in flow methods*

Immobilized enzymes are "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and, which can be used repeatedly and continuously" [169]. In other words, they are attached to an appropriate support over which the substrate can pass and be converted into a product [170].

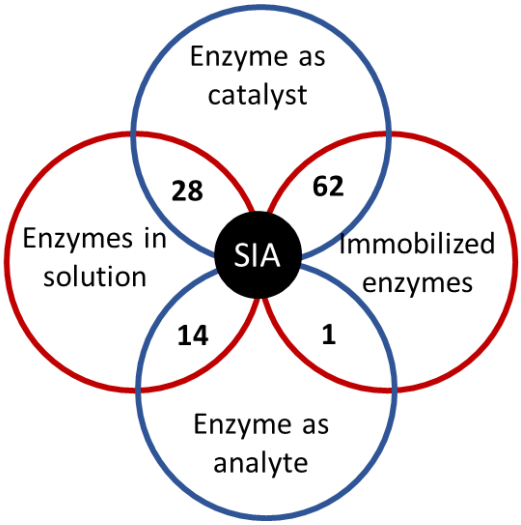

Figure 21: The role of the enzyme and its physical presence, from 2011[11].

Enzyme immobilization can be reasoned by several benefits, save of cost being one of the most important. Repeatable utilization of enzymatic reactors decreases significantly the consumption of enzymes, whose isolation is a costly procedure [165, 169]. Moreover, enzymes in solutions can easily lose their activity due to the environmental factors such as temperature or pH, and some authors claim the enhanced stability of the immobilized version under both the operation and storage conditions [171]. The rigidity and more feasible handling with the immobilized enzyme are also appreciated in bioanalysis. Preventing the possible incompatibilities in cascade reactions using multiple enzymes was also pointed out [171]. Also, due to the repeated use, a larger amount of the enzyme can be used for immobilization than if used in a solution, so that a more efficient substrate conversion can be achieved [168].

On the other hand, immobilization of an enzyme requires additional time and cost. The blockage or irreversible loss of activity, e.g. by a sample component, can increase the cost of analysis significantly [165]. The enzyme activity must be regularly controlled over the time of utilization.

There are multiple methods of enzyme immobilization for biotechnological and bioanalytical assays. They can be divided into reversible and irreversible [169]. Adsorption, ionic binding, affinity binding, chelation or metal binding, and disulphide bonds belong to the reversible ones,

[11] Adapted from [165]. Numbers represent the number of SIA applications with the two overlapped characteristics.

whereas covalent binding, microencapsulation, cross linking and entrapment are the irreversible ones [169]. Several reviews on this topic were published [171-173], which also report on the used support materials. From these, synthetic or natural polymers such as chitosan [174], agarose beads [175] or inorganic materials [176], e.g. silica, are generally used.

Moreover, integration of the enzyme into the working electrode, e.g. by carbon pasting [177] for amperometric detection is a common strategy of achieving a sensitive biosensor [98, 165].

Apart from the incorporation of an enzyme on the active surface of the electrode, another approach, suitable also for other than electrochemical detection techniques, is the use of flow through reactors. Packed beds and open tubular formats are the most common ones. Schematic representation of such reactors is depicted in Figure 22.

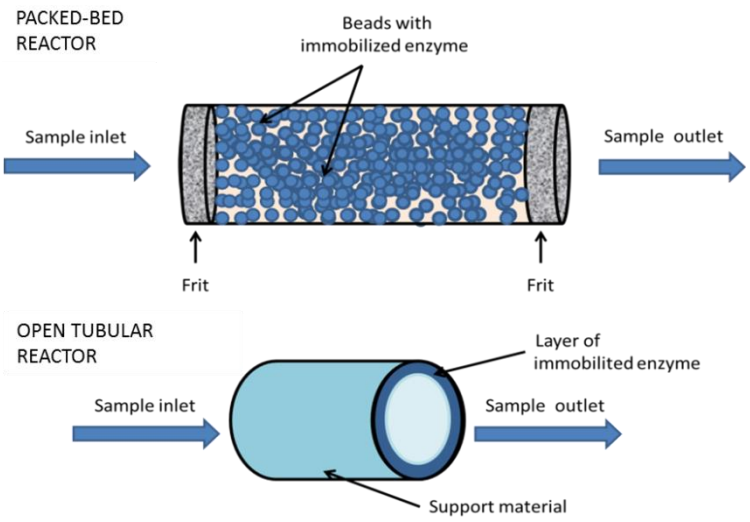

Figure 22: Examples of reactor with immobilized enzymes.

A packed-bed reactor is built by filling a tube by beads with immobilized enzyme. This type of the reactor brings the risk of high pressure in the system due to particle compaction [165]. On the other hand, it has the advantage of a large reactive surface. Open tubular reactors have the enzyme immobilized on the inner wall of a tube, integrated in the flow system. The apparent disadvantage of the lower enzyme quantity and hence substrate turnovers can be overcome by longer residential time of the sample in the reactor (low flow rate or stopped-flow methodologies) or the dimensions of the reactor [165].

Integration of an enzymatic reactor into a flow system can be done in several ways. In FIA, the reactors are placed downstream towards the detector, with the possibility of serial coupling of reactors for cascade reactions, where the product generated in one reactor passes through the second column with immobilized enzyme [178]. Simultaneous analysis of several analytes in one sample was done in parallel in a FIA system using multiple injection valves and different reactors for specific analytes [179]. In SIA, the reactor can be placed either before the detector, so that

detection of the product follows directly after the reaction [180]; in between the multiposition valve and the holding coil, so that a reagent can be added to the product of the enzymatic reaction [181]; or connected to a special port of the multiposition valve, so that the sample resides in the reactor for a defined time and is retracted back after the reaction [182]. The next generation of flow techniques, LOV, enables to build a new reactor with the beads, on which the enzyme is immobilized, for each assay directly in the desired channel of the monolithic manifold, with the possibility of on-column detection.

3.3.2.3 *Use of enzymes in solutions in flow methods*

Some authors recommend to use enzymes in a solution as this strategy assures a fresh portion of the enzyme for each assay [165]. Utilization of the enzymes in a homogeneous phase shows some advantage over the enzymatic reactors. Problems such as clogging of the packed reactor, carryover, deactivation of binding sites are omitted [77]. Also, a much simpler manifold can be utilized, without the need of preparing a special reactor [168]. However, using an enzyme in solution, the activity should be preserved by keeping the required conditions (pH, temperature, presence of acids/bases, organic solvents or heavy metals) during storage, preparation of the solution, and when at use. Temperature control of the system, reaction coil, or mixing chamber is often required to assure the optimum working temperature for the enzyme [180, 183]. SIA system for determination of propofol using an enzyme in solution with temperature control is discussed in chapters 4.2 and 5.1.

3.3.3 Reactions employing radiation in a flow system

3.3.3.1 *General remarks on applying radiation in analytical methods*

Radiation in several forms can be employed in different analytical procedures. Benefits of this approach can be enhanced by implementation of the effects of electromagnetic field and vibration to flow methods.

Some of the advantages of using ultrasound vibration as one of the auxiliary energies were outlined by Bendicho et al. [184]. These features can be applied also to the methods exploiting other forms of radiation and extended by the advantageous of flow methods:

a) Shortening of analysis in comparison with traditional, non-automated methods;
b) Possibility to decrease volumes of reagents;
c) Closed system – safety for the operator and less possibilities for sample contamination or loss of analyte(s);
d) Compliance with the principles of green analytical chemistry;
e) Lower cost of analysis with increased effectivity compared to the traditional methods.

The most typical forms of radiations used in combination with flow methods are microwaves, ultrasound and UV irradiation. Their application in combination with flow techniques is briefly described in the following chapters, with the focus on UV irradiation.

3.3.3.2 *Application of microwave irradiation in automated sample pretreatment*

Microwaves are a form of electromagnetic radiation of high frequency ranging from 0.3 to 300 GHz. Dipolar molecules (e.g. water) are re-oriented in the electric field of the microwaves. Since the electric field is altering, the molecules move. Thanks to this movement, heat is generated and transmitted through the sample [185]. The microwaves differ from the traditional sources of heat by faster temperature raise [185]. Microwave irradiation is mostly exploited for sample digestion, i.e. decomposing of a complex sample into simpler units by the means of heat, high pressure, and specific reagents for the respective time period [186].

Domestic ovens, generating microwaves of wavelengths around 12.2 cm, are often used in flow methods employing this form of radiation [187]. A chamber or a reaction coil is placed into the oven to attain the effects of microwaves on the sample in a flow system [188]. Both continuous and stopped-flow approach can be applied to assure sufficient dose of irradiation of the sample [189].

As stated by Smith et al., theoretically, all kinds of samples can be processed by microwave radiation [190]. Additional advantages of this approach are feasibility, safer work for the operator thanks to the closed environment (vapours of aggressive reagents), and low risk of sample contamination. Mineralization of biological samples can be shorten from hours to minutes when using microwaves [186].

A large number of methods implementing microwave-assisted digestion are focused on metal analysis. AAS or ICP-MS are mostly used for detection at such cases as these are the techniques typically combined with analysis of metals. On the other hand, bubbles generated by the radiation or Schlieren effect can interfere undesirably with spectrophotometric measurements [73].

Although digestion is the typical purpose to apply microwaves, examples of oxidation [187], extraction [188], or hydrolysis [189] in FIA systems assisted by microwave radiation can be also found in the literature. Comprehensive reviews on using microwaves in sample pretreatment [190], also in flow analysis [186], were published.

3.3.3.3 *Application of ultrasound in automated sample pretreatment*

Ultrasound is a vibration in a gas, liquid or solid with the frequency over 20 kHz. If the ultrasound is strong enough, bubbles (or cavities) are produced as the waves travel through a matter. The bubbles formation, their growth and collapsing is called cavitation [191]. This process is accompanied by a very high temperature and pressure [184].

In samples containing particles, the collapse of bubbles is accompanied by microjets towards solid surfaces. This promotes both the release of substances into the liquid phase and also helps to open bonds between the analyte and matrix components [192].

Ultrasound can be applied to the sample in two ways: using a chamber for ultrasound bath or an ultrasound probe. The probes might be advantageous, since the use of ultrasound bath shows some drawbacks: the non-uniformity of the energy distribution throughout the sample and the subsequent loss of power over time; the probe, on the other hand, can provide a more efficient cavitation in the sample by focusing the energy to a define sample zone [191].

Thus, application of ultrasound can bring improvements in various sample pretreatment techniques. Leaching is one of the most common application [191]. Extractions can be successfully carried out applying ultrasound: a water bath was used in a flow-batch method for determination of glycerol in biodiesel [193]. In this method, the analyte was extracted directly into the water mediating the vibrations and the method is completely reagent- and solvent-less, using only water. Both extraction and hydrolysis of paracetamol in suppositories were ultrasound-assisted and automated in a FIA system [194]. Enhancement of a chemiluminescence reaction of the luminol-H_2O_2-cobalt(II) system was carried out in a FIA system by immersing an ultrasonic probe for 4 s into water in a water bath with a reaction coil [195].

3.3.3.4 *Application of UV irradiation in sample pretreatment*

UV radiation is a spectrum of electromagnetic radiation characterized by wavelengths between 40 and 400 nm [196]. The UV radiation can be generated by deuterium, xenon or mercury vapour lamps, the latter being the most common ones thanks to their properties such as ease of operation, low cost, high efficiency of the energy conversion from electric to electromagnetic and emission preferably in the UV region [73]. Mostly, UV radiation is used to convert UV sensitive analytes to species detectable by the used detector [168]. The UV irradiation can fully replace the role of a derivatisation agent, and represents a more environment-friendly variant of derivatisation. This kind of energy can be further utilized for organic matter destruction, e.g. in analysis of environmental samples [196].

In flow methods, UV irradiation is implemented easily by coiling a tube around the lamp being the source of radiation as shown in Figure 23. Similarly to the implementation of microwaves, stopped-flow strategy can be applied to a system with a coiled reactor to achieve the necessary time of irradiation. Alternatively, a small chamber can be placed next to the lamp. The properties of the material building the coil or chamber must be considered; it must allow the radiation transmission to the sample [197]. Measurement of chemiluminescence or fluorescence is quite typical in methods with UV irradiation since this is related to the generation of reactive species [196].

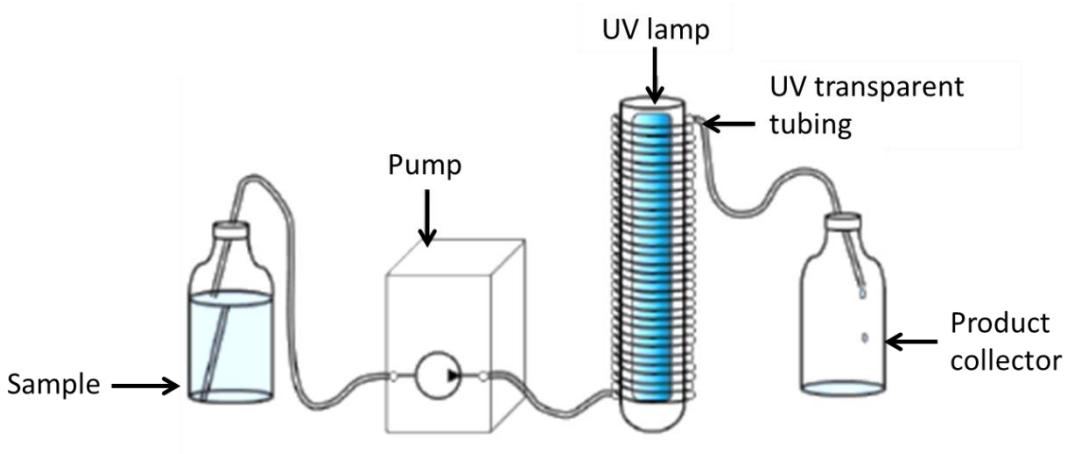

Figure 23: Example of a simple flow system with a UV lamp and a reaction coil[12].

A FIA system for hydride generation with AAS comprising a coiled reactor for on-line photooxidation with peroxodisulphate for determination of organoarsenic and organotin compounds in environmental samples was built by Tsalev et al. [199].

UV photodecomposition of organic arsenic species was carried out in a FIA system prior to hydride generation by addition of sulphuric acid and aqueous sodium borohydride and ICP-MS

[12] Adapted from [198].

detection. The system was built to verify the applicability of an automated hydride generating interface to ICP-MS for measurement of arsenic species in rice [200].

A SIA system with cold vapour AAS was developed for on-line monitoring of mercury in river water [201]. UV irradiation was used in this case to enhance the digestion of the sample. The digestion was done by mixing the sample with the bromide/bromate oxidation mixture and then passing through the UV irradiated reaction coil.

Tue-Ngeun et al. [202] used UV irradiation for digestion of dissolved organic carbon in freshwater samples with acidic peroxodisulphate. Then, the sample zone passed a phase separator and was captured by the acceptor stream of cresol red. In the same SIA system, dissolved inorganic carbon was determined: the sample reacted with sulphuric acid, and CO_2 passed again through the membrane into the stream of cresol red and measured. The values obtained by measuring the inorganic fraction were subtracted from the overall values to obtain the concentration of the dissolved organic carbon.

Coiled reactor for UV irradiation at the presence of peroxodisulphate was used in a FIA system for determination of malathion in waste water, grains and vegetables [203]. Phosphate, the product of this reaction, further enters another, two-step reaction, the product of which is then detected by a fluorescence measurement.

Pesticide asulam was determined using a multicommutation system [204]. After the irradiation of the sample, oxidation with potassium permanganate in sulphuric medium followed and subsequently, the generated chemiluminescence of the photoproducts was recorded.

UV photodegradation was applied in the determination of the antibiotic drug chloramphenicol. The photoproducts, unlike the original substance, show chemiluminescence upon reaction with potassium permanganate in sulphuric acid. The reaction was automated in a FIA system [205].

Application of UV irradiation to the pesticide metsulfuron methyl to generate a fluorescent product, and the study of conditions with an effect on fluorescence is described in chapters 4.5, 5.4 and 5.5.

3.3.4 Automation of liquid phase-based sample pretreatment techniques using flow systems

3.3.4.1 *General remarks on application of flow methods for automation of liquid extractions*

Liquid phase-based sample pretreatment techniques, or solvent extractions, are widely used in analytical chemistry. They are used not only in batch-wise manners but nowadays the importance of their automation is increasing, too. The main features of LLE and LPME were discussed in chapter 3.1.2. Reduction of solvent consumption is one of the most important advantages of modern, miniaturized sample pretreatment methods. Adding the advantages of flow techniques to the extraction process can help to eliminate some drawbacks of LPME techniques: long phase separation times can be shortened, errors-prone manual handling of small volumes can be avoided, precision and repeatability can be improved due to programmed operation, and health risks associated with an exposition of the operator to vapours of organic solvents are avoided thanks to the closed tubing manifold.

Solvent extractions have been performed in flow methods since early after their introduction. This chapter will review some approaches of the implementation of selected (non-dispersive LPME, DLLME, SDME) sample pretreatment methods in flow analysers.

3.3.4.2 *Automation of non-dispersive liquid - liquid (micro)extraction*

Using a flow manifold in automation of liquid - liquid microextraction is advantageous, which is documented by a high number of publications (over 100 in 2013) listed in a FIALab Database [206]. Representative examples of LPME automated in a flow manifold are listed in a chronological order in Table 3. The goal of LLE automation in flow techniques is to ensure a large contact area between the sample and acceptor solution and subsequently, the measurement of the analyte concentration, typically in the acceptor phase, in a simple and automated way.

The first methods for automated LLE were suggested in 1978 by Karlberg and Thelander [207] and by Bergamin [208]. In their works, the sample was injected into a stream of aqueous carrier in a FIA system. The aqueous phase was then divided into segments by the introduction of the organic phase. The extraction occurred while the altering segments of sample and extractant passed along a PTFE tube. Afterwards, the phases were separated in a specific unit.

A similar approach was adopted by Comitre and Reis, who employed a MCFIA system for the automation of LPME and determination of Mo or Pb in plant materials, respectively. The sample, reagents, and solvent were aspirated as very short plugs in several repetitions into an extraction coil, where the reaction and extraction took place. Then, the mixture proceeded into a unit, where

the phases were separated and the phase with the extracted analyte was further delivered to the detector [209, 210].

In 1996, Luo et al. presented a different approach with Bromothymol blue as a model substance. The authors took advantage of the affinity of organic solvents to the hydrophobic PTFE tubing to form a so-called wetting film [211]. In their method, the extraction solvent, sample, and a second solvent were sequentially aspirated in a SIA system. By flow reversal, the analyte was extracted into the film of benzene formed on the inner tubing wall and was then washed into the detector by 25% methanol – 1M NaOH solution. This approach does not require any additional unit in the system, such as a mixing or a phase separation units. However, careful choice of the solvents must be done with regards not only to the extractability of the analyte but also to the affinity to PTFE and the method repeatability. The same principle was used also in the determination of nitrophenols by Cladera et al. [212] and Cr(VI) by Nielsen and Hansen [213].

In 2004, Diniz et al. [214] used a glass chamber as extraction and separation unit. Mixing and sufficient contact between the two phases was assured by placing the chamber with a magnetic rod on a stirrer. After the stirring time and subsequent phase separation, water was pumped into the chamber to elevate the upper layer into the detection cell. In 2008, Anthemidis also used a chamber for separation after the extraction in an extraction coil. SIA system with additional peristaltic pump and injection valve to deliver the sample to the extraction chamber after it´s mixing with reagent in the confluence point were used. The system was synchronized with the sample uptake of the FAAS detector [215].

SIA systems with a pipette tip mounted to one port of the selection valve, serving for phase separation were used by Burakham et al. in 2005 [216]. The applied technique was named Lab-at-valve (LAV). The extraction took place in a coil, into which the solutions were sequentially aspirated. After flow reversal, the liquids were delivered into the separator and after discarding the aqueous phase, the organic part was propelled to the detector. The problem of non-stability of the baseline and affected repeatability can arise by the phase changes in the detector when an aqueous carrier is used.

Škrlíková et al. (2010) developed a universal Dual Valve-SIA (DV-SIA) system in our laboratory [217]. The system comprised two SIA systems, one serving for the aspiration of the reagents and extraction solvent (extraction unit, using water as carrier), the second one (detection unit, using the extraction solvent as carrier) only for handling the organic phase. A polypropylene microtube of 1.5 mL was used as a separation subunit. The solutions were aspirated and subsequently delivered from the extraction unit to the separation chamber. After phase separation, the organic solvent used for the extraction was aspirated in the detection unit for measurement. In this way, problems related to the presence of two phases in one system were circumvented. Formation of an organic film on the tubes, bubbles in the system or baseline shifting were avoided.

Table 3: Selected methods for LLE automation using a flow manifold.

Analyte	Matrix	Extractant	Flow technique	Mode	Detection	LOD [µg L^{-1}]	Ref.
Bromothymol blue	Standard	Benzene	SIA	Wetting film, back-extraction with MeOH+NaOH	UV-VIS	not given	[211]
Cu	Plant leaves	CCl_4	FIA	EC / PS	UV-VIS	5	[222]
Cr(VI)	Standard	MIBK	SIA	EC	ETAAS	0.0032	[213]
2-Nitrophenol	Waste waters	Butylchloride: n-octanol 66:34 (v/v)	SIA	Wetting film, back-extraction with NaOH	UV-VIS	36	[212]
3-Nitrophenol						46	
4-Nitrophenol						4.9	
Al	Natural and waste water	$CHCl_3$	FIA	Segmented flow / membrane separator	FL	6	[223]
				Segmented flow		2	
				Non-segmented flow		0.2	
Mo	Plants	Isoamyl alcohol	MCFIA	Segmention and mixing chamber	UV-VIS	4.6	[209]
Cu(II)	Bovine liver, river water	MIBK	MCFIA	Batch mode, stirring-assisted extraction	UV-VIS	20	[214]
Pb	Plants	CCl_4	MCFIA	EC / PS	UV-VIS	12	[210]
Diphenhydramine	Pharm.	$CHCl_3$	SIA	EC / LAV	UV-VIS	1900	[216]
Anionic surfactants	Water					480	

Methanol	Biodiesel	Xylene	FIA-SIA	Hydrophilic membrane	UV-VIS	2000	[220]
Pb	Natural waters, CRM	MIBK	SIA with IV and PP	EC / PS	FAAS	1.4	[215]
Solasodine	Plants	$CHCl_3$	SIA /	EC / LAV	UV-VIS	1410	[224]
Picric acid	Pharm., water	Ethylacetate	DV-SIA	Segmention and mixing chamber	UV-VIS	1060	[217]
Cu	Pharm., water	Amylacetate	DV-SIA	Air assistance	UV-VIS	20	[225]
Pb	Natural waters, urine, CRM	$CHCl_3$	SIA-LAV	Drop-in-plug	FAAS	1.8	[218]
Pb	Natural waters, CRM	$CHCl_3$	SIA	Chamber for counter-current extraction	FAAS	1.5	[219]

The system was firstly used for the determination of picric acid in pharmaceutical formulations and drinking waters. A modified system based on similar assembly applied to determination of thiocyanates in biological samples (saliva) is discussed further in chapter 4.3 and as attachment in chapter 5.2.

Mitani et al. (2015) [218] brought the sample and extraction solvent into contact by pumping chloroform (extractant) into the extraction chamber, similar as in LAV, and propelling the aqueous sample from an inlet in the bottom of the chamber through the plug of chloroform (drop-in-plug). Another manifold, suitable for both solvents lighter and denser than water, was proposed later by the same authors [219]. The solvent (denser than water) was pumped to the upper inlet of the extraction chamber and runs downwards in a channel engraved on the inner wall of the chamber. The sample was pumped from the bottom inlet of the chamber in opposite direction, so a counter-current extraction takes place. Thereafter, the organic phase was collected at the bottom of the chamber and propelled to the detector.

A different strategy is presented by utilization of membranes for LPME in flow systems. Two streams - sample and acceptor - are propelled towards a semipermeable membrane from opposite sites and the analyte is transported through it into the acceptor, as it was done e.g. by Araújo et al. [220].

More details on applications of membranes in automated LPME as well as on automation of other LPME techniques in flow are given in a review by Silvestre et al. [221].

3.3.4.3 *Automation of dispersive liquid - liquid microextraction*

In DLLME, the volume of the extraction solvent is decreased, and more organic solvents are typically used in the system, having the function of extractant, disperser, or eventually a modifier. In automation of DLLME, the aims are:

1. Use of a very small volume of an extraction solvent,
2. Creation of the cloudy solution,
3. Handling of very small volumes of the solvent (~ tens of μL) in a repeatable manner.

Several technically different approaches were suggested for the automation of DLLME. Automated methods simplify the use of extractant with density lower than that of water, which might be even more difficult to handle than extractant heavier than water. The role of dispersive solvent can be substituted by kinetic energy applied to the analytical mixture, which is in concordance with the requirements of green analytical chemistry.

In the works of Anthemidis and Ioannou from 2009 and 2011, a cloudy solution was created in one tube of a SIA manifold by confluence of the sample and the solvent mixture [226, 227]. The droplets of the organic phase containing the analyte (an on-line formed complex of the metal cation of interest) were retained on a home-made column with PTFE or PEEK flakes and the analyte was then eluted with methyl isobutyl ketone. The system enabled chelation, cloudy solution formation,

extraction, and phase separation in one step and it was effective thanks to the continuous flow and high sample to solvent ratio. The solvent density did not have to be considered. On the other hand, a column for phase separation and the third solvent for elution to the detector had to be used.

Recently, Alexovič et al. developed another approach. A plastic centrifugation microtube with a volume of 1.5 mL was used as an extraction chamber, into which the sample, reagent, and the extractant or extraction mixture were delivered. Two modes of mixing were applied in different works, by air bubbling [228] and by vigorous injection [229]. A DV-SIA system was employed to handle the aqueous and organic solvents separately. Later, the system was simplified to a single-valve (SV)-SIA system. Several ways were suggested to handle extraction solvents lighter than water: aspiration of the upper layer from the extraction solvent after phase separation [228], use of an auxiliary solvent of high density to achieve sedimentation of the organic phase [230], or most recently, the use of the microtube in an upside down position so that the conical part is at the top (Figure 24), which enables an easy collection of the phase with the extracted analyte [229]. This later flow-batch approach has the advantage of an easier configuration in comparison with the works from Anthemidis and Ioannou [226, 227] described above. Moreover, droplet retention on a sorbent with later elution was not required, thus the use of solvents is limited to the extractant. On the other hand, the sample volume and therefore the enhancement factor were limited by the volumes of the syringe and extraction unit. Also, the system is not self-washing, as in the works of Anthemidis and Ioannou.

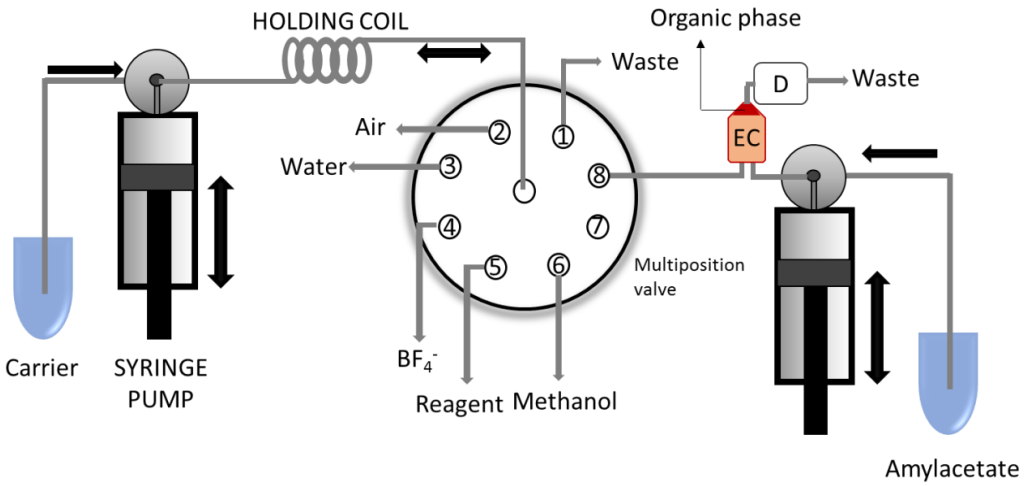

Figure 24: SV - SIA system for determination of boron[13].

Berton and Wuillaud used a FIA system for handling the sample and solvents mixed off-line [231]. The mixture was loaded onto a Florisil column and then eluted with acidified acetone solution to ETAAS detector. The use of an ionic liquid for the extraction of metals makes the developed methods more environmental-friendly.

[13] Adapted from [229].

Table 4: Selected methods for DLLME automation using a flow manifold.

Analyte	Matrix	Extractant/ Disperser	Flow technique	Mixing	Separation	Detection	LOD [µg L^{-1}]	Ref.
Cu, Pb	Natural waters	Xylene/MeOH	SIA	On-line in tube	PTFE turnings column, elution with MIBK	FAAS	0.04 (Cu) 0.54 (Pb)	[226]
Pb, Cd	Natural waters	Xylene/MeOH	SIA	On-line in tube	PTFE turnings column, elution with MIBK	ETAAS	10 (Pb) 2 (Cd)	[237]
Ag	River, sea and waste water,	1-dodecanol/ MeOH	SIA	On-line in tube	PEEK turnings column, elution with MIBK	FAAS	0.15	[227]
Co	Tap and river water, pharm.	CYPHOS® IL 101/acetone	FIA	Off-line	Florisil column, elution with 10% (v/v) HNO_3 acidified-acetone solution	ETAAS	0.008	[231]
Se(IV) Se(VI)	Garlic, natural waters	CYPHOS® IL/MeOH	FIA	Off-line	Florisil column, elution with 10% (v/v) HNO_3 acidified-acetone solution	ETAAS	0.015	[238]
SCN^-	Saliva	Amylacetate/ ACN/ CCl_4	SIA/ batch mode	High flow-rate injection	Different densities of solutions	UV-VIS	17	[230]
Th	Different waters, urine, mussel tissue	[Hmim][PF_6]/ MeOH	SIA	On-line in tube	Polyurethane foam column, elution with MIBK	FAAS	0.86	[239]
Cr(VI)	Tap, river, bottled water	Toluene/air	SIA/ batch mode	Air bubbling in EC	Different densities of solutions	Stopped flow UV-VIS	4.5	[228]
B	Tap and bottled water	Amylacetate/-	SIA/ batch mode	Vigorous injection	Different densities of solutions	Stopped flow UV-VIS	3	[229]

PAH	Natural waters	Trichlorethylene/ ACN	MSFIA-LOV	Injection into EU	Different densities of solutions	GC-MS	0.01-0.07	[234]
As	Wine	$[C_8mim][PF_6]$	FIA	Off-line	Florisil column, elution with 10% (v/v) HNO_3 acidified-acetone solution	ETAAS	0.005	[240]
Benzo(a) pyren	St. and tap water	n-Octanol/ ACN	LIS-MSC	In syringe	Different densities, chromatographic column	UV-VIS	0.7	[137]
Phenols	Well water	Xylene/ACN + n-propanol	MSFIA	In syringe	Different densities of solutions	UV-VIS (LWCC)	0.9	[233]
Rhodamin B	Waters, soft drinks	n-octanol/ n-propanol	LIS	In syringe	Different densities of solutions	UV-VIS	7	[232]
Al	Seawater	n-hexanol/EtOH	LIS	In syringe	Different densities of solutions	FL	0.22	[241]
Cr(VI)	Waters	n-hexanol/-	LIS	In syringe with m.s.	Different densities of solutions	UV-VIS	0.27	[235]
Al	Sea and pond water	n-hexanol	LIS	In syringe with m.s.	Different densities of solutions	FL	0.22	[142]
DBAS	Water	5% n-hexanol in $CHCl_3$/-	LIS in upside down position	In syringe with m.s.	Different densities of solutions	UV-VIS	6	[236]

IBMK: isobutylmethylketon, MeOH: methanol, EtOH: ethanol, $CHCl_3$: chloroform, PTFE: polytetrafluorethylene, $[Hmim][PF_6]$: 1-hexyl-3-methylimidazolium hexafluorophosphate, CYPHOS® IL: tetradecyl(trihexyl)phosphonium chloride, PAH: polycyclic aromatic hydrocarbons, EU: extraction unit MSC- multisyringe chromatography, FL: fluorescence, DBAS: disulfine blue active substances, m.s.: magnetic stirring

Maya et al. (2012) demonstrated DLLME of benzo(a)pyrene using the later denoted LIS technique for the first time with subsequent separation of the extracted analyte on a monolithic column [137]. In further works for the determination of rhodamin B, copper, and phenols, respectively, the system was fully automated and compact, and allowed mixing, extraction, phase separation and even detection inside the syringe [140, 232, 233].

Hyphenation of in-syringe DLLME performed in a MSFIA system with GC was demonstrated by Clavijo et al. [234]. Horstkotte et al. then proposed dispersion and mixing using the kinetic energy of a stirring bar inside the syringe thus performing magnetic stirring-assisted DLLME [142, 235]. Although utilization of a solvent with density higher than water as typical in DLLME might not be feasible to perform inside an upright syringe, this concern was solved later by using the syringe in upside-down position [236]. This later configuration enabled to use chloroform as an extraction solvent, to empty the syringe (containing the stirring bar) completely by the air inside the syringe, and to perform the extract washing.

The selected works with different proposed DLLME automation modes and some key features of the methods are listed in Table 4. DV-SIA systems with an auxiliary solvent for DLLME of thiocyanates, with two different detection cell positions, were compared. The results were presented at the ICFIA conference in 2012. The work is commented in chapter 4.3.

3.3.4.4 *Automation of single drop microextraction by flow techniques*

There are significantly less works devoted to the automation of SDME in a flow system than other non-dispersive or dispersive LPME techniques, probably due to the seemingly cumbersome manipulation with a single drop. The stability of a small volume of the acceptor phase during the preconcentration must be assured and physicochemical characteristics should be carefully considered, e.g. miscibility of the sample and acceptor solutions according to the SDME mode (head-space or direct immersion), and the affinity of the acceptor phase to the support material must be kept in mind before building a flow manifold.

The first attempts of automation date back to 1995, when Liu and Dasgupta published a study of a liquid drop exploited as a sampling interface, used for determination of NH_3 in a SIA system [5]. Two concentrically placed tubes were used in the work, the inner one for the drop delivery and support, the outer one as a sample line. After the analyte collection time, the drop was withdrawn into a SIA system and further processed. The geometry and physicochemical processes of the analyte collection were described and the effects of relevant parameters such as humidity, drop size and sample flow directions were explained in details.

Another exploration of the drop possibilities, so called drop-in-drop technique, was presented in 1996 by H. Liu and Dasgupta [14]. In this work, a drop of the sample solution coloured by a reagent was formed around the drop (1.3 μL**)** of chloroform. After the extraction time, the drop was replaced by a colourless washing solution and the absorbance was measured. The drop was used

not only as the acceptor phase but also as a windowless detection cell, so that any possible signal bias caused by the drop transportation within the system or interaction of the drop with the cell walls was omitted.

In 2008, Pena et al. presented a SIA system directly coupled with ETAAS detector for a single drop immersed in a sample [242]. The system comprised a vial with the sample, in which an immersed drop was generated, and later withdrawn by the furnace sampler arm. The external chamber (vial) was placed on a magnetic stirrer to enhance the analyte transfer into the drop. Although a peristaltic pump was used in the system, the sample volume and thus the method sensitivity was limited by the vial volume.

An extraction chamber for SDME in a SIA system was suggested by Anthemidis and Adam [243]. The chamber comprised a glass capillary for the drop support. The sample was pumped from the bottom of the chamber by an additional peristaltic pump during the extraction time. The drop was then aspirated back into the system and later pumped through injection valve into the graphite furnace for detection in ETAAS.

A system similar to DV-SIA was assembled by Timofeeva et al. [244]. One pump, filled with the acceptor solution, was used to generate a drop above the sample of concrete in the sample vial, connected to one of the ports of the selection valve. After the analyte (NH_3) collection in the acceptor phase, the drop was pumped into a mixing chamber. The second pump was used to aspirate reagent via the second valve and to propel it into the mixing chamber, where the reaction to form a coloured product took place. The mixing was assured by the air. In this method, the vials containing solid samples were replaced manually.

In chapters 4.4 and 5.3, a HS-SDME without any additional chamber, automated in a LIS system is described. Vacuum was generated and magnetic stirring was exploited to enhance the analyte transfer into the drop. This idea was adopted by Mitani et al., who used a similar system with additional pump and valve for the determination of mercury [144]. Some examples of SDME automation in flow manifolds are listed in Table 5 in chronological order.

Table 5: Selected methods for SDME automation using a flow manifold.

Analyte	Matrix	Acceptor phase	Flow technique	Mode	Drop size [µL]	Detection	LOD [µg L^{-1}]	Ref.
NH_3, SO_2	Standards	H_2SO_4	SIA	Drop in flowing gaseous sample	4.5	UV-VIS	not given	[5]
SDS	Standards	$CHCl_3$	FIA	Drop-in-drop	1.3	UV-VIS	740	[14]
Cr(VI)	Natural waters	Toluene	SIA	Immersed drop	3	ETAAS	0.2	[242]
Cd	Natural waters	DIBK	SIA	Drop in a flowing liquid sample	60	ETAAS	0.01	[243]
Hg(II)	Natural waters	Pd^0 suspension	LIS	Head-space	25	ETAAS	0.48	[144]
NH_3	Concrete	H_3PO_4	DV-SIA (SWIA)	Head-space	5	UV-VIS	30 *	[244]

SWIA: stepwise injection analysis

* µg kg^{-1}

3.3.5 Automation of solid phase-based sample pretreatment techniques using a flow system

3.3.5.1 *Generals remarks on the application of flow methods in automation of solid phase-based microextraction techniques*

Automation of solid phase-based microextraction in flow systems is an interesting field in the flow analysis community considering that the number of publications listed in FIA database [206] devoted to this technique was higher than 200 in the year 2013 with mainly solid phase extraction (SPE) being the target of automation. The explanation of the high interest might be the applicability of these techniques for different purposes (matrix removal, preconcentration) for different analytes on one hand, and search for automation of laborious manual procedures (taking into account the protocols for e.g. SPE or MEPS) on the other hand. Various formats and materials of sorbents and different flow techniques with a plenty of system configurations have been described. Representative examples of possibilities of flow techniques in automation of SPE are described in the following chapter and listed inTable 6.

3.3.5.2 *Examples of solid phase-based microextraction automated in a flow system*

A multitude of different kinds of sorbent chemistries and formats were incorporated into a flow manifold to perform the preconcentration and purification of the analyte. Some modes of SPE automation were derived from the wetting-film extraction, such as the utilization of a tube of a flow manifold as a solid surface for extraction. A knotted PTFE coil was used as a phase for adsorption of a complex of Cr(VI) before its elution with ethanol and propelling to an ETAAS detector [213]. A similar approach was chosen by de Aquino et al. [245]. In their work, the walls of a glass chamber acted as the extraction phase, and the analyte (Zn) was retained at high pH, at which the silanol groups were deprotonated. The analyte was then extracted from the glass walls by an organic solvent containing a ligand binding to the analyte. Although this approach does not require sorbent particles, they were adopted in majority of methods for solid phase sample pretreatment methods.

In order to avoid the loss of sensitivity caused by elution of the analyte preconcentrated on a sorbent, Miró et al. measured the absorbance or reflectance of the analyte while still being retained on a membrane or in a microcolumn, respectively [246, 247]. A FIA system was employed in both works to ensure the sorbent conditioning, sample loading and rinsing, and elution. An optosensing device was mounted directly to the solid surface of the sorbent, and this approach enabled the measurement of nitrite at trace levels.

In our working group, Šatínský and co-workers contributed significantly to the field of SPE automation. In 2002, a FIA system with a microcolumn filled with carboxyl–modified silica was used for the determination of salbutamol in both pharmaceutical formulation and urine [248]. Later on, SIA systems were employed in SPE automation. An elegant separation of two substances without a chromatographic column was done by retention of the non-polar one on a C18 sorbent and percolation of the polar one through the sorbent without retention [249]. Coupling of RAM for increased selectivity [61, 250] and anion exchanger sorbents [251] was also successfully carried out in SIA systems.

Integration of two SPE columns into one system for parallel determination of UV filters was shown by León et al. [252]. While one sample was processed on one SPE cartridge, the previous one has been already analysed on a coupled HPLC system. Such coupling of an analyser system directly to a sample pretreatment system enables full exploration of the advantages of sample pretreatment automation as the analytes can be eluted from the preconcentration cartridge onto the chromatographic column.

A different strategy for SPE automation was presented by Oliveira et al. [253] who employed MSFIA and several solenoid pumps to commute and direct the flow streams either to a polymeric resin or to bypass it. The method was applied for screening of phenolic pollutants. MSFIA was also used in connection with LOV platform, which enables the utilization of renewable sorbents. The beads slurry is aspirated into a LOV channel and after the conditioning steps, the sample is loaded. Thereafter, the analytes are eluted with a proper solvent directly into the loop of an injection valve and proceed further onto an analytical column. The sample preparation in a flow system is synchronized with the LC analysis [254]. Methods based on a similar approach were applied to the determination of UV filters and riboflavin by Oliveira et al. [255, 256].

The possibility to build a multimodal column in a flow manifold was shown by Boonjob et al. [257]. The authors used a MSFIA manifold with a selection valve and an injection valve. The column was built in the short loop of the injection valve and the slurries and solutions were aspirated through the ports of the selection valve. The eluate was pumped directly to the coupled chromatographic system. The described system enabled to carry out automated multiresidue assays.

A single valve was used in a SIA system of Karakosta et al. [258]. The analytes were retained on and eluted from a cartridge mounted to one of the lateral ports of the valve and collected to a vial. The eluted analytes were aspirated in the next step from the same vial and delivered onto a chromatographic column coupled to the system.

A conical column filled with Cyanex 923 (a mixture of straight-chain alkylated phosphine oxides, mainly (92.4%) with normal hexyl and octyl groups) sorbent was mounted in a SIA system for cadmium preconcentration with posterior hydride generation and detection by atomic fluorescence spectrometry [259]. The system comprised both SIA and FIA components with the proportional injection valve.

A simpler FIA system with anion exchanger column was used for extraction of arsenic species from groundwater before hydride generation AAS [260]. A cartridge mounted to the ports of an injection valve was used for cadmium preconcentration before FAAS detection. The sample was loaded by means of a peristaltic pump and eluted after valve switching, in an opposite direction, by means of a syringe pump, to the detector [261].

Recently, coupling of SPE with separation and detection within one SIC system was presented [262]. The sample was pumped through the SPE cartridge connected to the lateral port of a one selection valve and the central port of the second valve. The matrix was washed out to the waste and the retained analytes were eluted directly onto the chromatographic column connected to a lateral port of the second valve. Excellent validation parameters were obtained with this method for eight sulphonamides separated on a pentafluorophenylpropyl fused-core column.

In this dissertation, a work exploring on-line SPE in a flow-batch system coupled to a photodegradation and detection chamber is described and commented in chapters 4.5 and 5.4. The first coupling of MEPS and a separation column within one SIC system is described and discussed in details in chapters 4.6 and 5.6.

Table 6: Selected methods for SPE automation using a flow manifold.

Analyte	Matrix	Technique	Sorbent, format	Detection	Remark	Ref.
Cr (VI)	NIST ref. material	SIA	PTFE tube inner walls	ETAAS	Analyte adsorbed on the coiled tube, eluted with MIBK	[213]
NO_2^-	Tap and natural waters	FIA	PTFE with C18 or sulfonate / discs	Diffuse reflectance	Measurement on the sorbent	[246]
NO_2^-	Tap and natural waters	FIA	C18 / disc	Solid-phase absorptiometry	Measurement on the sorbent	[247]
Zn	Pharm.	MSFIA	Glass tube inner walls	UV-VIS	Adsorption on glass at high pH	[245]
Salbutamol	Pharm., urine	FIA	Carboxyl group modified silica gel	UV-VIS	-	[248]
Ascorbic acid, rutin trihydrate	Pharm.	SIA	C18 / column	UV-VIS	Dissolution test, separation of two substances without column	[249]
Salbutamol	Serum and urine	SIA	C18, carboxyl group modified silica gel / column	CL, FL	Comparison of two detection techniques	[263]
Dodecylamine	Diesel fuels	FIA	Aminopropyl isolute /column	CL	-	[264]
Quinlukast	Serum	SIA	RAM / column	UV-VIS	-	[61]
Fe (total)	River water, dogfish muscle	MPFIA	Polymer support with iminodiacetic groups /discs	UV-VIS	Chelating of iron on the disc	[265]
Phenols	CRM	MSFIA	Amberlit XAD-4	UV-VIS	Reaction with 4-aminoantipyrine	[253]
Cd	Tea samples	FIA/SIA	Conical column of Cyanex 923	AFS	On-line hydride generation	[259]

NSAID residues, benzofibrate	Waste water, urine	MSFIA	Renewable Oasis HLB column	UV-VIS	Column built in a LOV platform (BI), coupling to HPLC	[254]
Amilorid	Urine	SIA	WCX-carboxy group / column	FL	-	[251]
Propranolol	Plasma	SIA	RAM	FL	Reaction after extraction	[250]
Morphine	Human urine	SIA	C18 column	UV-VIS		[266]
Phenols	Waters and environ. samples	MSFIA	Polystyren-divinylbenzene	UV-VIS	Coupling to HPLC	[267]
UV filters	Urine	SIA	C18, DEA columns	UV-VIS	Parallel utilization of two SPE columns, coupling to HPLC	[252]
UV filters	Waters	MSFIA	Oasis® HLB / Renewable	DAD	Column built in a LOV platform (BI), coupling to HPLC	[255]
Riboflavin	foodstuffs	MSFIA	MIP / Renewable	UV-VIS	Column built in a LOV platform (BI), coupling to HPLC	[256]
Herbicides	Soil extracts	MSFIA	MIP + Oasis®/renewable	UV-VIS	Column built in a valve loop, bimodal SPE, coupling to HPLC	[257]
Cd, Pb	Water, CRM	FIA	Oasis® HLB	FAAS	-	[261]
As(III), total As	groundwater	FIA	SAX column with triethylaminopropyl	AAS	On-line hydride generation	[260]
Captopril	Urine	SIA	Oasis® HLB	UV-VIS	Coupling to HPLC	[258]
Sulphonamides	River water	SIC	SAX column with 3-trimethylamino-2-hydroxypropyl	UV-VIS	Coupling of SPE and separation in one system	[262]

MIBK: methyl isobutyl ketone, Pharm.: pharmaceutical formulations, CL: chemiluminescence, FL: fluorescence, NSAID: non-steroidal anti-inflammatory drugs, DEA: diethylaminopropyl, CRM: certified reference material, WCX: weak cation exchanger, BI: bead injection, SAX: strong anion exchanger

4 RESULTS AND DISCUSSION

4.1 LIST OF PUBLICATIONS INCLUDED IN THE THESIS

1. C.C. Acebal, H. Sklenářová, J. Škrlíková, ***I. Šrámková***, V. Andruch, J.S. Balogh, P. Solich, Application of DV-SIA manifold for determination of thiocyanate ions in human saliva samples, Talanta 96 (2012), 107-112.

2. ***I. Šrámková***, C.G. Amorim, H. Sklenářová, M.C.B.S.M. Montenegro, B. Horstkotte, A.N. Araújo, P. Solich, Fully automated analytical procedure for propofol determination by sequential injection technique with spectrophotometric and fluorimetric detections, Talanta 118 (2014), 104-110.

3. ***I. Šrámková,*** B. Horstkotte, H. Sklenářová, P. Solich, Automated in-syringe single-drop head-space micro-extraction applied to the determination of ethanol in wine samples, Analytica Chimica Acta 828 (2014), 53-60.

4. C.C. Acebal, M. Grünhut, ***I. Šrámková***, P. Chocholouš, A.G. Lista, H. Sklenářová, P. Solich, B.S. Fernández Band, Application of a fully integrated photodegradation-detection flow-batch analysis system with an on-line preconcentration step for the determination of metsulfuron methyl in water samples, Talanta 129 (2014), 233-240.

5. P. Bolinová, ***I. Šrámková***, H. Sklenářová, C.C. Acebal, B.S. Fernández Band, P. Solich, Study of the effect of organic solvents on the fluorescence signal in a sequential injection system, Analytical Methods 6 (2014), 9392 – 9396.

6. ***I. Šrámková***, P. Chocholouš, H. Sklenářová, D. Šatínský, On-line coupling of micro-extraction by packed sorbent with sequential injection chromatography system for extraction and determination of betaxolol in human urine, submitted to Talanta.

4.2 COMMENT ON PUBLICATION 1

Fully automated analytical procedure for propofol determination by sequential injection technique with spectrophotometric and fluorimetric detections

In this work, two methods for propofol determination in pharmaceutical formulation were developed and compared. For quantification, two different detectors were used: a spectrophotometer and a fluorimetric detector comprising photomultiplier with excitation and emission filters.

A simple SIA system with a water bath and a debubbler device was used to perform the spectrophotometric determination. Since propofol is a phenolic substance, a colour reaction with 4-aminoantipyrine (4-AAP) catalysed by horseradish peroxidase was chosen for the spectrophotometric determination. Several facts had to be considered before the method development and optimization. First, propofol as a lipophilic compound is administered as an oil-in-water emulsion, i.e. the excipients would contain hydrophobic substances and thus pure aqueous solution could not be prepared. Second, since the used reaction was catalysed by an enzyme, its denaturation should be avoided. Therefore, to release the substance from the formulation and to free it for the reaction with 4-AAP to yield a colour product, ethanol was used to dilute the emulsion in a 1 to 10 ratio. The colour product was determined at a wavelength of 485 nm. The enzyme was used in a form of a solution prepared freshly every day, which ensured that it could be used without any significant activity change during one working day and it was not altered by the content of ethanol in the sample solution. Also, using the flow manifold, the enzyme consumption was minimized.

The reaction conditions were optimised. First, the concentrations of reagents were tested and the optimum temperature was found in batch conditions and then these were transferred to a flow system, comprising also a water bath to ensure that the holding coil, accommodating the reaction mixture, was placed at the optimum temperature of 37°C. The mixture of ethanolic and aqueous solutions and increased temperature, however, led to bubbles formation, which worsen the measurement repeatability and precision. A de-bubbler device was therefore added to the system prior to the flow detection cell. The solution passed along a gas permeable membrane of the device and bubbles were released through the membrane pores in its upper compartment and only a consistent plug of the reaction product passed through the detection cell.

The system with fluorimetric detection consisted of only the basic SIA components. Since propofol is a naturally fluorescent substance, no derivatisation was required. Only ethanol was used as reagent to break the emulsion and to release propofol. Using filters for specific wavelengths cut-offs for propofol excitation and emission, no interferences of the emulsion constituents were observed in the ten times diluted samples.

Similar detection and quantitation limits were obtained in both systems. Lower sample throughput was achieved with the enzymatic reaction, however, it was shown that similar limits can be obtained using enzymatic reaction for spectrophotometric detection, which is commonly less sensitive than the fluorimetric one. Two simple methods for the determination of an active substance in a complex pharmaceutical matrix were developed and validated.

4.3 COMMENT ON PUBLICATION 2

Application of DV-SIA manifold for determination of thiocyanate ions in human saliva samples

The Dual Valve-Sequential Injection Analysis system was developed at our laboratory as a universal tool for the automation of different LPME techniques. Its applicability was proven for solvents both lighter and denser than water, as mentioned before in chapters 3.3.4.2 and 3.3.4.3. It was also submitted as patent application and in 2014 also patented under the name "A device of sequential injection analysis for liquid - liquid extraction" (Czech Patent No. 304296) [268].

In this work, the system was used to perform on-line reaction, extraction, and detection of thiocyanates in human saliva samples. The determination of thiocyanates ions was based on the formation of an ionic pair with the cationic dye Astra Phloxine at pH 3 and its extraction to amyl acetate, which is a solvent with density lower than water. Typically, the type of solvent used for extraction (lower or higher density) is projected in the position of the tube in the extraction unit for aspiration of the organic phase with the extracted analyte.

The system was built of an extraction unit, a detection unit and an extraction cell (EC), into which the solutions from the two remaining units were delivered. The first unit, comprising a syringe pump and a multiposition valve, served for the aspiration of air, sample, buffer, reagent and the extraction solvent and their delivery to the EC. The detection unit was designed to aspirate the extraction solvent which was also used as a carrier serving for delivering of organic part with the extracted ion associate to the flow detection cell. In this way, only organic solvent passed through the flow cell circumventing any disturbances in the measured signal. The aspiration of the organic solvent with the extracted analyte was achieved by careful adjustment of the position of the aspiration tube.

The organic layer from the EC with the extracted ion pair then passed through the flow cell and the absorbance at wavelength of 550 nm was recorded. The parameters to be optimised were the pH value at which the ion pair is formed, the concentrations, and the volumes of reagents and extraction solvent, as well as the phase separation time and positioning of tubes in the EC.

The method was applied to the determination of thiocyanates ions in human saliva samples, in which this analyte can be found at increased levels especially in smokers and where it represents a risk, as SCN^- can be metabolised to cyanates. Using the DV-SIA system in this application, any problems related to handling of aqueous and organic phases in one system were avoided, such as bubbles or baseline shift caused by different optical properties of different phases.

In an additional work, the method for thiocyanates determination was modified and DLLME was used as a sample pretreatment carried out in a DV-SIA system. In DLLME, the volume of the

extraction solvent is reduced thanks to the use of dispersive solvent. However, precise aspiration of microliter volumes of organic phase lighter than water might be cumbersome if the surface to volume ratio is relatively large and a careful adjustment of the tubes positioning is necessary. This difficulty was overcome by using carbon tetrachloride as an auxiliary solvent to increase the density in the mixture of amyl acetate as the extractant and acetonitrile as the dispersive agent [230]. Thus, the organic solvent could be separated at the conical bottom of the microtube used as EC and its collection for absorbance measurement was more feasible by placing the aspiration tube at the bottom of the EC. The possibility to decrease the dispersion by changing the position of the flow cell in the system was studied. Figure 25 demonstrates the difference in the system configuration. The results were presented as a poster at the 12th International Conference of Flow Analysis in 2012 in Thessaloniki, Greece (listed in chapter 5.7).

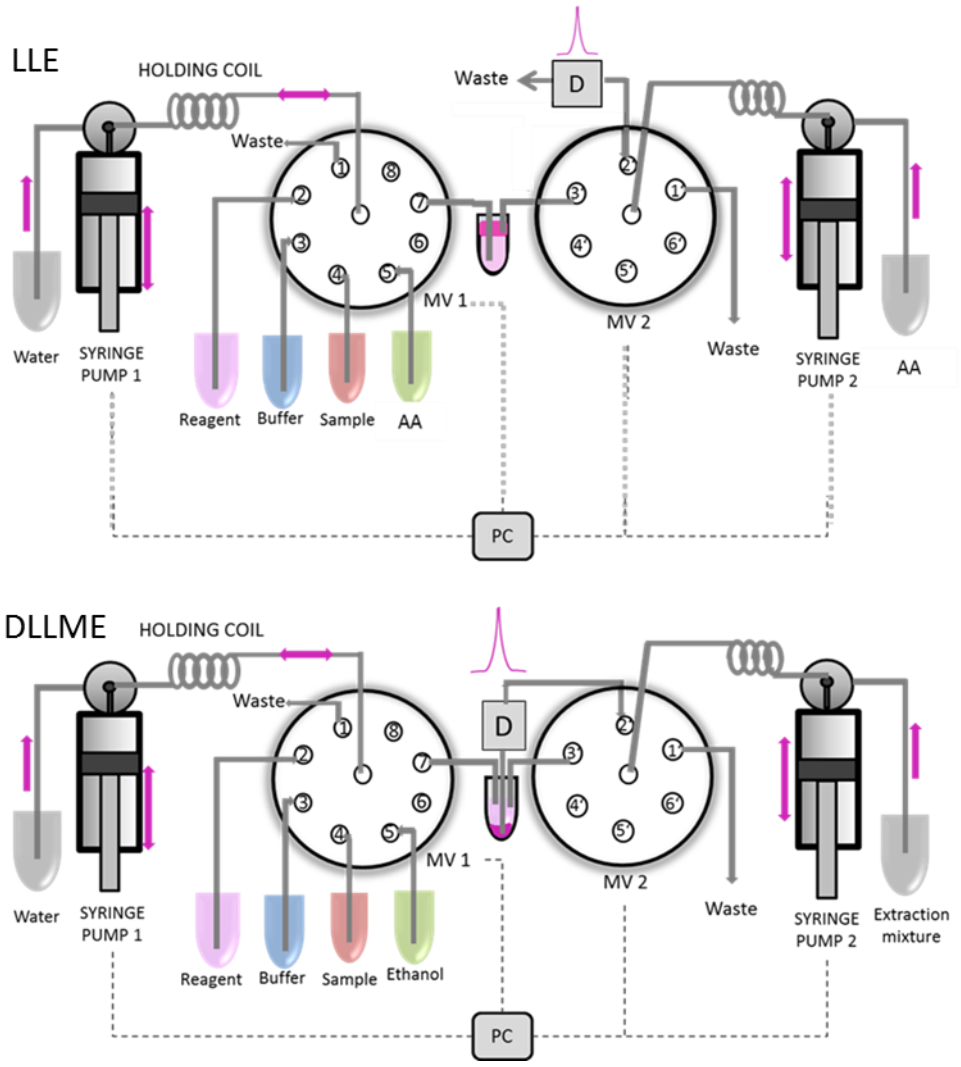

Figure 25: Comparison of the assemblies for two different LPME methods.

4.4 COMMENT ON PUBLICATION 3

Automated in-syringe single-drop head-space micro-extraction applied to the determination of ethanol in wine samples

Automation of SDME, especially HS-SDME, in a flow manifold is not as common as the other LPME techniques, although this technique has a significant advantage of selectivity for volatile substances. This is probably due to the fact that the formation of a stable, reproducible drop and its measurement is a challenging task. Only four of the works reviewed in chapter 3.3.4.4 describe automation of HS-SDME and typically, relatively complex manifolds were used.

In the present work, the sometimes difficult formation of the drop was elegantly solved using a new approach. The drop was positioned at the inlet of the syringe barrel by precise programming of the sample and reagents flow. After aspiration of air (for head-space), buffer and sample inside the syringe, 20 μL of the reagent for drop formation was aspirated and delivered to the syringe barrel by aspiration of air and water plugs after the reagent. An important step to assure a stable drop position was the increase of hydrophilicity by introducing a glass capillary into the channel connecting the head-valve and the syringe barrel.

It is noteworthy that no organic solvents were used in this work for the microextraction, but potassium dichromate in sulphuric acid. Since the extraction medium and the sample are not in contact in head-space extraction, an aqueous reagent could be used instead of an organic solvent and a reaction to increase the sensitivity was performed simultaneously with the analyte extraction.

Regarding the efficiency of head-space extraction, mixing and decreased pressure were used in this work to support the mass transfer from the sample bulk into the head-space. Mixing was realized by placing a magnetic stirring bar inside the syringe and a motor with two neodymium magnets next to it. It was found that the measurement repeatability was improved by the mixing, because it helped to homogenise the syringe content. Creating of vacuum was accomplished by moving of the syringe piston downward when the multiposition valve was turned to a closed port, which is one of the elements of novelty of this method.

The system was coupled to a VIS detector, measuring the decrease of absorbance of the reagent (potassium dichromate) due to its reduction to chromium(III) by ethanol. Measuring the absorbance decrease instead of the increase of chromium(III) absorbance had the advantage of higher sensitivity, although the accuracy at higher ethanol concentration level was compromised.

Parameters such as vacuum, time of reaction, acid concentration were studied. The developed method was proven to be applicable to the analysis of volatile substances as shown on the determination of ethanol in wine samples. Unlike alternative methods for the analysis of volatile substances, e.g. gas diffusion in a flow manifold using additional devices such as gas diffusion

membranes or multiple pumping devices for the donor and acceptor streams, a spare, compact instrumentation was developed in this work.

The limit of detection of the method was 0.025% (v/v) and the measurement repeatability lower than 5%. Apart from publication in the scientific journal Analytica Chimica Acta, the work was presented at The 18th International Conference on Flow Injection Analysis in Porto, Portugal in 2013 in form of a poster and was awarded The Best Poster Award.

4.5 COMMENT ON PUBLICATIONS 4 AND 5

Application of a fully integrated photodegradation-detection flow-batch analysis system with an on-line preconcentration step for the determination of metsulfuron methyl in water samples

Study of the effect of organic solvents on the fluorescence signal in a sequential injection system

This work was a part of the project Mobility, No. 7AMB12AR008, supported by the Ministry of Education, Youth and Sport of the Czech Republic and by the Ministry of Science, Technology and Productive Innovation of Argentina and was done in a cooperation with the colleagues from the Institute of Chemistry, University of the South, Bahía Blanca, Argentina.

The task was to develop an automated method for the determination of metsulfuron methyl (MSM), a sulphonyl urea pesticide, in water. The fluorescence properties of the analyte after UV irradiation were the basic characteristics important for the method development. The behaviour of naturally fluorescent substance (quinine) and MSM upon irradiation in a flow system with fluorescence detection and the effect of several factors were evaluated in an additional work, published in the journal of Analytical Methods. The type of organic solvent used for preparation of the solution, the irradiation time, the pH value, and the composition of the carrier stream were the main variables influencing the fluorescence of the studied substances.

The actual determination of the analyte was carried out in a flow-batch manifold, coupling several analytical procedures in one system. A column filled with C18 sorbent was coupled to the system for the analyte preconcentration. After the on-line SPE procedure, the eluate was directed to the mixing chamber by means of a solenoid valve. Activating another valve, sodium hydroxide solution was delivered into the chamber, providing optimum pH for the photodegradation of the analyte. For homogenisation of the chamber content, stirring was integrated. The chamber was placed directly in the measuring site of a fluorimeter. Two quartz windows were integrated in the chamber, placed at a right angle to each other, allowing to use the chamber as a detection cell. The configuration and positioning of the chamber also enabled to use the light beams from the detector for photodegradation. Then the signal of the photoproduct was measured directly in the chamber. The method protocol was written in the LabVIEW program.

The conditions for the UV decomposition in a flow system and the effect of several factors were studied in the context of the above mention task. The type of organic solvent for the analyte elution, irradiation time and wavelengths, slid widths, the pH value, and the composition of the carrier stream were the main variables influencing the fluorescence of the MSM decomposition product.

After the optimization, the method performed fully automated sample purification and analyte preconcentration, photodecomposition and detection in one system.

The method´s analytical performance parameters were evaluated and selectivity for MSM among pesticides with various chemical structures was proven. The method was applied to MSM determination in spiked surface waters of Bahía Blanca region (Buenos Aires, Argentina) and its suitability for this purpose was proven with the achieved LOD of 0.28 μL L^{-1}, which is within the limits of Argentina´s regulations.

The novelty of the system lies in several points: 1. Sample clean-up as well as analyte derivatisation and detection were fully automated and integrated in one flow-batch system, 2. UV radiation from the detector light source was used for photodegradation, i.e. avoiding the use of any derivatisation reagent, which fulfils one of the principles of green analytical chemistry, 3. Placing the chamber directly inside the detector implies that any further movement of the analyte from the point of derivatisation to the point of detection is left out thus any dispersion and subsequent loss of sensitivity are avoided. The potential of this method is also in its universality, i.e. other pesticides can be determined using the same manifold by changing the excitation and emission wavelengths.

4.6 COMMENT ON PUBLICATION 6

On-line coupling of micro-extraction by packed sorbent with sequential injection chromatography system for extraction and determination of betaxolol in human urine

The idea behind this work was to develop a simple, fully automated method for MEPS directly coupled to low pressure chromatography for the determination of betaxolol in urine. As stated elsewhere, the automation of MEPS and its coupling with a suitable detection technique remains a critical point [269]. A MEPS cartridge was chosen in this work as a sorbent format, which should guarantee uniform sorbent filling and thus better reproducibility than lab-made columns. Also, the usability time of the cartridge is typically longer than with classical SPE format. In addition, broadening the scope of flow techniques in automation of various sample pretreatment techniques by automation of MEPS was evaluated.

Automation of MEPS by SIA brings a significant simplification in comparison with the manual performance. Since the use of precise and repeatable volumes and flow rates are essential in MEPS performance, the advantage of computer-controlled flow programming, the main characteristic of sequential injection analysis, is here fully taken and especially useful, bringing complete automation and ensuring excellent repeatability. Also, a continuous loading of a large sample volume can be done in a flow manifold, unlike in the manual or other automated modes.

The developed system was assembled of a SIC manifold and an additional eight-port valve. A MEPS with a C18 sorbent was connected to one of the ports of the multiposition pump. The typical steps for MEPS procedures were performed on-line with optimized solutions: conditioning with 50% methanol, equilibration with 15% acetonitrile, and loading of urine sample. Then the matrix was washed out with 15% acetonitrile. All solutions were aspirated from their respective ports on the multiposition valve and passed through the MEPS into waste by reverse flow and switching the valve to the respective port. Then, a solution of 30% acetonitrile was propelled through the MEPS, eluting the retained substances from the sorbent. By selecting the respective port on the second multiposition valve, this fraction was pumped directly onto a chromatographic monolithic column where the analyte was separated from the rest of the matrix not washed out during the sample pretreatment step (MEPS). The analyte was detected by fluorimetric detection, using 220 nm as excitation and 305 nm as emission wavelengths, respectively.

The method was applied to the determination of betaxolol in urine samples. Measurement of blank urine showed that no other substances were co-eluted with the substance of interest. The method was validated and real samples were measured. The manuscript describing the method development and results has been submitted for publication to the scientific journal Talanta.

Additionally, a similar method was developed for the determination of propranolol and presented as a poster at the International Symposium of Luminescence Spectrometry in Rhodes, Greece (chapter 5.7).

5 SUPPLEMENT

5.1 PUBLICATION 1

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Fully automated analytical procedure for propofol determination by sequential injection technique with spectrophotometric and fluorimetric detections.

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Fully automated analytical procedure for propofol determination by sequential injection technique with spectrophotometric and fluorimetric detections

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ABSTRACT

In this work, an application of an enzymatic reaction for the determination of the highly hydrophobic drug propofol in emulsion dosage form is presented. Emulsions represent a complex and therefore challenging matrix for analysis. Ethanol was used for breakage of a lipid emulsion, which enabled optical detection. A fully automated method based on Sequential Injection Analysis was developed, allowing propofol determination without the requirement of tedious sample pre-treatment. The method was based on spectrophotometric detection after the enzymatic oxidation catalysed by horseradish peroxidase and subsequent coupling with 4-aminoantipyrine leading to a coloured product with an absorbance maximum at 485 nm. This procedure was compared with a simple fluorimetric method, which was based on the direct selective fluorescence emission of propofol in ethanol at 347 nm.

Both methods provide comparable validation parameters with linear working ranges of 0.005–0.100 mg mL^{-1} and 0.004–0.243 mg mL^{-1} for the spectrophotometric and fluorimetric methods, respectively. The detection and quantitation limits achieved with the spectrophotometric method were 0.0016 and 0.0053 mg mL^{-1}, respectively. The fluorimetric method provided the detection limit of 0.0013 mg mL^{-1} and limit of quantitation of 0.0043 mg mL^{-1}. The RSD did not exceed 5% and 2% ($n=10$), correspondingly. A sample throughput of approx. 14 h^{-1} for the spectrophotometric and 68 h^{-1} for the fluorimetric detection was achieved. Both methods proved to be suitable for the determination of propofol in pharmaceutical formulation with average recovery values of 98.1 and 98.5%.

1. Introduction

Sequential Injection Analysis (SIA) is a technique which is valued for its simplicity, easy control, versatility, repeatability, easy manipulation with solutions in a closed system, and the possibility of automation of complex analytical protocols. All these features make this technique suitable for the analysis of samples with complex matrices, such as pharmaceutical formulations or food [1], where a fully automated sample pre-treatment would bring a significant benefit.

Several methods using SIA system as a tool for analysis of different pharmaceutical formulations have been suggested, measuring the drug content or evaluating various pharmaco-technological parameters stated in Pharmacopoeias such as dissolution [2] or liberation; even applications to semi-solid formulations such as ointments have been reported [3].

Emulsions are often used as pharmaceutical dosage form due to their capacity to dissolve and stabilize lipophilic compounds, while achieving a high applicability and bioavailability of the active substance. They are a heterogeneous mixtures of two immiscible liquids, one forming microdroplets within the other liquid. Emulsions can be administered either by the oral, topic, or parenteral route. If a pharmaceutically active substance possesses lipophilic properties and has to be administered intravenously, oil-in-water emulsion is generally prepared as a dosage form.

Usually, a pharmaceutical emulsion represents a complex matrix for analysis, as it contains, apart from the active substance, two different solvents, generally water and vegetable oil as main constituents and further additives such as antimicrobials, anti-oxidants and surfactants which are necessary to maintain the required stability of the active substance and the matrix over the declared time. Both, the main emulsion constituents as well as the additives can significantly affect the analysis and therefore,

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separation or extraction of the substance of interest is often required. Potential co-extraction of matrix constituents together with the active substance can require a selective reaction or detection technique. Enzymatic reactions can further be carried out to improve the selectivity or reaction rate.

Also, the oil content in the sample matrix increases the risk of analysis performance deterioration, since it can stick to hydrophobic surfaces and lead to cross over. Therefore, a more thorough and thus time consuming cleaning step is required. Additionally, emulsions are generally turbid, which disables the direct use of spectrophotometry or fluorimetry as the most commonly used detection techniques. In consequence, a tedious and time consuming sample pre-treatment process prior to analysis is needed in many analytical methods to avoid matrix effects, which can require as much as 80% of the total analysis time [4,5].

Organic solvents can be effectively used to overcome the formerly mentioned problems as their addition can lead to a homogeneous solution by breaking the surfactant micelles, which permits the use of all optical detection techniques [6].

Although SIA systems are primarily applied to handling with aqueous solutions, the usage of organic solvents in automated flow systems has been reported, especially for sample pre-treatment, e.g. solid-phase extraction, [7] liquid–liquid extraction [8] or when a specific detection techniques such as atomic spectrometry are used [9]. While sticking on the hydrophobic surfaces can be desirable to coat the tubing walls with an organic film to perform extractions [10], it can also be a source of problems in analysis as it may impair the spectrometric measurement due to a different refractive index compared to aqueous solutions [11]. Therefore, analytical methods comprising an organic solvent are usually more complex than others where only aqueous solutions are used, requiring an additional/external component and a more laborious clean-up step, leading to the decrease in sample throughput and larger effluents production. Also, organic solvents can represent a limitation for implementation of specific reagents, such as enzymes, in the analysis, since they can affect their activity [12].

The application of enzymatic reaction in the analytical procedures has been studied very intensively [13]. The use of an enzyme generally provides the method with higher selectivity and offers a green alternative to inorganic catalysers which might possess toxic properties [14].

For the analysis of highly hydrophobic substances employing an enzymatic reaction, ionic liquids (IL) were suggested [15] as an alternative to organic solvents that can affect the enzyme activity. However, the cost of analysis represents a significant drawback for the combination of enzyme and IL in one analytical procedure.

In this work, the application of an enzymatic reaction for the determination of a highly hydrophobic drug propofol (2,6-diisopropylphenol) in a complex emulsion matrix in the presence of ethanol as a dissolving agent was presented.

Propofol is applied in medicine as an intravenous anaesthetic drug. Its chemical structure is not related to any other anaesthetic [16]. It is valued for its pharmacodynamic properties such as a rapid onset and offset of anaesthesia and a fast recovery of the patients without severe side effects due to a fast elimination from the human body [17]. The drug has lately increased attention after several death cases reports related to its application [18,19].

Due to the physical properties of propofol such as being an oily liquid at room temperature and an octanol: water partition coefficient of 6761:1 [20], the drug is administered in the form of an oil-in-water emulsion as a bolus in intravenous injection.

Although there are many works dealing with propofol determination in body fluids in the literature, only a limited number of papers were found referring to its determination in pharmaceutical formulation. The first method for propofol determination in bulk form was proposed in 1991 [21] and is based on the second derivative UV spectroscopy and HPLC.

Almost 10 years later, Kariem and Abounassif [22] developed a colorimetric method for propofol determination in emulsion dosage form. In this method, propofol reacted with 2,6-dichloroquinone–4-chlorimide (DCQ) and the reaction product was developed within 15 min.

Pickl et al. [23] used a more sophisticated instrumentation to determine propofol in emulsions, allowing very low detection limits. Headspace-solid phase microextraction was used as a sample pre-treatment technique prior to GC-MS analysis.

Here, the fully automated spectrophotometric method based on SIA for the determination of propofol in emulsion matrix without any sample preparation requirement but simple dilution is described and compared to a simple fluorimetric determination. The proposed method is focused on the handling of emulsion to enable the quantification of the analyte in such complex drug formulation as a matrix using SIA system.

The first method is based on an enzymatic reaction where horseradish peroxidase (HRP) was chosen as a catalyst of the enzymatic reaction applied in its determination, since it exhibits selectivity towards phenolic compounds [24]. Subsequently, the oxidation product reacts with 4-aminoantipyrine to give a coloured product, which can be spectrophotometrically measured at its absorbance maximum of 485 nm. This method was compared with respect to sensitivity, linear range, recovery and repeatability with a simple automated method with fluorimetric detection.

The development, optimization, and the achieved analytical performances and figures of merit of both methods are discussed in detail. The complexity of the first method including the problems arising from the handling of both aqueous and organic solutions in one system is described.

2. Material and methods

2.1. Reagents and solutions

Analytical grade reagents were used to prepare all solutions throughout the study. All aqueous solutions were prepared in ultra-pure water provided from a Millipore Milli-Q RG system (EMD Millipore Corporation, Billerica, MA, USA).

Peroxidase from horseradish (HRP), Type I, and propofol standard ($\geq$ 97%) were purchased from SAFC™ (Steinheim, Germany). Hydrogen peroxide was purchased from Fluka (Buchs, Germany). 4-Aminoantipyrine (4-AAP), sodium hydroxide and potassium phosphate were purchased from Sigma-Aldrich (Steinheim, Germany).

Propofol 1% MCT/LCT "Fresenius" i.v. emulsion (Fresenius Pharma, Graz, Austria) was used for recovery evaluation as reference material. The emulsion constituents and additives are soybean oil, purified egg lecithin, medium long chain saturated triacylglyceroles, glycerol, oleic acid, sodium hydroxide and purified water (aqua pro-injectione).

For spectrophotometric batch experiments, a propofol stock solution of approx. 1.4 mg L^{-1} was prepared by the following procedure: 60 μL of the substance was dissolved in 20 mL of ethanol 96% (V/V) and then diluted with water to 50 mL to reach a final ethanol concentration of 40% (V/V). Propofol working solutions were prepared by dilution of the stock solution with 40% (V/V) ethanol.

A 0.2 mol L^{-1} phosphate buffer was prepared from dihydrogen potassium phosphate and adjusted to pH 7.4 by addition of 0.2 mol L^{-1} sodium hydroxide solution.

Solutions of 4-AAP, H_2O_2, and HRP were prepared dissolving the adequate amounts of individual substances in buffer solution.

For SIA measurements with spectrophotometric detection, propofol was dissolved in 96% (V/V) ethanol. Reagents solutions were prepared dissolving the appropriate amounts of each substance in water, and buffer was used as a carrier.

For both batch and SIA studies, H_2O_2 and 4-AAP solutions were prepared daily prior to use. The propofol stock solution was used for preparation of fresh working solutions. The HRP solution was stable for over 3 days.

For the SIA measurements with fluorimetric detection, propofol stock solution of approx. 1.4 mg L^{-1} was prepared dissolving 60 μL of the substance in ethanol 96% (V/V). The working solutions were prepared diluting the stock solution with ethanol 96% (V/V). Ethanol was also used as a carrier.

The sample solution was treated in the same way as the propofol standard solution.

All prepared solutions were stored in dark at 4 °C.

2.2. Apparatus

For batch spectrophotometric experiments, spectra were acquired using an HP diode array spectrophotometer Agilent 8453 UV–vis. All SIA experiments were performed using a commercially available FIAlab® 3500 system (FIAlab® Instrument Systems Inc., Bellevue, USA, http://www.flowinjection.com). It consists of a Cavro syringe pump equipped with a 5 mL glass syringe with a rotary three-way head valve to connect the syringe either with the solution reservoir (carrier, IN) or the tubing manifold (OUT) and an 8-port Cheminert selection valve. All connections were made using PTFE tubing of 0.75 mm i.d. The central port of the selection valve was connected to the OUT port of the syringe head valve via a holding coil (HC) of approx. 1.5 m length. Lateral ports of the selection valve were used for solution discharge to waste (port 1), aspiration of sample and reagents (ports 2–6) and propelling the reaction mixture to a detection flow cell (port 7) of 1 cm optical path length (Z-cell, PEEK). The detailed manifold configuration for spectrophotometric detection is indicated in Fig. 1.

In the system with spectrophotometric detection, the holding coil was placed into a vessel of a thermostat to maintain the temperature at 40 °C for the enzymatic reaction.

A USB 2000 spectrophotometer (Ocean Optic Inc., Dunedin, USA, http://www.oceanoptics.com) was used for signal detection. A Mikropack DH-2000 Deuterium–Tungsten Halogen lamp was used as a light source. Both instruments were coupled to the detection cell via optical fibres of 400 μm diameter (I.D.) A home-made de-bubbling device, shown in the Fig. 1, was used further. It consisted of two pieces of PMMA (3 × 2 × 1 cm). The first comprised a milled flow channel of 20 mm in length, 3 mm in width, and 1 mm in depth, which was sealed with one layer of gas permeable PTFE tape. The second piece was used to seal the cell by the help of four metal screws and had four holes for air exit. It was placed in between the selection valve and the detection flow cell, fixed by means of commercially available fittings.

For the second system, FIAlab 3500 system equipped with a Flow Through Photomultiplier based Detector (PMT-FL, FIAlab®) was used. The detector comprises a photomultiplier for data readout, a commercial fluorescence quartz flow cell with a cuvette support and fibre optic connection to the same UV light source as described above. Wavelength selectivity was achieved using optical filters. A UV 330 band pass filter with wide wavelength interval of 140 nm was used for excitation and a 295 Long Pass Filter with cut off at any wavelength under 265 nm was used for emission light filtering (Edmund Optics, Barrington, New Jersey, USA).

Control of the whole flow system as well as data acquisition and data collection evaluation was carried out using FIAlab software for Windows, version 5.9.290 (FIAlab®).

2.3. SIA – spectrophotometric procedure

The operational protocol is given in Table 1. It started with the aspiration of 600 μL of the carrier (buffer), from the reservoir at a flow rate of 50 μL s^{-1}, followed by the aspiration of H_2O_2, propofol, HRP, and 4-aminoantipyrine solutions (50 μL of each reagent) at the same flow rate. Solution mixing was improved using four flow reversals under the flow rate of 50 μL s^{-1} in the holding coil, heated to 40 °C. By this step, efficient mixing and heating were ensured and the peak shape and signal repeatability were improved.

The reaction was allowed to proceed in the holding coil (HC) for another minute to enhance the reaction product yield. In the following step, the mixture was propelled through the de-bubbling device and the detection flow cell to waste at a flow rate of 25 μL s^{-1}.

2.4. SIA – fluorimetric procedure

Fluorimetric determination of propofol was done using a very simple control programme, which started with the aspiration of 1000 μL carrier (ethanol 96%, V/V) from the reservoir at a flow rate

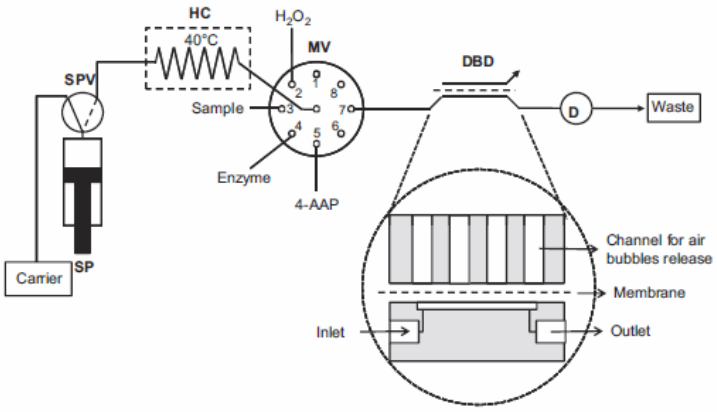

Fig. 1. Automated SIA system for determination of propofol in emulsion with spectrophotometric detection. SP: syringe pump, SPV: syringe pump valve, HC: holding coil, MV-multiposition valve, DBD: de-bubbling device, and D: detector.

Table 1
Operation protocol for spectrophotometric determination of propofol.

Step	Port	Flow rate ($\mu L\,s^{-1}$)	Operation	Description
1	–	100	Aspirate 600 μL	Syringe pump Valve in position; aspiration of the carrier
2	2	50	Aspirate 50 μL	Syringe pump Valve out position; aspiration of hydrogen peroxide
3	3	50	Aspirate 50 μL	Aspiration of sample
4	4	50	Aspirate 50 μL	Aspiration of enzyme
5	5	50	Aspirate 50 μL	Aspiration of 4-AAP
6	6	100	Aspirate 50 μL, Dispense 50 μL	Mixing, repeated 4 times
7	–			Delay 1 min
8	7	25	Empty	Propelling to the detection cell

of 80 $\mu L\,s^{-1}$, followed by 50 μL of propofol standard or sample dissolved in ethanol 96% (V/V) from the selection valve and propelling it towards the detector at a flow rate of 50 $\mu L\,s^{-1}$ while the fluorescence emission signal was registered. By the appropriate selection of excitation and emission filters, interferences of the emulsion components were significantly reduced, as described in the Results Section.

3. Results and discussion

3.1. Preliminary batch experiments

The optimum reaction conditions were investigated in batch using propofol standard solution before transferring the reaction procedure to the SIA system. This was done due to the complexity of the reaction including four components (HRP, propofol standard or sample solution, H_2O_2 and 4-AAP solutions).

The parameters to be optimized in batch were the concentrations of reagents, the temperature, and the reaction time. Once the chemical parameters were optimized in batch, hydrodynamic parameters such as solution volumes, flow rate and mixing mode had to be studied in the SIA system.

For batch, equal volumes of all reagents were used to perform these studies. All the reagents were prepared by dissolution in 0.2 mol L^{-1} phosphate buffer, adjusted to pH 7.4, which is the reported activity optimum of HRP, and as further proven by a preliminary test (data not shown). The value is also in good agreement with an earlier report [25]. The ethanol content was intended to be kept as low as possible in order not to affect the enzyme activity. Thus, propofol was at first dissolved in pure ethanol and then diluted with water to set the final ethanol concentration to 40% (V/V). This was the lowest concentration, for which two non-miscible phases were not observed. Later experiments revealed that for the analysis of a propofol emulsion, 96% ethanol was required to eliminate the matrix effects, as described in Section SIA – spectrophotometric method.

After scanning the whole spectrum range (200–700 nm), 485 nm was chosen as optimum wavelength representing maximum absorbance. It should be pointed out that after reaching the reaction's steady-state the second maximum was found at 436 nm. However, the reaction kinetics studied at this wavelength was slower and the blank value significantly higher, indicating that this absorbance corresponds to a side product, most likely, the oxidation of 4-AAP with H_2O_2.

3.1.1. Study of the enzyme concentration

HRP was prepared and used in solution to ensure a fresh portion of the catalyst for each run [14]. The concentration of the enzyme was investigated in the range of 0.16–5.10 mg mL^{-1}, with a multiplying factor of 2. Other solutions were prepared in the following concentrations: $c_{H_2O_2}$ = 18 mmol L^{-1}, $c_{4\text{-AAP}}$ = 1.36 mmol L^{-1}, and $c_{propofol}$ = 0.2 mg mL^{-1}. The reaction was performed at room temperature and the absorbance was measured after 20 min. An excess concentration of the substrate (propofol) was used to ensure that the enzyme activity was not dependent on the substrate amount but only on the enzyme concentration.

The signal increased up to 2.56 mg mL^{-1} while for higher concentration of HRP, a similar absorbance was observed, and so further experiments were performed with 2.56 mg mL^{-1} of the enzyme.

3.1.2. Study of the reaction time

After the optimum enzyme concentration was found, the influence of the reaction time was studied under the same conditions mentioned in the previous paragraph. All reagents were mixed at room temperature in a test tube. Then, an aliquot was taken and the absorbance was measured. This was repeated every 5 min over the next 45 min. The signal increased during the first 15 min and then remained stable without significant changes. So, 20 min was chosen to ensure that the reaction time is long enough to reach the steady state.

3.1.3. Study of the temperature and reaction time correction

Since the rate of enzymatic reactions is highly temperature dependent, it was necessary to choose an adequate reaction temperature. The conditions were identical as in the previous experiments: c_{HRP}=2.56 mg mL^{-1}, $c_{H_2O_2}$ = 18 mmol L^{-1}, $c_{4\text{-AAP}}$ = 1.36 mmol L^{-1}, and $c_{propofol}$=0.2 mg mL^{-1}. The test tube was placed in a thermostat for temperature control. The influence of temperature was studied in the range of 25–45 °C, with 5 °C increments. A signal increase was observed up to 40 °C, while beyond that the signal decreased rapidly, which can be attributed to the thermic denaturation of the enzyme.

Regarding this observation, the reaction time was re-examined, setting the temperature to 40 °C. The results revealed that at a higher temperature, the reaction reached the steady state after 10 min. Therefore, further batch experiments were performed at 40 °C with a reaction time of 10 min.

3.1.4. Study of the 4-AAP and H_2O_2 concentration

The 4-AAP concentration was studied in the range of 6.0–11.0 mmol L^{-1} with an increment of 1 mmol L^{-1}. The highest response was obtained when a concentration of 8 mmol L^{-1} was used (Fig. 2A), so this concentration was chosen for all following experiments.

The influence of the concentration of hydrogen peroxide was tested between 16.0–20.0 mmol L^{-1}. As demonstrated in Fig. 2B, the reaction with 18 mmol L^{-1} H_2O_2 yielded the highest signals, so this concentration was adopted for next trials.

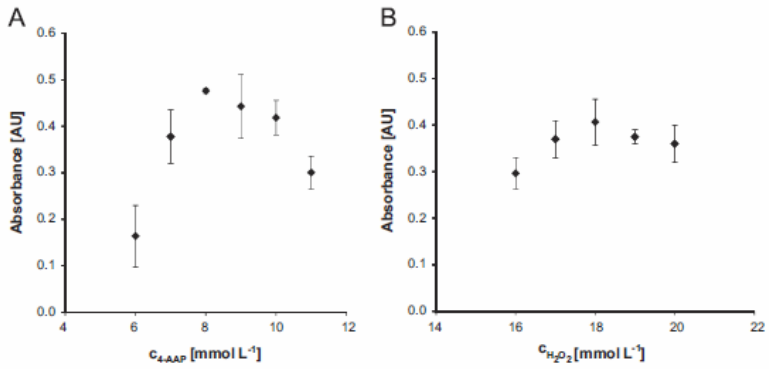

Fig. 2. Study of reaction conditions in batch; general conditions: c_{HRP}=2.56 mg mL^{-1}, $c_{propofol}$=0.2 mg mL^{-1}, T=40 °C, t=10 min (A) study of 4-AAP concentration; $c_{4\text{-AAP}}$=6.0–11.0 mmol L^{-1}, and (B) study of H_2O_2 concentration, $c_{H_2O_2}$ = 16.0–20.0 mmol L^{-1}.

3.2. SIA – spectrophotometric method

Once the reaction conditions were established, they were transferred to the SIA system for automation of propofol determination.

In the automated system, other parameters had to be optimized. The crucial role in the analysis performance had the sequence of reagents aspiration, mixing conditions, and the reaction time. Heating could not be omitted because it increases significantly the reaction rate, as confirmed by batch experiments.

A thorough penetration of, first, the sample with HRP and its substrate H_2O_2, and then the chromogenic reagent was intended. To accomplish this requirement, the following sequences of aspiration were tested: 1st H_2O_2–sample–HRP–4-AAP; 2nd H_2O_2–4-AAP–sample–HRP; 3rd H_2O_2–HRP–sample–4-AAP. The most satisfying results in terms of peak height and peak shape were obtained when the solutions were aspirated in the 1st order. This was accomplished by the sample aspiration in between the zones of H_2O_2 and HRP solutions to react first with the analyte.

Regarding the volume of the solutions, range from 25 μL to 50 μL was tested for each reagent. With the smaller one we did not obtain peaks of symmetric shape, probably due to high level of dispersion in the carrier.

Peaks of favourable shapes were obtained with the reagents´ volume of 50 μL, thus this volume was chosen for further experiments. The behaviour with higher volumes was not investigated, since large volumes caused very high consumption of reagents, especially the enzyme.

The optimal temperature was ensured placing the HC into a double-wall thermostatic glass beaker connected to a thermostat with a water bath and the temperature set to 40 °C.

In the SIA system, buffer solution was used as a carrier ensuring a stable pH value (optimal for the enzyme activity, i.e. 7.4). Solutions (H_2O_2, 4-AAP, and enzyme) were prepared in water and for sample preparation 96% (V/V) ethanol was used to obtain higher solubility of propofol in real sample (emulsion). Using these conditions the emulsion matrix was completely dissolved in ethanol, which was important to achieve high recovery in case of formulation analysis.

One consequence of increasing ethanol content (in comparison to batch) and especially at increased temperature is the air bubbles formation inside the flow system. This affected the spectrophotometric measurements in the SIA system significantly. This problem was overcome by placing a simple de-bubbling device depicted in Fig. 1 right before the detection flow cell. It showed that the bubbles were effectively removed and the spectrophotometric detection was not affected by a baseline drift.

The main attractiveness of flow techniques is that a reaction steady state does not have to be reached as long as high repeatability can be achieved by careful optimization and timing. Due to the fact, that a reaction time of 10 min was required to reach steady-state in the previously described batch method, special effort was given to achieve high repeatability and sensitivity within a shorter time in the automated method.

To achieve efficient zone mixing for a homogeneous solution and to achieve a better repeatability, four flow reversals were performed at a flow rate of 50 μL s^{-1} using 50 μL zones and were followed by an additional reaction time of 5 min.

However, this mode did not bring the expected results. Double peaks and unacceptable repeatability were observed, indicating that the solutions were not yet thoroughly mixed. Increasing the number of flow reversals did not improve the results either. Using a higher flow rate of 100 μL s^{-1} for mixing, the shape of the recorded peaks improved considerably. It was found that applying the flow reversals over a period of 1 min was sufficient for complete mixing of all aspirated zones.

In the second step, the time of reaction after stopping the carrier flow was examined. It was observed that a reaction time of 5 min lowered the measurement repeatability while not increasing the method's sensitivity significantly. Based on these observations, only one additional minute of stop flow was applied. The peak heights at a given analyte concentration and repeatability achieved using the optimized conditions were within the expected limits and no carry-over between the individual measurements was observed.

3.2.1. Figures of merit of spectrophotometric method

Linearity was obtained over the range of 0.005–0.100 mg mL^{-1} for the enzymatic method with spectrophotometric detection. LOD value was calculated as three times the standard deviation of ten blank measurements divided by the calibration curve slope. LOQ value was then calculated as 3.3-fold LOD. In the spectrophotometric detection technique values of 0.0016 and 0.0053 mg mL^{-1} expressed LOD and LOQ, respectively. This method revealed good repeatability, with RSD not exceeding 5% (Table 2). Regarding the analysis time, a single run was completed within 5 min. For the spectrophotometric determination, only 50 μL of ethanol (as a sample diluent) and 50 μL of the enzyme solution (as the reagent of highest cost) were consumed. Less than 1 mL of waste was produced in a single run.

Bubble formation which aroused from mixing of organic and aqueous solutions was effectively overcome using a simple

Table 2
Summary of analytical parameters of the spectrophotometric and fluorimetric methods.

Method	Spectrophotometry	Fluorimetry
Slope ($mL\,mg^{-1}$)	3.054 ± 0.161	$2.865 \times 10^6 \pm 0.308 \times 10^6$
Intercept	0.016 ± 0.004	$26.90 \times 10^3 \pm 723.9 \times 10^3$
Correlation coefficient	0.993	0.997
Linear range ($mg\,mL^{-1}$)	0.005–0.100	0.004–0.243
LOD ($mg\,mL^{-1}$)	0.0016	0.0013
LOQ ($mg\,mL^{-1}$)	0.0053	0.0043
Repeatability (RSD%, $n=10$)	4.45 (0.075 $mg\,mL^{-1}$) 4.29 (0.125 $mg\,mL^{-1}$)	1.66 (0.050 $mg\,mL^{-1}$) 0.83 (0.200 $mg\,mL^{-1}$)
Recovery (%)	103.8 (0.050 $mg\,mL^{-1}$) 94.7 (0.075 $mg\,mL^{-1}$) 95.7 (0.100 $mg\,mL^{-1}$)	97.5 (0.025 $mg\,mL^{-1}$) 98.9 (0.050 $mg\,mL^{-1}$) 99.1 (0.075 $mg\,mL^{-1}$)
Sample throughput (h^{-1})[a]	14	68

[a] Expressed for single injection.

membrane device. Additionally, although the sample matrix was complex, direct determination with only dilution as a sample pretreatment was possible due to the selective enzymatic reaction. To evaluate potential interferences, the effect of the sample matrix (soybean oil, purified egg lecithin, medium long chain saturated triacylglyceroles, glycerol, oleic acid, and sodium hydroxide) was tested at three different concentration levels (0.050, 0.075 and 0.100 $mg\,mL^{-1}$) using the standard addition method. Recovery values ranged from 94.7% to 103.8% with the average recovery of 98.1%. These results (Table 2) did not show any interference of the additives from the pharmaceutical matrix with the proposed method. The spectrophotometric method was found to fulfil the requirements for all tested parameters and additionally, higher selectivity of the enzymatic reaction could be expected (that is important mainly in case of real samples of biological material).

3.3. SIA – fluorimetric method

Since fluorimetric detection has been often used for propofol determination [27, 28], fluorimetric determination in the SIA system was carried out for comparison with the developed spectrophotometric determination. For this determination, the sample was prepared in ethanol 96% (V/V). Therefore, the possibility of reducing the organic waste production using water as a carrier was examined. However, a strong baseline drift was observed in this case which affected the detection considerably. Comparing the results obtained with standard solutions and real samples of the same concentration level (as declared by the producer of the pharmaceutical formulation), significantly different results were observed. For this reason, ethanol was used as a carrier, which improved the repeatability and analyte recovery (evaluated with real samples) significantly.

Hence, the primary radiation from 260 nm was applied and emission at $\lambda \geq 295$ nm was measured. This led to recovery values near to 100%, so the matrix effects were eliminated by the wavelength selection.

3.3.1. Figures of merit of fluorimetric method

Fluorimetry as a commonly used detection in other propofol determinations was tested in the SIA system, too. The linear range of 0.004–0.243 $mg\,mL^{-1}$ for the fluorimetric method was found. LOD and LOQ values were 0.0013 and 0.0043 $mg\,mL^{-1}$, respectively. The analysis time of 1 min was needed and about 1.05 mL of waste was produced in a single run. The fluorimetric determination required approx. 1 mL of ethanol per analysis as environmentally harmless and economic organic solvent. The fluorimetric method showed excellent repeatability, with RSD less than 2%.

The recovery values in case of spiked matrix of pharmaceutical emulsion ranged from 97.5% to 99.1% with average recovery of 98.5% (Table 2). Simple fluorimetric determination revealed similar values of all tested parameters which were within the required limits. Only low selectivity of this detection decreases its application in real biological samples analysis.

3.4. Comparison of spectrophotometric and fluorimetric methods

The parameters characterizing the analytical performance of both methods were evaluated and compared in Table 2.

Linearity of both tested methods was found in similar range. The fluorimetric method showed broader range towards the higher concentration levels. Limits of detection and quantitation were comparable and the described values were more than three orders of magnitude smaller than the content of propofol in the pharmaceutical formulation declared by the producer, being 10 $mg\,mL^{-1}$.

The time of analysis per run was longer for the complex spectrophotometric method which included the enzymatic reaction. However, this did not lead to higher solvent and reagent consumption, compared to other flow methods. Low waste volume generation was proved in both methods. As for repeatability, the RSD values of the spectrophotometric method were found to be a little bit higher but did not exceed the common values found in automated determinations using different flow techniques. The difference in RSD values was caused by higher number of steps in the spectrophotometric system compared to the simple fluorimetric one.

The recovery values from the tests with real samples (pharmaceutical emulsion matrix) showed a slightly wider range in case of spectrophotometric method but the average values were found to be very similar. The data obtained from real sample measurement proved that the suggested spectrophotometric method did not exhibit the problems that are highly likely to occur when an organic solvent and/or highly hydrophobic substance is handled in a flow system, such as carry-over, Schlieren effect or the need of an external device.

Comparing both methods (spectrophotometric and fluorimetric detections), it can be seen that the more complex one (spectrophotometric) provided a sensitivity comparable to the simple fluorimetric detection. Higher selectivity is expected due to the enzymatic reaction. As the recovery measurements revealed, in both cases, the components of the studied emulsion sample did not influence the analysis.

3.5. Comparison with the formerly reported methods

Most of the analytical parameters were found to be better than the first reported method for propofol determination in pharmaceutical formulation [21]. The detection limits were almost 100 times lower; also higher recovery and better repeatability were achieved with the automated SIA methods.

In comparison with the manual spectrophotometric method reported by Kariem and Abounassif [22], we observed an about four times higher LOD. However, only one organic solvent (ethanol) was used in the SIA method, unlike in their method, where two solvents (2-propanol and dimethylsulphoxid) were necessary to perform the analytical reaction. Moreover, the analysis time is

about three-times shorter and the determination is carried out fully automatically.

Pickl [23] presented a highly sensitive method, enabling the detection of very small quantities of analyte in the sample (emulsion). This was possible due to the use of a very sensitive but very costly detection technique (mass spectrometry) which is not accessible to all laboratories. Sample pre-treatment was carried out prior to analysis (solid phase microextraction). This step, however, increased the analysis time significantly.

Other separation methods with spectrophotometric and fluorimetric detection were designed for propofol determination in biological fluids matrix. They provided low detection limits, required for such kind of samples. In [26], SPE pre-concentration step is followed by HPLC/UV detection with total analysis time of 20 min. The HPLC/fluorimetry method developed by Boulieu at al. [27] required less than 6 min for the elution of propofol, using 500 μL of the sample and producing 3.6 mL of waste in a single injection. Similarly, an HPLC method with fluorimetric detection was proposed by Cox et al. [28]. Double liquid–liquid extraction using 1.5 mL of acetonitrile–methanol mixture was performed to increase the sensitivity. The following separation step took then almost 10 min, with almost 15 mL of organic waste per analysis. In comparison to these methods, the proposed SIA procedure offers a faster, simpler, more economic and especially automated alternative for the determination of propofol.

In comparison with formerly published methods, this work resulted in lower detection limits. SIA is a technique based on flow where the steady state is not reached, unlike in batch methods. Other methods might have reached lower detection limits than the proposed method; however, the SIA method offers other advantages (low solvent consumption, feasibility, automation, high sample throughput, and easy manipulation with solutions).

4. Conclusion

The use of a fully automated system with spectrophotometric detection for the determination of a highly hydrophobic substance in a complex matrix, and the application of the selectivity of an enzymatic reaction to eliminate sample matrix effects, was demonstrated. The obtained results were described and compared with an automated method with fluorimetric detection in terms of analytical performance.

Both methods and the used analyser system stand out by simplicity, rapidness, and high sensitivity, and were proven to be applicable to the determination of the lipophilic analyte propofol in emulsion taking advantage of both organic solvent and an enzymatic reaction.

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5.2 PUBLICATION 2

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Application of DV-SIA manifold for determination of thiocyanate ions in human saliva samples

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ABSTRACT

An automated, simple and inexpensive double-valve sequential injection analysis (DV-SIA) spectrophotometic method with online liquid–liquid extraction, for the determination of thiocyanate has been developed. The method has been based on the formation of an ion associate between thiocyanate and Astra Phloxine in acidic medium, and the subsequent extraction with amylacetate. The absorbance of the extracted ion associate was measured at 550 nm.

The calibration function was linear in the range 0.05–0.50 mmol L^{-1} and the regression equation was $A = (1.887 \pm 0.053)[SCN^-\ \text{mmol}\ L^{-1}] + (0.037 \pm 0.014)$ with a correlation coefficient of 0.995. The precision of the proposed method was evaluated by the relative standard deviation (RSD) values at two concentration levels: 0.20 and 0.50 mmol L^{-1}. The obtained results were 1.0 and 2.8%, respectively, for the intra-day precision, and 4.2 and 3.8%, respectively for the inter-day precision. The calculated detection limit was 0.02 mmol L^{-1}.

The developed method has been successfully applied for determining thiocyanate ions in human saliva samples.

1. Introduction

Thiocyanate is usually present in human saliva in low amounts, but its concentration could be increased as the result of the digestion of glucosinolate-containing vegetables such as cabbage, turnip, tomato, or by the intake of thiocyanate-containing food such as milk and cheese [1,2]. It is also present in drugs that are used in the treatment of thyroid problems and arterial hypertension. Another important source of this ion arises from tobacco smoke, because thiocyanate is the main metabolic product of cyanide.

Though not as toxic as cyanides, thiocyanate in chronically increased levels can be harmful for life and its determination is of great interest [3,4].

A considerable number of methods for monitoring the level of thiocyanate are available in the literature in which different techniques were applied including amperometry [5], fluorimetry [6] and flame-atomic absoption spectrometry [7]. Several thiocyanate-selective electrodes have been reported over the last years [8,9]. Separation techniques such as electrophoresis and chromatography were applied to thiocyanate determination in biological samples [1,10].

Although some of these techniques have low detection limits and a very good selectivity along with the ability to perform multielemental analysis, spectrophotometry is still very popular because of its speed, simplicity and instrumentation availability. Many spectrophotometric methods have been developed to determine thiocyanate based on the Stugart reaction [11], on the well known Koning reaction [12] and on the quantitative oxidation of thiocyanate using permanganate [13]. Thiocyanate also forms colored complexes with copper and 2,2-dipyridyl-2-quinolylhydrazone that are extractable with chloroform [14], and with the ion-pairing reagent 1-(3,5-diamino-6-chloropyrazinecarboxyl) guanidine hydrochloride monohydrate that are extracted with 4-methyl-2-pentanone [15]. Another method that has been published was the reaction of the analyte with chloramine-T in the presence of iron (III) chloride as catalyst to give cyanogen chloride, which reacts with a mixture of γ-picoline (4-methylpyridine) and barbituric acid to form a soluble violet-blue product [16].

Thiocyanate ions interact with a poymethine dye, 1,3,3-trimethyl-2-[3-(1,3,3-trimethyl-1,3-*H*-indol-2-ylidene)propenyl]-3*H*-indolium chloride (Astra Phloxine, AP) to form an ion associate

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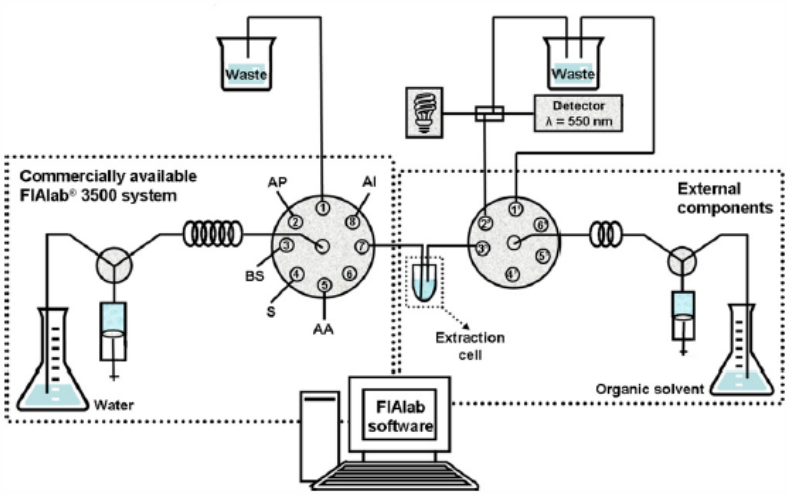

Fig. 1. Schematic view of the DV-SIA manifold for online liquid–liquid extraction and spectrophotometric determination of thiocyanate. AP, Astra Phloxine; BS, buffer solution; S, sample; AA, amylacetate; Al, air input.

in acidic medium. In general, the merits of polymethine dyes over other classes of analytical reagents include the stability of their solutions over time and their high values of absorbance [17]. AP has been applied in our laboratory as reagent for the determination of various analytes [18–20] but, to the best of our knowledge, it has not been previously employed in thiocyanate determination.

In spite of the different techniques that have been employed to determine SCN^-, often the pre-treatment of the sample is required. One of the most commonly and widely used sample pre-treatment techniques in analytical chemistry is liquid–liquid extraction (LLE), due to its simplicity, flexibility, selectivity and, in some cases, suitability to achieve the analyte preconcentration. Likewise other sample manipulation, the manual implementation of this technique introduces errors that affect the accuracy and precision of results. The application of the automation and miniaturization to perform LLE contributes greatly to improve the quality of the results and leads to significant reduction in solvent consumption and an inherent decrease in waste generation. In addition, the risks for the operator and sample contamination are minimized and the sampling throughput is significantly enhanced. The search for new and improved methods are thus still of great interest in analytical chemistry and has been the focus of many recent developments. In fact, several articles were published dealing with the use of LLE in flow systems [21].

A simple, user-friendly and universal dual-valve sequential injection (DV-SIA) system with the online incorporation of LLE cell into the SIA manifold has been designed [22,23]. The design of the SIA manifold was based on the separation of extraction and detection units, avoiding some common problems in such kind of flow systems due to the different affinity of the organic and aqueous phase to the walls of the PTFE tubing, and bubble formation. One of its main advantages is the variability of samples and organic solvents that could be used and analyzed in the same system. Furthermore, the system was constructed only with commercially available components, which makes it accessible and easy to be assembled in any other laboratory.

In this work, the DV-SIA system was employed to the spectrophotometric determination of thiocyanate ions. The reaction between the analyte and AP, the extraction of the formed ion associate and the subsequent determination was carried out on-line. The automated method was successfully applied for the determination of SCN^- in saliva samples.

2. Experimental

2.1. Reagents

Solutions were prepared using analytical grade reagents and ultra pure water Millipore Milli-Q RG (Millipore, USA). Toluene 99.8% (HPLC grade, Fluka, Germany) and Amylacetate ≥99% (Sigma Aldrich, Germany) were used as organic solvents.

A 1.0×10^{-2} mol L^{-1} Astra Phloxine (AP) (Jiacheng-Chem Enterprise Ltd., China) solution was prepared by dissolving 0.1963 g of the dye in 0.5 mL of methanol and filled up with water to 50.0 mL. A 2.0×10^{-3} mol L^{-1} solution was prepared by diluting 5.0 mL of this solution to 25.0 mL with water.

A 1.0×10^{-2} mol L^{-1} NH_4SCN stock solution was prepared by dissolving 0.0381 g of ammonium thiocyanate purum p.a. (Chemapol, Czech Republic) in 50.0 mL of water. The working solutions were prepared by appropriate dilution of the stock solution with water.

The pH of the medium was adjusted with an ammonium acetate buffer solution, pH 3.0. The buffer solution was prepared by mixing 49.65 mL of a 1.0 mol L^{-1} CH_3COOH solution with 0.35 mL of a 1.0 mol L^{-1} NH_4OH solution.

2.2. Apparatus

The DV-SIA system divided into two parts (extraction and detection part) is depicted in Fig. 1. A commercially available FIAlab® 3500 system (FIAlab® Instrument Systems Inc., Bellevue, USA) consisting of a syringe pump (syringe volume 5 mL) and a central eight-port Cheminert selection valve belongs to the extraction part.

The central port of the selection valve was connected to a PTFE holding coil (0.75 mm i.d. and 150 cm length). The extraction cell consisted of a 1.5 mL polypropylene tube connected to both extraction and detection parts of the system.

External six-port Cheminert selection valve (Valco Instrument Co., Houston, USA), a holding coil 34 cm in length and an

Table 1
SIA procedure for the extraction and determination of thiocyanate ions in DV-SIA.

Step	Port selection	Flow rate ($\mu L\,s^{-1}$)	Operation	Description
1	–	200	Aspirate 600 μL	Syringe pump in the valve in position; aspiration of carrier
2	8	50	Aspirate 10 s	Syringe pump in the valve out position. Aspiration of air
3	2	50	Aspirate 50 μL	Aspiration of AP (2×10^{-3} M)
4	3	50	Aspirate 10 μL	Aspiration of buffer solution pH 3
5	4	50	Aspirate 50 μL	Aspiration of standard solutions/samples
6	5	80	Aspirate 350 μL	Aspiration of amylacetate
7	7	120	Empty	Empty syringe pump into the extraction cell for the extraction and self-separation of phases
8	–	100	Aspirate 600 μL	External syringe pump in the valve in position; aspiration of amylacetate as carrier
9	3′	100	Aspirate 30 μL	External syringe pump in the valve out position. Aspiration of the extracted ion associate to fill the port by the measured sample
10	1′	100	Dispense 100 μL	Cleaning the external holding coil
11	3′	50	Aspirate 100 μL	Aspiration of the extracted ion associate
12	2′	50	Empty	Empty the external syringe pump into the detector and measurement of absorbance

1-8–ports of selection valve in extraction part of the DV-SIA system.
1′–4′-ports of selection valve in detection part of the DV-SIA system.

external 5 mL syringe pump (FIAlab® Instrument Systems Inc., Bellevue, USA) constituted the detection part of the system. The absorption spectra were recorded by the fibre-optic charge-coupled detector USB 2000 (Ocean Optics Inc., Dunedin, USA), supplemented with a micro-volume Z-flow cell of 20 mm optical path length and the VIS light source LS-1 tungsten lamp (Ocean Optics Inc., Dunedin, USA).

FIAlab® for Windows software, version 5.9.290, was used to control the units of both parts of the SIA set-up and to perform the data acquisition.

PTFE tubes of 0.75 mm i.d. were employed in the SIA manifold, except for the aspiration of the carrier solution, which was made of 1.5 mm i.d. PTFE tubing.

2.3. Optimization of the DV-SIA system

The physical and chemical parameters of the DV-SIA system for the thiocyanate determination were optimized. The aspiration sequence, AP aspiration volume (30–70 μL) and AP concentration (1×10^{-3} to 5×10^{-3} mol L^{-1}) and the buffer aspiration volume (0–30 μL) were tested to find the most favourable extraction conditions. Additionally, the variables of the extraction procedure like the method of mixing, the aspiration time of the air used for mixing (2–14 s) and the flow rate to deliver the aspirated solutions into the extraction cell (80–140 $\mu L\,s^{-1}$) were thoroughly studied.

The sample volume was kept constant at 50 μL. All measurements were done in triplicate and average values with RSD (%) were evaluated.

2.4. DV-SIA procedure

Table 1 shows the SIA procedure employed to carry out the determination of thiocyanate. Firstly, 600 μL of water, used as the carrier, were aspirated at 200 $\mu L\,s^{-1}$ in the extraction part. After switching to the Valve Out position, an air plug was aspirated into the holding coil during 10 s at 50 $\mu L\,s^{-1}$, followed by 30 μL of a 2×10^{-3} mol L^{-1} AP solution, 10 μL of buffer solution (pH 3), 50 μL of NH_4SCN working solutions (0.05–0.50 mmol L^{-1}) or sample solution and 350 μL of amylacetate.

Next, all aspirated zones were delivered into the Extraction cell at 120 $\mu L\,s^{-1}$. The air was used for efficient mixing of the solutions and to speed up the extraction of the ion associate into the organic phase. The phases were then self-separated (3 s delay) due to their different densities, leaving the organic phase with the extracted compound in the upper part.

Then, the aspiration of 600 μL of amylacetate, using as a carrier, was carried out in the detection part. The valve position was changed and the aspiration of 30 μL of the extracted ion associate was performed in order to wash and fill port 3′ with the sample. The excess volume was pushed into port 1′, used as an auxiliary waste, by 100 μL of the organic solvent.

To carry out the measurement of the absorbance, 100 μL of the sample were aspirated and transferred to the detector by the port 2′ using the remaining volume of the carrier. The signal was recorded at 550 nm. The extraction of blank and sample solutions is depicted in Figs. 2 and 3, respectively, and a real-time procedure for the sample extraction is demonstrated with a short video file in Supplementary data (Video 1).

Finally, the extraction cell was emptied aspirating the residual solution through port 7 and discarding it through port 1 to the waste. In order to prevent sample cross-contamination, the extraction part of the SIA system was washed with water between determinations (washing step was included in the control program).

Fig. 2. Extraction procedure of blank solution measurement. The slight color of the upper organic phase is caused by low amount of extracted AP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

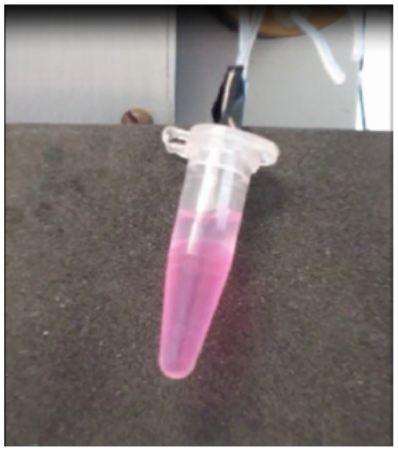

Fig. 3. Extraction procedure of sample solution measurement. The ion associate formed is extracted into the upper organic phase.

2.5. Preparation of the samples

Human saliva samples were collected from 4 healthy volunteers between the breakfast and the lunch time in three non-consecutive days. The sample was taken in previously weighted plastic tubes and after that, the tube was reweighted to calculate the amount of sample to be analyzed. Then, the sample was diluted to 7.0 mL with water and centrifuged 10 min at 5000 rpm. The supernatant solution was collected and 50 μL was aspirated to the SIA system for the analyte determination.

For the recovery study, samples were spiked with 62.5 μL of 1×10^{-2} mol L^{-1} NH_4SCN solution using automatic pipette and the same protocol was followed. The recoveries were calculated according to the AOAC definition [24].

3. Results and discussion

3.1. Preliminary studies

To investigate the adequate conditions for the thiocyanate-AP interaction and the suitable extraction solvent, some preliminary studies were carried out in batch conditions.

To set the acidity of the medium to carry out the reaction, the optimum pH value was studied. For this, ammonium acetate buffer solutions of pH values between 3.0 and 8.0 were used, and varied in increments of 1.0 unit. Briefly, in a test tube, 0.5 mL of 1×10^{-3} mol L^{-1} NH_4SCN, 4.0 mL of ammonium acetate buffer solution and 0.5 mL of 1×10^{-3} mol L^{-1} AP were manually mixed with 3.0 mL of amylacetate. After the separation of the phases, the absorbance of the organic extract was measured at 550 nm against the reagent blank. The highest difference between the sample and the blank signals was obtained using the buffer solution of pH 3. Furthermore, at pH values lower than 3 the interference of some anions, such as nitrates, can be observed; and at pH values higher than 6, the absorbance of the blank increased. Thereby, pH 3 was chosen for further experiments.

In order to obtain the best extraction efficiency with the lowest blank signal, several organic solvents were tested. Amylacetate and toluene met these requirements, but in case of toluene the formation of air bubbles in the extraction cell was observed. Therefore, amylacetate was selected for the extraction in the DV-SIA system.

3.2. Optimization of the DV-SIA system

3.2.1. Optimization of the aspiration sequence

Different reagent sequences were tested at two thiocyanate concentration levels, keeping the aqueous phase (dye, buffer and sample solutions) together at the beginning or at the end of the aspiration sequence. The optimum values were selected taking into account the compromise between the highest value of the analytical signal and the lowest value of the % RSD, obtained for two standard concentrations (0.50 and 1.0 mmol L^{-1}).

Three possible combinations for the aqueous phase were tested when the organic solvent was aspirated at the end of the sequence: (1) sample–buffer–dye, (2) dye–sample–buffer, and (3) dye–buffer–sample. The best results were obtained with the last-mentioned combination, when the aspiration of the AP solution was carried out in the first place and buffer solution was aspirated between the dye and the sample solution.

Keeping the chosen combination of aqueous solutions, the aspiration of the solvent at the beginning of the sequence was tested, but an ineffective mixing between the solvent and the aqueous phase was observed. Thus the aspiration of the organic solvent after all aqueous solutions was the selected aspiration sequence (dye–buffer–sample–amylacetate).

3.2.2. Optimization of the volume of reagents

The optimization of the volume of reagents was carried out using three concentrations of the thiocyanate standard. To choose the optimal value of the reagent, a criterion was established to achieve the highest difference between three concentration levels (0.05, 0.25 and 0.50 mmol L^{-1}) and the best repeatability of the measurement.

The volume of the carrier was adjusted in order to keep the total volume to be transferred to the extraction cell constant.

The volumes of the ammonium acetate solution and the AP solution were studied in 5 μL increments, in ranges of 0–30 μL and 30–70 μL, respectively. The volumes of 10 μL of buffer and 30 μL of the dye were selected.

3.2.3. Optimization of the AP concentration

The effect of dye concentration was investigated in the range 1×10^{-3} to 5×10^{-3} mol L^{-1} of AP. In this case, the linearity of the calibration function was used as the criterion of choice and a 2×10^{-3} mol L^{-1} concentration was picked out. At lower concentrations, the linearity was affected and similar absorbance values were obtained for the higher standard concentrations, meaning that the concentration of the dye was not high enough to interact with the thiocyanate present in standard solution. At higher AP concentrations RSD % values were increased.

3.2.4. Optimization of the extraction procedure

To ensure the best mixing of aqueous and organic phases in the extraction part, three methods of mixing were studied: (1) unidirectional – sample and reagents passed directly through the holding coil to the extraction cell, (2) flow reversals in the holding coil and (3) mixing with air bubbles in the extraction cell. In the last case, zone of the air was aspirated followed by all reagents and bubbling in the extraction cell ensured effective mixing of all solutions [23]. The value of the slope and the linearity of the analytical curve were established as main criteria to choose among the methods.

At first, a comparison between (1) and (2) was made and better results were found by repeating flow reversals three times using 20 μL increments. Thus, different flow rates for the mixing in the coil, and the subsequent movement to the extraction cell were tested (80–140 μL s^{-1}). The optimal value (140 μL s^{-1}) was compared with mixing the solutions by aspirating an air zone (3). Significant difference was not found when evaluating the slope and

linearity. Taking into account the analysis time, mixing with air zone was chosen for further determinations.

The quantity of air that was necessary to accomplish the adequate mix of the solutions in the extraction cell was investigated. For this, the time of aspiration was optimized between 2 and 14 s with a flow rate of 50 μL s^{-1}, finding 10 s as the optimum value. Lower air aspiration times were insufficient to carry out mixing and extraction of the ion associate and some troubles with the separation of the phases were observed. At higher aspiration times, significant changes in the measured signal were not observed.

The flow rate used to dispense all aspirated zones into the extraction cell was also optimized between 80 and 140 μL s^{-1} in increments of 20 μL s^{-1}. The best results were achieved with a flow rate of 120 μL s^{-1}, and successive increase did not influence the obtained results.

The volume of the organic solvent used for the extraction was 350 μL following previous experience [22]. Lower volumes of solvent were not suitable owing to the SIA configuration.

3.3. Analytical performance

The analytical performance was evaluated by the calibration function, limit of detection (LOD), limit of quantitation (LOQ), sample throughput, intra-day and inter-day precision, and selectivity.

3.3.1. Analytical curve, LOD, LOQ and sample throughput

Using the proposed DV-SIA system and the optimized values for physical and chemical parameters, an analytical curve for the thiocyanate determination was constructed from seven data points over the range of 0.05–0.50 mmol L^{-1}. The regression equation was $A = (1.887 \pm 0.053)\,[SCN^-\ mmol\ L^{-1}] + (0.037 \pm 0.014)$ with a correlation coefficient of 0.995.

The LOD was 0.02 mmol L^{-1} and LOQ was 0.07 mmol L^{-1} calculated from the calibration function. The sample throughput was 5 h^{-1} for samples aspirated in triplicate and including the washing step.

3.3.2. Precision

The intra-day and the inter-day precision were checked by the RSD (%) values at two concentration levels: 0.20 and 0.50 mmol L^{-1}. The intra-day precision was carried out performing 10 determinations and the results were 1.0 and 2.8%, respectively. The inter-day precision was evaluated measuring the same concentrations by triplicate over 10 days. The results for both concentration levels were 4.2 and 3.8%, respectively.

The obtained RSD values showed good repeatability and inter-day precision of automated extraction procedure and were comparable with the values obtained when applying other methods found in the literature [25–27].

3.3.3. Selectivity

Human saliva is produced by salivary glands and it is composed of 98% water, but also contains macromolecules, antibacterial compounds, proteins and inorganic ions. The effect of various ions (possible interferents) on the extraction and determination of the analyte was tested. For this purpose, a 0.2 mmol L^{-1} SCN^- solution was used. The tolerable amount for each ion was evaluated as SCN^-: interferent ion ratio that resulted in an error that did not exceed ±5%. The tested species and the tolerance limits are summarized in Table 2. Nitrates significantly interfered with the determination of thiocyanate, but in case of real samples nitrate levels are about 100-times lower than thiocyanate concentrations, for this reason, in such conditions, it does not interfere with SCN^- determination [10]. Although phosphates can react with AP [20], they can barely be extracted with amylacetate giving a tolerance level higher than the quantity of phosphates that can be found in saliva samples.

Table 2
Effect of some possible interfering ions on the extraction and determination of SCN^-.

		Tolerance level (mM)	Error (%)
Anions	$S_2O_3^{2-}$	20	3.1
	Cl^-	60	2.3
	$H_2PO_4^-$	70	−1.6
	Br^-	2	−1.2
	SO_4^{2-}	200	−4.7
	F^-	300	−2.3
	HCO_3^-	200	−4.2
Cations	Ni^{2+}	2	−1.1
	Pb^{2+}	30	−1.9
	Zn^{2+}	2	−2.0
	Co^{2+}	2	−2.6
	Cu^{2+}	1	−0.7
	Ca^{2+}	30	−2.1
	K^+	200	−4.2
	Mg^{2+}	200	−2.3
	Fe^{2+}	2	−0.6
	Cr^{3+}	20	−3.1

3.4. Analysis of real samples

The proposed method was applied for determination of thiocyanate in human saliva samples. To study the thiocyanate concentration levels at different day-times, saliva sample was collected from two volunteers early in the morning before breakfast and brushing teeth (1), before lunch (2), and after lunch (3). The results were expressed as milligrams of SCN^- per gram of saliva sample. The added amount of standard solution was recalculated considering the sample weight. As can be seen in Table 3, a significant decrease of the SCN^- concentration and the recoveries values occurred after lunch, probably because of the changes in the concentration of the different ions in the saliva as a result of the digestion process. On the other hand, sampling in the morning proved recovery values in the range acceptable for biological fluids (100 ± 10%).

Hence, the concentration of thiocyanate was determined in saliva samples collected between the breakfast and the lunch time.

Table 4 showed the obtained thiocyanate concentration for the samples and the results of the recovery study. The variation of the obtained results can be attribute to the strong relation between the SCN^- ions and the individual saliva composition (the thiocyanate concentration is different for each person). This fact can also be observed in the values found in the literature [2,8,27]. Additionally,

Table 3
Thiocyanate determination in real saliva samples at different day-times.

	Early morning		Before lunch		After lunch	
	Concentration (mg g^{-1})	R (%)	Concentration (mg g^{-1})	R (%)	Concentration (mg g^{-1})	R (%)
A	0.105 ± 0.011	101.0	0.178 ± 0.014	104.1	0.088 ± 0.010	77.3
B	0.141 ± 0.009	94.2	0.140 ± 0.008	84.3	0.133 ± 0.014	78.1

A, B: person A, person B; Confidence limit $x = st/\sqrt{n}$, where s means standard deviation, t is Student coefficient for $n-1$ degrees of freedom, $n = 3$; R%, recovery percentage, $\%R = ((Cf - C)/Ca) \times 100$, where Cf = measured analyte concentration in the spiked sample; C = measured analyte concentration in the non-spiked sample; Ca = added analyte concentration.

Table 4
Thiocyanate determination in real saliva samples.

		A	B	C	D
Day 1		0.102 ± 0.010	0.067 ± 0.024	0.116 ± 0.005	0.165 ± 0.041
	Added ($mg\,g^{-1}$)	0.026	0.022	0.065	0.033
	Found ($mg\,g^{-1}$)	0.134 ± 0.029	0.134 ± 0.024	0.206 ± 0.054	0.179 ± 0.066
	R (%)	104.5	86.8	113.4	110.8
Day 2		0.104 ± 0.009	0.102 ± 0.020	0.089 ± 0.009	0.127 ± 0.014
	Added ($mg\,g^{-1}$)	0.052	0.041	0.033	0.035
	Found ($mg\,g^{-1}$)	0.140 ± 0.004	0.119 ± 0.003	0.139 ± 0.013	0.137 ± 0.009
	R (%)	92.3	83.3	114.0	85.4
Day 3		0.107 ± 0.014	0.062 ± 0.007	0.140 ± 0.014	0.168 ± 0.030
	Added ($mg\,g^{-1}$)	0.034	0.052	0.028	0.050
	Found ($mg\,g^{-1}$)	0.161 ± 0.013	0.078 ± 0.006	0.142 ± 0.016	0.242 ± 0.042
	R (%)	114.0	80.3	84.3	111.0

Each measurement was done in triplicate.
A, B, C, D: person A, person B, person C, person D; *R* (%): recovery percentage.

a small variation could be observed for the same person at different days and this is possible to attribute to changes in the diet.

In spite of that, the obtained values are within the range of values found in the literature for the determination of this ion in saliva samples [2]. The recovery percentages varied between 80% and 114%. It is worth to notice that the values were almost equal for the same person, and it is related to the unique saliva composition of each person and the diet.

4. Conclusion

The DV-SIA system was successfully used for extraction and determination of thiocyanate ions in human saliva samples. Optimal chemical and flow conditions were found. Repeatability of the automated extraction procedure was 2.8%. Day-time of sampling was studied in detail to achieve accurate results proved by recovery tests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.01.021.

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5.3 PUBLICATION 3

I. Šrámková, B. Horstkotte, H. Sklenářová, P. Solich,

Automated in-syringe single-drop head-space micro-extraction applied to the determination of ethanol in wine samples.

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Automated in-syringe single-drop head-space micro-extraction applied to the determination of ethanol in wine samples

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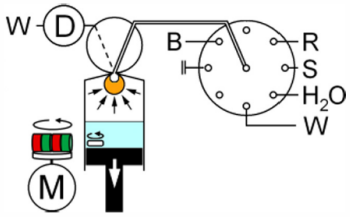
HIGHLIGHTS

- In-syringe automation of single-drop head-space micro-extraction was carried out.
- A new mode of achieving in-syringe stirring is presented.
- In-syringe vacuum applicability was demonstrated.
- High sensitivity of ethanol determination in wine samples was achieved.
- The work reports the smallest consumption of dichromate reagent for this task.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel approach of head-space single-drop micro-extraction applied to the determination of ethanol in wine is presented. For the first time, the syringe of an automated syringe pump was used as an extraction chamber of adaptable size for a volatile analyte. This approach enabled to apply negative pressure during the enrichment step, which favored the evaporation of the analyte. Placing a slowly spinning magnetic stirring bar inside the syringe, effective syringe cleaning as well as mixing of the sample with buffer solution to suppress the interference of acetic acid was achieved.

Ethanol determination was based on the reduction of a single drop of 3 mmol L^{-1} potassium dichromate dissolved in 8 mol L^{-1} sulfuric acid. The drop was positioned in the syringe inlet in the head-space above the sample with posterior spectrophotometric quantification.

The entire procedure was carried out automatically using a simple sequential injection analyzer system. One analysis required less than 5 min including the washing step. A limit of detection of 0.025% (v/v) of ethanol and an average repeatability of less than 5.0% RSD were achieved. The consumption of dichromate reagent, buffer, and sample per analysis were only 20 μL, 200 μL, and 1 mL, respectively. The results of real samples analysis did not differ significantly from those obtained with the references gas chromatography method.

1. Introduction

Miniaturized liquid–liquid extraction (LLE) techniques have been extensively developed lately with the intention to decrease solvent consumption and time while increasing the enrichment factor. In this context, single-drop micro-extraction (SDME) was

Abbreviations: DC, direct current; FT, flow techniques; GD, gas diffusion; HC, holding coil; LLE, liquid-liquid extraction; MBL-GD, membrane-less gas diffusion; P, pervaporation; R, reagent; SDME, single-drop micro-extraction; SIA, sequential injection analysis; SV, selection valve; ETFE, ethylene tetrafluoroethylene.

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introduced in 1996 [1,2]. In immersed SDME, a sole drop of a few microliters of solvent is generated and held inside a liquid sample by means of a syringe and injection needle and later retracted. Later, proposed variations differ in the way of bringing the sample into contact with the solvent such as continuous-flow SDME [3] or head-space SDME (HS-SDME) [4]. A comprehensive review on SDME development and application is given elsewhere [5].

For volatile analytes, HS-SDME is a method of choice and a powerful alternative to other pre-treatment methods since direct contact of sample and solvent is omitted and coupling with mostly GC instruments enables automation of the entire procedure. In HS-SDME, the needle of a micro-syringe penetrates the septum of a sample vial and a drop of the extraction solvent is exposed in the HS above the sample. After a defined time for analyte enrichment, the drop is retracted and injected into the GC for analysis.

Generally in LLE, solvents immiscible with the sample have to be chosen, while in HS-SDME, an aqueous drop can be used in case that the octanol:water distribution coefficient of the volatile analytes is low [6–8]. In addition, the acceptor phase drop can be used not only for analyte preconcentration but also as a derivatization reagent [6–10].

The rate of mass transfer from the sample into the head–space and further into the acceptor phase is crucial for the method sensitivity. Increased temperature or agitation are mostly used for its acceleration. Another approach, although still less explored, is the partial or total evacuation of the sample vial, which has been applied to solid-phase micro-extraction from gas-phase [11].

Up-to-date, SDME has been used only in a few cases as a procedure for analyte enrichment from liquid [2,3,12] or gaseous samples [6,7,13] using non-separation analytical flow techniques (FT). This is in clear contrast to other techniques of LLE, which have been automated using FT and reviewed elsewhere [14].

In FT, the use of the syringe void as reaction vessel, i.e. the aspiration of reagents and sample into the syringe, is normally strictly avoided. However, if syringe cleaning prior to each analysis is acceptable, new applications of in-syringe automation open the way to SIA due to the possibility to handle sample volumes of several milliliters in a closed and optionally stirred reaction vessel. Another important advantage over the classically used separation vessels is its inner volume adaptability [15]. Using a computer-controlled syringe pump, the potential of automation of LLE "in-syringe" protocols has been proven [16–18].

In this work, automated in-syringe HS-SDME was studied for the first time for the quantification of ethanol in wine, employing the well-known oxidation of ethanol by acidic dichromate solution.

The same non-selective reaction has previously been used for the analysis of ethanol employing FT as an alternative to the traditionally used GC. While direct mixing was applied for distillates [19], special units coupled to flow system were used for either trans-membrane gas diffusion (GD) [20,21], membrane-less GD [22,23] or pervaporation [24] to achieve the required selectivity. Alternative methods including Ce(IV) in combination with GD [25], direct IR measurement [26] or IR after extraction of ethanol into chloroform have been proposed [27]. The use of colorimetric reactions in FT for ethanol determination is given in Table 1. Enzymatic methods are summarized elsewhere [28].

Here, only a simple flow analyzer system following the typical design of sequential injection analysis (SIA) [29] was employed for automated solvent-free in-syringe HS-SDME, including an in-drop reaction of the analyte. A single drop of only 20 μL of the reagent was positioned above the sample and after a short incubation time, the reduction of dichromate by ethanol was quantified by spectrophotometric detection. The method shows the advantage of high flexibility of SIA, based on using the same simple instrumental assembly for different analytical procedures just by changing the computer-controlled operation protocol.

2. Material and methods

2.1. Reagents

All solutions were prepared with ultra-pure water. Reagents were of "for analysis" grade and purchased from Sigma–Aldrich (Prague, Czech Republic).

Aqueous stock solutions of 19.6% (v/v) ethanol, 0.25 mol L^{-1} of potassium dichromate, and 8 mol L^{-1} sulfuric acid were prepared and stored in the dark at room temperature. For study of interferences, further aqueous stock solutions of 15.0 g L^{-1} glycerol, 0.3 g L^{-1} acetic acid, 5.0 g L^{-1} glucose, 5.0 g L^{-1} fructose,

Table 1
Overview about reported methods for colorimetric determination of ethanol in flow systems.

FT	Separation[a]	Reagent	Sample[b]	Working range (% (v/v))	LOD (% (v/v))	Repeatability (% RSD)	Detection wavelength (nm)	Required vol. of reagent (mL)	Injected vol. of sample (mL)	Sample throughput (h^{-1})	Ref.
SIA	–	200 mM $K_2Cr_2O_7$ in 4 mol L^{-1} H_2SO_4	D	1–6	0.09	<1	600	0.2	0.1	19	[19]
FIA	MBL-GD	30 mM $K_2Cr_2O_7$ in 1.5 mol L^{-1} H_2SO_4	B, D, W	0.5–30	0.27	0.5	590	1	0.3	16	[23]
SIA + FIA	MBL-GD	200 mM $K_2Cr_2O_7$ in 4 mol L^{-1} H_2SO_4	L, M	5–50	0.64 / 2.7	3.7	590	0.2	0.4	17/25	[22]
FIA	GD	40 mM $(NH_4)_2Ce(NO_3)_6$ in 0.3 mol L^{-1} HNO_3	B, D, W	0.1–10	0.03	<1.3	415	3	0.1	20	[25]
FIA	MBL-GD	150 mM $K_2Cr_2O_7$ in 6 mol L^{-1} H_2SO_4	G	3–80	0.9	<4.9	590	1.78	1.33	24	[32]
FIA	GD	200 mM $K_2Cr_2O_7$ in 4 mol L^{-1} H_2SO_4	D, W	1–20	0.5	3.7	600	2.5	0.5	20	[21]
FIA	GD	300 mM $K_2Cr_2O_7$ in 4 mol L^{-1} H_2SO_4	F	5–25	0.18	2.1	600	0.62	0.5	29	[20]
FIA	P with heating at 60 °C	54 mM $K_2Cr_2O_7$ in 8 mol L^{-1} H_2SO_4	W	1–20	0.5	3	600	15	2	6	[24]
SIA	HS-SDME with pressure decrease	3 mM $K_2Cr_2O_7$ in 8 mol L^{-1} H_2SO_4	W	0–1.5, non linear	0.025	<4	447	0.020	0.1	12	here

[a] GD: Gas diffusion, HS-SDME: Head-space single-drop microextraction, MBL-GD: membrane-less gas diffusion, P: Pervaporation.
[b] B: Beer, D: Distilled spirits, F: Fermentation brew, G: gasohol fuel, L: Liquor, M: Medical product, W: Wine.

2.0 g L^{-1} potassium metabisulfite, 100 mg L^{-1} gallic acid, 10 mg L^{-1} resveratrol and 0.5 M sodium phosphate buffer, pH 12, were prepared. A chromogenic reagent (R) was prepared daily from $K_2Cr_2O_7$ and H_2SO_4 stock solutions. The reagent proved to be stable for over 24 h. Apart from the buffer, all working solutions were prepared daily by appropriate dilution of the stock solutions.

2.2. Instrumentation

A rotary selection valve (SV, type Cheminert, 12U-0484H) from Valco Instruments (Houston, TX, USA) and a syringe pump (SP, type Cavro XL) from Tecan (San Jose, CA, USA), equipped with a 5 ml glass syringe, were used and controlled via RS232 connection.

A miniature fiber-optic spectrophotometer from Ocean Optics Inc. (Dunedin, FL, USA), type USB4000, was used for detection. A bright white LED was used as light source. Spectrophotometer and LED were connected via 1 mm id optical fibers from FIALab® to a micro-volume detection flow cell with 1 cm optical path length made of Ultem polymer from the same company.

The analyzer configuration is schematically shown in Fig. 1 with all tubing dimensions indicated. Tubing connections were made of 0.8 mm id PTFE with the only exception being the detection cell inlet and outlet where PTFE tubing of 0.5 mm id was used.

The syringe inlet was modified as given in Supplementary material 1 placing a short piece of glass capillary (2 mm od, 1.5 mm id, 3 mm length) from the inner side of the syringe barrel to yield better wettability of the liquid inlet and by this, the stabilization of the liquid drop. To decrease the dead volume further, a piece of PTFE tube of 0.8 mm id was inserted from above.

Lateral ports of SV were connected to waste reservoir, sample, reagent, water, and air, and standard solutions. The central port was connected to the IN port of the SP by a short holding coil (HC, 25 cm). The OUT port of the syringe pump was connected to the detection flow cell and further to waste. All ferrules and fittings were made of ETFE and Acetal, and purchased from FIALab® company.

2.3. In-syringe stirring

In order to achieve high repeatability, homogeneous mixing of sample, buffer, and water was required. As an adequate technique, in-syringe stirring, as formerly described [16,17], was applied. In this work, only homogenization of the solutions was aimed, so slow rotation was sufficient. This allowed the use of a different approach for the induction of the rotation of a magnetic micro-stirring bar (3 mm in length, 1 mm diameter) placed inside the syringe. Two neodymium magnets were fixed on the axis of a simple direct current (DC) motor powered by a USB port of the control PC and the rotation velocity was adjusted to approximately 300 rpm with a precision potentiometer. The motor was placed as close to the syringe as possible, forcing the stirring bar inside the syringe to turn. The configuration is given in Fig. 1 and as a photo in Fig. 2. The slow motion and small size of the stirring bar was sufficient to homogenize the solutions inside the syringe within seconds but was slow enough to avoid disturbances of the liquid surface or splattering.

2.4. Data acquisition and evaluation

The used detection wavelength was the maximum absorbance of chromate in the visible range at 447 nm. A reference wavelength of 570 nm was used to compensate unspecific light intensity fluctuations. An integration time of 10 ms and data averaging over

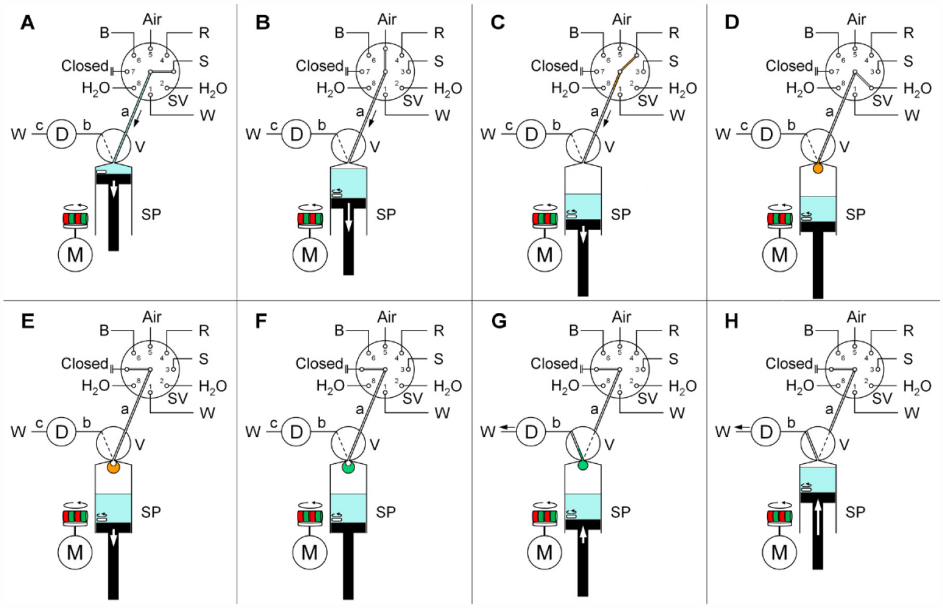

Fig. 1. Scheme of analyzer system configuration and operation for head space extraction of ethanol. a: holding coil between valve and syringe pump (25 cm, 0.8 mm id) b: PTFE tube to detection flow cell (7 cm, 0.5 mm id), c: PEEK tube to waste (10 cm, 0.5 mm id) D: Detection flow cell, M: DC-Motor with magnets, SP: Syringe pump, SV: Rotary selection valve, V: Rotary syringe head valve. B: Buffer, R: Chromate reagent, S: sample, W: Waste. Indicated operation procedure (not all steps shown) A: Aspiration of sample, B: Aspiration of Air, C: Aspiration of chromic acid (R), D: Filling the holding coil with water and generation of droplet for head space extraction and enrichment time, E: Application of negative pressure, F: Enrichment time, G: Release of negative pressure, H: Dispense for measurement and then syringe emptying through detection flow cell to waste.

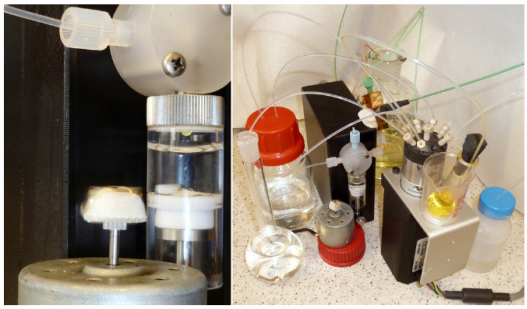

Fig. 2. Photo documentation of drop exposure with stirring assembly (Left) and analyzer system (Right).

15 single measurements were applied allowing an effective measuring frequency of 5 Hz. Spectral smoothing over 15 pixels of the diode array sensor was carried out.

Instrumentation control and data processing were done using FIALab software 5.9.321. Posterior data smoothing and peak-height evaluation were done with MS-Excel. The obtained signals were of rectangular shape and the average height of the signal plateau was taken for data evaluation. All experiments were carried out as a minimum in triplicate.

2.5. Operation protocol

The analytical method is given as Supplementary material 2; all steps are indicated with numbers. The procedure started by syringe cleaning aspirating three-times 0.8 mL of water through syringe port IN and dispensing it to waste through syringe port OUT (step 1 + 2). When the detection cell was filled with water after cleaning, blank measurement was performed [3].

Secondly, 500 μL of air were aspirated to avoid contact of the sample with the top part of the syringe barrel [4]; 200 μL of this air zone was used to empty the detection cell and connecting tubes completely at reduced flow rate [5].

Then, 1 mL of sample was aspirated slowly into the syringe [6], followed by 200 μL of buffer [8] and three-times 33 μL of water [10], each separated into segments by air bubbles (7 + 9) to achieve a better cleaning of the holding coil from any rest of sample.

Mixing of solutions by stirring was achieved during the next step (emptying the HC at low flow rate steps 11 + 12). Another 100 μL of air were aspirated to overcome the mechanical backlash of the syringe pump [13].

Finally, 20 μL of reagent [14], 15 μL of air [15], and 145 μL of water [16] were slowly aspirated. These volumes were carefully adjusted in a way that the reagent drop would not enter the syringe completely but would stay attached to the syringe inlet, only about 3 mm over the liquid surface.

Negative pressure was applied by turning the selection valve to the permanently closed port 7 and by performing an aspiration step equal to 250 μL [17]. With the main volume of air (500 μL) inside the syringe and only a very small volume of air in the HC (15 μL), the liquid drop would hardly move but it would be blown up slightly by the expansion of the air in the holding coil. The pressure inside the syringe can be estimated to be approx. 2/3 of an atmosphere.

After an enrichment and reaction time [18], the negative pressure was released by moving the syringe piston to its original position [19]. Then, the droplet was slowly pushed through the detection flow cell to register the occurred change of color due to chromate reduction [20] followed by the complete and rapid emptying of the syringe [21].

2.6. Reference method

Gas chromatography was used for comparison of the results obtained with the proposed analyzer system for real wine samples, diluted 1:10 with water. GC analysis was performed on a GC Shimadzu 17a Ver.3 instrument from Shimadzu Europa GmbH (Duisburg, Germany) using a 30 m long, 0.32 mm i.d., Alltech®, AT™-624 column (Grace, Worms, Germany) with 1.80 μm poly [(phenyl)(cyanopropyl) dimethyl]siloxan stationary phase. An injection volume of 0.5 μL and maximum splitting ratio of 1:150 were applied. The temperature profile was: 0–3 min at 40 °C, then rising between 3 and 8 min to 140 °C with a gradient of 20 °C min^{-1}. Injector and detector temperatures were set to 280 °C and 300 °C, respectively. Isopropanol was used as internal standard at a concentration level of 1% (v/v). Data acquisition and evaluation were carried out with Data Apex CSW v.17 software.

3. Results and discussion

3.1. System configuration and method development

Since all solutions had to pass the HC, the avoidance of any contact between the reagent drop and other solutions was crucial. Each one would have caused bias signals either by dilution (water), partly neutralization (buffer), or untimely and unselective reduction of dichromate by any organic compound (sample).

The sample was supposed to give the highest memory effect on the walls of the HC; therefore, it was aspirated first, followed by buffer solution and three small segments of water for cleaning. A high flow-rate for sample aspiration was chosen to keep the contact time between sample and HC as short as possible.

In off-line HS-SDME, the tip of a micro-syringe needle has to provide an adequate support to achieve a stable and reproducible liquid drop of the extraction solvent. In the same way, the stabilization of the reagent drop in the syringe inlet was crucial in this work. By using the unmodified commercial syringe initially, it was observed that the aqueous reagent drop would easily leak down on the inner side of the syringe and fall into the sample or would not enter the syringe far enough to provide a significant contact surface with the head-space atmosphere. Introducing a short glass capillary as hydrophilic material in syringe inlet, it was found that a stable drop could be created (Supplementary material 1). The length of the waste tube showed to have a significant influence on the expulsion of the reagent drop through the detector. When the detector cell and the connected tube were emptied with air, remaining water on the inner wall of the PTFE tubes would form pellicles driven by surface tension and air-segments, which increased the pressure required for drop expulsion. This problem was overcome by making the tubes to detector and waste as short as possible and applying a reduced flow rate for the drop expulsion (step 5).

Although the procedure comprises a large number of steps, all of them were proven to be necessary. So, the repeated cleaning of the HC with slugs of water (steps 9 and 10) was necessary to avoid even minor cross-over between samples, which was evaluated by the addition of fluorescein dye to the standard solution. The filling of the HC after aspiration of the reagent drop was needed to avoid that the drop would fall into the sample at vacuum application. It was observed that at pressure decrease, the expansion of the air volume remaining in the HC was sufficient to expose the reagent solution as a drop at the syringe inlet.

3.2. Reagent and buffer

The reaction kinetics of dichromate reduction is considerably enhanced in the presence of sulfuric acid. Therefore, the

concentration of sulfuric acid in the reagent was studied in the range from 1 to 8 mol L^{-1} using both 1 and 10% (v/v) of ethanol standards. The results and experimental conditions for both concentration ranges are given in Fig. 3.

No significant reduction was observed for 1 and 2 mol L^{-1} and for 4 mol L^{-1}, only a 10% (v/v) standard yielded a significant signal decrease. The highest sensitivity was achieved for acid concentration of 8 mol L^{-1}, being the maximum concentration tested. This concentration was used further as a compromise between the reaction sensitivity and work safety.

Two modes of detection were considered being either the measurement of dichromate, i.e. the decrease of the analytical signal with higher ethanol content, or the increase of the absorbance of Cr(III) as the reaction product.

Following dichromate reduction implies that the blank signal yields the highest signal and since higher signals than 1.5 AU were considered to be not reliably measurable, the initial dichromate concentration must not be higher than 3 mmol L^{-1}. This means a low chromium consumption and waste production. Due to a six-fold higher molar absorbance of dichromate compared to Cr(III), this mode also yielded a higher sensitivity. On the other side, dichromate availability for the reduction would be a limiting factor so that non-linearity and a higher analytical error could be expected than when measuring Cr(III), where the reagent could be used in large surplus but with minor sensitivity. Finally, the first option using 3 mmol L^{-1} K_2CrO_7 was adopted to achieve a faster and more sensitive method.

The size of the drop contributes significantly to the extraction efficiency. For HS-SDME combined with GC, the droplet volume is normally <5 μL since a larger volume can lead to peak tailing [30]. In this work, the drop volume had to be chosen mainly under considerations of practical handling. For a larger drop, lower sensitivity can be expected, since it could not enter the syringe completely without risking its fall. Also, a longer reaction time would be necessary to achieve equal reduction of the larger quantity of dichromate. For a smaller drop, signal repeatability decrease would possibly occur, e.g. by dilution with any remaining liquid film in the detection cell, tube, or syringe head valve, drop formation, or evaporation among others. A drop of 20 μL of the reagent was used throughout avoiding the above described problems while the possible volume range was 15–30 μL.

An alkaline buffer solution was useful in order to avoid volatility of organic acids and by that, their possible interference with ethanol evaporation and oxidation. Considering acid concentrations in the range of 1–2% in wine at a pH of 3 to 4 and a volume of 1 mL of sample, 0.2 mmol of buffer capacity was considered to be sufficient to increase the sample pH to neutral value. Testing the mixture with wine samples led to final pH values between 6 and 7. As well soluble, a 0.5 mol L^{-1} Na_3PO_4/Na_2HPO_4 buffer was chosen and 200 μL were used for interference study (see Section 3.5).

3.3. Enrichment time

The enrichment time was the second crucial parameter affecting the method sensitivity and linearity. Obviously, the optimum time would be different for low and for high ethanol contents. Therefore, the time studied for two different ranges of ethanol concentrations was 5–160 s for a low range (0.1–2.0% (v/v)) and 5–60 s for a high range (10.0–40.0% (v/v)). The criterion for the choice of the enrichment time was to achieve not more than 40% reduction of the initial chromate concentration.

As it can be seen in Fig. 4, the blank value decreased, in approximation, linearly over the studied time, which was considered to be the result of droplet growth by water adsorption.

As expected, the analytical response being the difference between blank and standard signals followed a saturation behavior as the chromate was consumed. As enrichment times, 40 s and 10 s were adopted for further experiments for lower and higher concentration range, respectively. Due to the saturation behavior, non-linear calibration was performed as described in detail in Section 3.6.

3.4. Application of vacuum and agitation

It was demonstrated that vacuum application can speed up the kinetics of mass transfer from the bulk sample into the head space, resulting in higher efficiency of HS-SDME [11]. A very interesting feature of using an extraction vessel of adaptable volume is the possibility of vacuum application without an external vacuum pump. In this work, decrease of the pressure in the syringe was achieved by aspiration of a certain volume through the selection valve to the permanently closed position 7 (step 17). The effect of vacuum was studied using different volumes (0, 125 μL, 250 μL, and 375 μL) to yield pressures of approximately 100%, 80%, 67% and 57% of one atmosphere, respectively. The results and experimental conditions are given in Supplementary material 3. Again, two standard solutions of 1 and 10% (v/v) of ethanol were used. The results confirmed the initial statement since the signal height compared to normal pressure for the 10% (v/v) standard decreased by about 7%, 8% and 12% with pressure decrease to 80%,

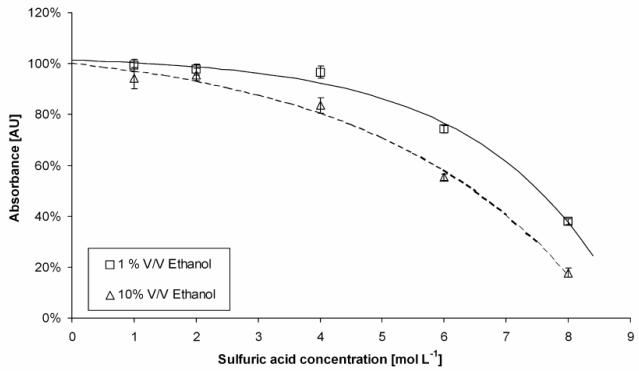

Fig. 3. Effect of sulfuric acid concentration on the signal decrease in relation to the blank value. Conditions: 3 mmol L^{-1} $K_2Cr_2O_7$, applied volume for pressure decrease: 250 μL, enrichment time: 40 s for 1% (v/v) of ethanol, 10 s for 10% (v/v) of ethanol.

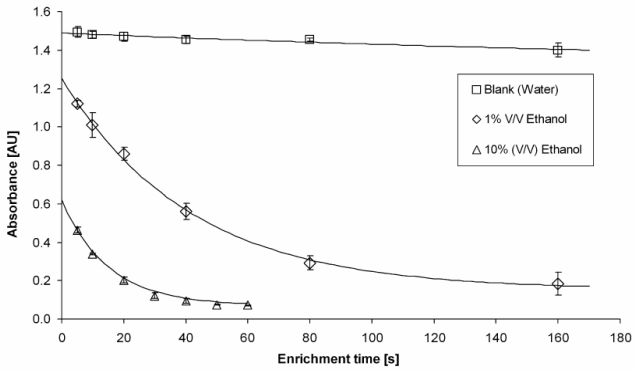

Fig. 4. Effect of enrichment time on the signal decrease given as absorbance. Conditions: 3 mmol L^{-1} $K_2Cr_2O_7$ in 8 mol L^{-1} H_2SO_4, applied volume for pressure decrease: 250 μL.

67%, and 57% atmosphere, respectively. For a 1% (v/v) standard solution, the signal decrease was about 10%, 29%, and 33%, respectively, while for the blank signal (data not shown) there was only a slight decrease of about 4% over the studied range.

The mass transfer of ethanol from the bulk sample into the head space is considered to be facilitated by sample agitation [30]. In this work, in-syringe mixing of sample with buffer and water used for cleaning was required to achieve a reproducible surface of the liquid.

3.5. Interferences study and use of buffer

The proposed system was applied to wine samples. Several compounds in wines could potentially interfere with the analysis. Most likely are substances which are volatile and oxidizable, which increase the sample viscosity or show affinity to hydrophobic surfaces such as the tubing material PTFE and cause sample sticking, or which could change the volatility of ethanol/its solubility in the sample.

Among the substances of highest content in wine apart from ethanol, organic acid (acetic) glycerol, carbohydrates (glucose and fructose) and antioxidants (natural: gallic acid, resveratrol; added by producer: sulfite) were studied as possible interferences. For this study, a set of standards was prepared being the blank solution (ultra-pure water), a 1%(v/v) ethanol standard corresponding approximately to a 1:10 diluted wine sample, two solutions of the potential interfering compound in a concentration equal to higher concentration range reported for wine as well as a 1:10 dilution. Two equal solutions but one with the addition of 1%(v/v) of ethanol were prepared with the substances, which are found in wine at high concentrations regarding ethanol (acetic acid, glycerol, glucose, and fructose) to evaluate the effect on the volatility of the analyte. The results and experimental conditions and concentrations are given in Table 2.

For acetic acid, the solutions were measured twice, i.e. with and without buffer solution, in order to evaluate the effect of the buffer on the suppression of the potential interference of volatile organic acids. The study revealed that sole acetic acid does not show a significant interference at the tested concentrations, while there was a significant increase of the methods sensitivity to ethanol of almost 27% if buffering was omitted. This is apparently caused by changing the acidity (and not the ionic force) of the solution. Thus, for all further measurements, 200 μL of phosphate buffer were used.

No interferences were observed from sugars and antioxidants. Also, no significant difference was observed between the blank solution and a 1.5 g L^{-1} solution of glycerol. However, with the mixed solution of 1% (v/v) ethanol and 15 g L^{-1}glycerol, a 24% signal lower than it was with the 1% (v/v) standard was measured. Nevertheless, the glycerol concentration in wine normally does not surpass 10 g L^{-1} [31] and in this case considering the applied

Table 2
Study of interferences. Conditions as given in Table 3.

Compound	Concentration (g L^{-1})	No addition of ethanol; signal in respect to blank ± SD (%)	Addition of 1% (v/v) ethanol; signal in respect to the standard ± SD (%)
Acetic acid	0.03	98.9 ± 5.0	98.3 ± 8.2
Acetic acid	0.3	99.5 ± 4.0	100.7 ± 8.8
Acetic acid[a]	0.03	98.8 ± 2.8	73.0 ± 4.7
Acetic acid[a]	0.3	98.8 ± 2.3	71. 8 ± 5.9
Glycerol	1.5	101.2 ± 3.4	88.1 ± 7.9
Glycerol	15.0	90.5 ± 3.1	76.3 ± 2.0
Glucose	0.5	98.5 ± 3.2	104.2 ± 11.9
Glucose	5.0	97.2 ± 3.3	93.0 ± 9.0
Fructose	0.5	103.0 ± 2.5	100.1 ± 2.6
Fructose	5.0	106.3 ± 3.3	98.0 ± 4.8
Sulfite	0.002	97.9 ± 4.6	–
Sulfite	0.02	95.6 ± 4.9	–
Gallic acid	0.01	100.2 ± 4.0	–
Gallic acid	0.1	88.8 ± 2.6	–
Resveratrol	0.001	105.2 ± 1.6	–
Resveratrol	0.01	103.6 ± 5.5	–

[a] No buffer used.

sample dilution by factor of 10, a concentration level of 1 g L^{-1} glycerol would correspond to the real concentration, which proved not showing any interference. The same conclusion can be taken for gallic acid, where a signal decrease of approx. 11% can be observed with the more concentrated solution, which represents a ten-time higher concentration than the one in wine after sample preparation (dilution). Also, from the reported concentration of gallic acid in red wine (10–100 mg L^{-1}) [32], the highest one was chosen for interference testing.

3.6. Analytical performance

From the optimization studies, the following parameters were chosen for method validation with the characteristics listed in Table 3: aspiration of 250 μL from the closed port to decrease the pressure in the syringe, 20 μL drop of 3 mmol L^{-1} potassium dichromate in 8 mol L^{-1} sulfuric acid, and 40 s enrichment time.

Three calibrations were measured in the range of 0.25–1.50% (v/v) of ethanol allowing the measurement of tenfold diluted wine samples. As discussed in Section 3.3, the signal showed a saturation behavior described by following equation: signal = $a - b \times [1 - \exp(-[\text{ethanol}]/c)]$; the parameters are listed in Table 3. Here, a is the parameter that gives the blank signal, b is the maximum possible signal decrease, and c is a kinetic parameter.

The overall signal repeatability expressed as a relative standard deviation (RSD; $n = 34$) was <5.0% (considering the measurement of blank, standards and samples). The limit of detection (LOD) was calculated as three-times the standard deviation of the blank divided by the negative ratio of b and c parameters of the calibration equation. This value equals to the initial slope of the calibration curve, i.e. the slope at blank concentration. The limit of quantification (LOQ) was then calculated as ten-times the standard deviation divided by the initial slope. Due to the curvature of the calibration function, the analytical error will increase with higher sample concentrations. The possibility of in-syringe agitation enabling automated and homogeneous sample dilution is therefore of interest.

The measurement frequency was 12 h^{-1}, i.e. 5 min per analysis including the washing step. However, standard and samples were measured in triplicate in this work, reducing the effective sample throughput. The reagents consumption was very low. Only 20 μL of the dichromate in sulfuric acid, 200 μL of buffer solution and 1 mL of standard/sample were required per analysis, i.e. the actual volume of wine sample, diluted with water 1:10, was only 0.1 mL.

3.7. Real sample analysis

The developed method was applied for the analysis of eight real samples, consisting of two white, two rosé and four red wines. The method resulted to be too sensitive for reliable measurement of the undiluted samples even when the enrichment time was 0 s. Therefore, the wine samples were diluted at 1:10 with water and the enrichment time was set to 40 s, being the chosen time for the lower concentration range. Samples were analyzed both by the proposed analyzer system and, for comparison, by the GC method described in Section 2.6. The obtained results are summarized in Table 4. The ethanol contents found by both the proposed SIA method and the reference GC method were in good agreement with the contents declared by the producers with the exception of sample 8 where a difference of 0.9% (v/v) was found. Comparing the results obtained by both the methods, a good average concordance of 100.1 ± 5.6% was found between the concentration values. This proves the suitability of the proposed method for the intended application.

3.8. Discussion and outlook

A large number of papers proposing miscellaneous methods of determination of ethanol as an analyte of high interest especially in food and forensic analysis were proposed. Most of them employed the same reaction as used here with the difference in the specie quantified. Opting for the quantitation of dichromate instead of Cr (III) in this work was reasoned in Section 3.2; the main advantages over other methods was a significantly lower concentration and smaller volume of the toxic reagent and higher sensitivity (Table 1). Using the same reaction, an SIA method with direct sample injection was reported by Fletcher et al. [19]. Although a wide working range was achieved, the method suffered from the need of sulfuric acid in high concentration as a carrier and which led to problems with Schlieren.

The Schlieren effect and interferences were overcome by contactless separation such as by so-denoted membraneless gas-diffusion (MBL-GD) [22,23,33]. Such problems were also omitted in this work; the reagent drop did not come into contact with any other liquid in the flow cell of the detector since it was emptied during the cleaning step and the achieved sensitivity is at least ten-times higher than in the cited works.

Using GD, MBL-GD, or pervaporation [24], more complex analyzer systems with an external chamber were required. Although MBL-GD was demonstrated to be potentially superior to classical GD [23], flow rates of donor and acceptor phases must be carefully adjusted and pressure release must be guaranteed at filling the respective diffusion units [22,33]. Here in contrast, the use of a syringe pump as reactor adaptable in size and simultaneously the liquid handling system represents a versatile assembly following the simplest SIA design without any external vessel. Therefore, the potential problems related to the use of membranes such as damage by the used reagent [24] or clogging are also omitted while achieving miniaturization and simplification of the analyzer system. Compared to all the above mentioned works, the proposed in-syringe SIA system brings the advantage of

Table 3
Analytical performance and method characteristics.

Figures of merit	Obtained value
Calibration curve equation	Signal = a − b × [1 − exp (−[Ethanol]/c)]
a	1.528 ± 0.058 AU
b	1.278 ± 0.089 AU
c	0.476 ± 0.066% (v/v)
LOD	0.025 ± 0.084% (v/v)
LOQ	0.084 ± 013% (v/v)
Repeatability (RSD)	<5.0%
Injection frequency	12 h^{-1}

Conditions: 3 mmol L^{-1} $K_2Cr_2O_7$ in 8 mol L^{-1} H_2SO_4; buffer solution: 0.5 mol L^{-1} sodium phosphate, pH 12; applied volume for pressure decrease: 250 μL, enrichment time: 40 s.

Table 4
Real sample analysis. Conditions as given in Table 3.

Sample no.	Type	Declared (% (v/v))	Values obtained by SIA method (% (v/v))[a]	Values obtained by GC method (% (v/v))[b]
1	White	10.0	9.3	9.9
2	White	11.0	10.4	11.0
3	Rosé	9.5	9.2	9.8
4	Rosé	11.0	11.5	11.3
5	Red	11.0	11.4	10.5
6	Red	11.0	11.3	10.7
7	Red	11.5	11.5	11.3
8	Red	12.5	11.7	11.6

[a] Average RSD of sample's peak heights: 8.6%.
[b] Average RSD of peak areas corrected by the internal standard area: 3.3%.

higher sensitivity, significantly lower consumption of the reagent, and simplicity.

General advantageous features of in-syringe SIA for HS-SDME were the straightforward application of negative pressure to the sample to increase the analyte transfer into the gas phase and portability of the system enabling field monitoring. Furthermore, there is the potential of coupling the system with a separation technique such as chromatography [34] or capillary electrophoresis [35] e.g. for organic analytes and even in-syringe analyte derivatization in combination with HS-SDME could be performed. Taking into account that in-syringe sample dilution and standard addition have been demonstrated [16,17], there is a high potential for future applications of the method presented.

4. Conclusion

In this work, the first exploitation of in-syringe analysis as a tool for automation of HS-SDME was presented. A simple and versatile analyzer system was proposed allowing full automation of on-line extraction of the analyte without any organic solvent and on-site measurement of the enriched acceptor phase. Application of negative pressure without the use of any external device was shown to improve the efficiency of head-space enrichment of the analyte as well as in-syringe mixing of sample and buffer solution. The proposed analyzer and method yielded very low reagent consumption and very high sensitivity were obtained.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.04.031.

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5.4 PUBLICATION 4

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Application of a fully integrated photodegradation-detection flow-batch analysis system with an on-line preconcentration step for the determination of metsulfuron methyl in water samples

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ABSTRACT

This work presents the development of a fully automated flow-batch analysis (FBA) system as a new approach for on-line preconcentration, photodegradation and fluorescence detection in a lab-constructed mixing chamber that was designed to perform these processes without sample dispersion. The system positions the mixing chamber into the detection system and varies the instrumental parameters according to the required photodegradation conditions. The developed FBA system is simple and easily coupled with any sample pretreatment without altering the configuration.

This FBA system was implemented to photodegrade and determine the fluorescence of the degradation products of metsulfuron methyl (MSM), a naturally non-fluorescent herbicide of the sulfonylurea's family. An on-line solid phase extraction (SPE) and clean up procedure using a C18 minicolumn was coupled to the photodegradation-detection mixing chamber (PDMC) that was located in the spectrofluorometer. An enrichment factor of 27 was achieved.

Photodegradation conditions have been optimized by considering the influence of the elution solvent on both the formation of the photoproduct and on the fluorescence signal.

Under optimal conditions, the calibration for the MSM determination was linear over the range of 1.00–7.20 $\mu g\,L^{-1}$. The limit of detection (LOD) was 0.28 $\mu g\,L^{-1}$; the relative standard deviation was 2.0% and the sample throughput for the entire process was 3 h^{-1}. The proposed method was applied to real water samples from the Bahía Blanca's agricultural region (Bahía Blanca, Buenos Aires, Argentina). This method obtained satisfactory recoveries with a range of 94.7–109.8%.

1. Introduction

Since the development of automated analytical methods, different applications have been performed to transform batch processes into automated processes. After the development of segmented flow analysis (SFA) [1] and flow injection analysis (FIA) [2], flow-analysis methodologies have been implemented in a variety of analytical techniques [3–7]. Among these techniques, flow-batch analysis (FBA) systems draw upon the useful features of batch and multi-commutation approaches [8]. FBA also offers an excellent alternative to traditional processes for the automation of analytical procedures because of its flexibility and versatility (multi-task characteristics). The main component of a FBA system is the mixing chamber in which different processes, such as sample conditioning, analyte-reagent addition and mixing, are performed. Moreover, one of the main advantages of using the mixing chamber is that compounds of interest can be detected in the chamber, avoiding the transport of material to the detector and the resulting dispersion. Several analytical applications have been developed using the FBA methodology [9].

Because of the public concern with the environmental and human effects of analytical methods, new sample preparation techniques have been developed [10] to incorporate procedures that reduce the use of hazardous chemicals. Therefore, photoradiation is a promising alternative to chemical reagents. Flow methodologies have already shown high potential for the automation of photochemical derivatization methods. These methods are being exploited for the quantification of various analytes upon photodegradation [11,12].

Metsulfuron methyl (MSM) is a sulfonylurea selective systemic herbicide for the post-emergent control of broadleaf weeds and brush, and MSM is added to diverse crops, such as autumn-winter

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cereals, rice, maize, and soybean. MSM is moderately persistent in water and highly mobile in soils and has the potential to enter surface waters from field drainage or runoff water. The persistence and mobility of this compound may cause serious damage to the environment and human health. Because of its widespread use in global agricultural production, particularly in the region surrounding Bahía Blanca, Argentina, the MSM level in water sources intended for both animal and human consumption must be monitored.

Many studies have focused on determining the concentration of this herbicide using different techniques. One of the first attempts to determine the concentration of MSM at trace levels in natural waters employed a gas chromatography technique with electron capture detection [13]. Because of the chemical properties of MSM, high-performance liquid chromatography (HPLC) is one of the most used techniques [14–16] combined with off-line preconcentration techniques (dispersive liquid–liquid microextraction, DLLME, or solid phase extraction, SPE) and spectrophotometric detection (DAD). Recently, ultra-high-performance liquid chromatography (UHPLC) with mass spectrometry detection [17] was employed to determine the concentration of this herbicide. Other methods, such as capillary electrophoresis [18,19], bioassays [20], and enzyme immunoassays [21], have often been use in previous studies. Additionally, the photochemical behavior of metsulfuron methyl in aqueous and organic media has been reported [22]. Since MSM is a naturally non-fluorescent herbicide, Coly et al. [23] determined the concentration of this analyte through photochemically induced fluorescence (PIF) detection in an aqueous micellar mobile phase using a flow injection analysis (FIA) system for the detection of MSM after off-line preconcentration employing successive extraction steps. Another study performed by López Flores et al. [24] proposed a flow injection system to preconcentrate and determine the concentration of the MSM photoproduct instead of MSM using solid-phase fluorescence spectroscopy. A suitable solid support was placed inside a flow cell to preconcentrate and retain the photoproduct for detection.

Because of the low levels of MSM present in environmental samples, a preconcentration step is required even for methods with high sensitivities [25]. Furthermore, a clean-up process is also necessary because of the complexity of the sample. Among the extraction techniques, SPE is the most widely used to preconcentrate MSM because of its easy implementation and high preconcentration capabilities. In addition, SPE is able to be automated, which allows a reduction in the analysis time and prevents exposure of operator to potential hazards. In previous studies, however, this process was performed primarily off-line.

The aim of this study was to develop a simple flow manifold to fully automate SPE-photodegradation-detection. The new FBA system for MSM determination implemented both the photodegradation process and the detection step in the mixing chamber that was placed instead of the cell holder in the spectrofluorometer. The proposed system was employed to determine trace levels of MSM in different water samples. For this purpose, an on-line solid-phase extraction (SPE) procedure was coupled to the developed manifold. Generally, photodegradation in flow systems is performed in a reactor coiled around an UV lamp and photoproducts are then, propelled to the detector [11,12,26]. To the best of our knowledge, this study provides the first demonstration of a photodegradation and detection procedures being performed in a flow-batch mixing chamber (PDMC) employed as a photoreactor and as a cell.

2. Experimental

2.1. Reagents

Solutions were prepared using analytical-grade reagents and ultra-pure water (18 MΩ cm^{-1}). Methanol, MeOH (99.8%, HPLC grade, Fluka, Germany) and acetonitrile, ACN (≥99%, Sigma Aldrich, Germany) were used as the organic solvents.

A 140 mg L^{-1} MSM (Sigma-Aldrich, Germany) stock solution was prepared in ACN and stored in a dark bottle at 4 °C. Working solutions were prepared daily by appropriately diluting the stock solution.

An appropriate volume of a 0.10 mol L^{-1} sodium dodecyl sulfate (SDS) (Anedra, Argentina) solution was added to the MSM working solutions to obtain the critical micelle concentration (CMC).

The pH of the medium was increased using a 0.10 mol L^{-1} NaOH (Merck, Germany). A 0.025 mol L^{-1} NaOH solution was prepared by diluting the concentrated solution. The pH of the samples was decreased using a 0.01 mol L^{-1} HCl (Merck, Germany) solution.

To evaluate possible interfering substances, solutions of sulfometuron methyl (SMM), chlorsulfuron methyl (CSM), ethoxysulfuron (ETS), nicosulfuron (NCS), deltamethrin, cypermethrin, malathion, fenitrothion and α- and β-endosulfan (all from Sigma-Aldrich, Germany) were prepared in the adequate solvent and diluted with water.

The SPE minicolumn (30 mm × 4 mm i.d.) was constructed by packing a glass cylindrical tube with 200 mg of the sorbent Polygoprep 60-80 C18 (Macherey-Nagel, Germany) with a particle size of 63–100 μm, and the column was sealed with cotton frits at both ends.

2.2. Apparatus and software

The spectrofluorometric measurements were performed on a Jasco® FP 6500 spectrofluorometer. The fluid was pumped with a Gilson® Minipuls 3 peristaltic pump. NResearch® three-way solenoid valves were used to handle all of the solutions in the system.

Tygon® tubes were used in all of the pumping channels. Tubes with an i.d. of 1.14 mm were used for ACN, water and NaOH, whereas tubes with an i.d. of 1.30 mm were used for the sample and waste channels. The remaining tubing was made of Teflon® (0.5 mm i.d.).

The PDMC was constructed with Teflon® and was designed in our laboratory to prepare the solutions and perform the photodegradation and detection in the same location. Detection windows of the PDMC were made of quartz. A lab-constructed stirrer system was designed to improve the mixing inside the PDMC. An electronic actuator (EA) connected to a Pentium® 4 microcomputer was used to control the peristaltic pump, solenoid valves and stirrer system. A schematic diagram of the proposed FBA system is shown in Fig. 1.

The software used to control the FBA system was developed in the LabVIEW® 5.1 visual programming language. The developed software controlled the solenoid valves, peristaltic pump and magnetic stirrer.

2.3. Flow-batch analyzer system

The PMDC could be used to perform photodegradation (using the lamp in the spectrofluorometer) as well as the detection cell for spectrofluorometric measurements. As shown in Fig. 2, the lab-constructed PMDC was designed with two inlets for the incoming solutions and one output for emptying. The PMDC was equipped with two quartz windows at a 90° angle to each other, which were located at the bottom of the chamber to ensure that the photodegradation and detection could be accomplished for the final expected volume (0.90 mL). The inner volume was 1.5 mL. Additionally, a small magnetic stirring system was constructed using a cooler fan motor obtained from an Intel® microprocessor (DC12V, 0.06A) and placed below the PDMC.

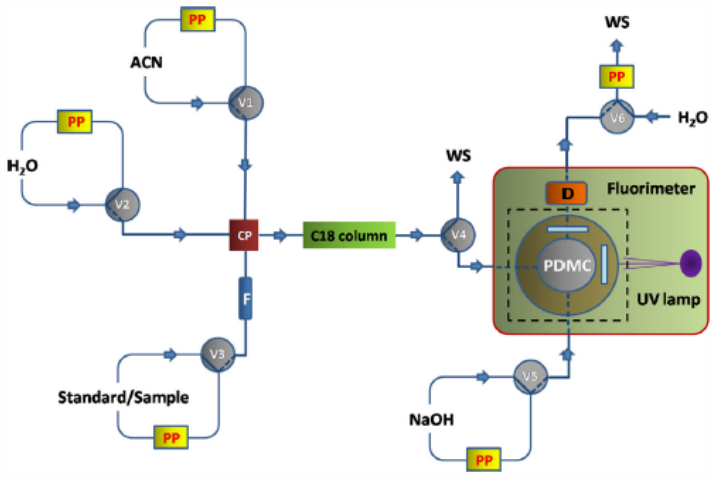

Fig. 1. FBA system for preconcentration, photodegradation and fluorescence determination of MSM. *V1–V6*: solenoid valves; F: syringe filter; CP: confluence point; PP: peristaltic pump; PDMC: photodegradation-detection mixing chamber; D: detector; and WS: waste.

To clearly explain the entire procedure, the process was divided into two parts: the *preconcentration procedure* and the *photodegradation-detection procedure*. Table 1 shows the flow rates and valve-switching time intervals for the complete procedure.

2.3.1. Preconcentration procedure

The preconcentration component of the system consisted of three channels: C_1, C_2 and C_3. These channels correspond to ACN, water and the sample, respectively. The flow rate of ACN and water was 1.22 mL min^{-1}; and the flow rate for the sample was 1.37 mL min^{-1}. The direction of the flow in each channel was controlled by a three-way solenoid valve. When the corresponding valve (i.e., V_1, V_2 and V_3) was switched OFF, the solution was recycled to the respective flask; when the valve was ON, the solutions were pumped to the minicolumn. Additionally, a filter (0.45 μm) was introduced in the C_3 channel to filter the sample prior to preconcentration in the C18 minicolumn. A fourth valve (V_4) was placed after the minicolumn. When this valve was in the OFF position (in the conditioning and loading steps), the solutions that passed through the minicolumn were directed toward the waste. When this valve was ON, ACN (eluting step) was directed toward the PDMC.

The C18 minicolumn was washed with 2.50 mL of ACN and 3.70 mL of water. Thus, the V_1 and V_2 valves were sequentially switched ON for 120 s and 180 s, respectively. After this time, 13.7 mL of the standard solutions or sample was pumped through the C18 minicolumn by switching the V_3 valve to the ON position, and the MSM was retained. The minicolumn was then washed with 2.50 mL of water by switching the V_2 valve to the ON position for 120 s.

Finally, the elution of MSM was performed using 0.50 mL of ACN. In this case, the V_1 valve was switched to the ON position for 46 s, that include pre-elution time (time required to displace the volume of water that reminds in column and channels) and the time for elution and filling the PDMC (25 s). During pre-elution time, V_4 remained in the OFF position while during elution and filling step the PDMC was switched ON to direct the flow to the PMDC.

2.3.2. Photodegradation-detection procedure

As mentioned previously, when the V_4 valve was in the ON position, the eluted MSM (in ACN) flowed to the PMDC. In this component, the system consisted of two channels: C_5 and C_6. These channels had flow rates of 1.22 mL min^{-1} and 1.37 mL min^{-1}, respectively. In C_5, NaOH flowed to the PMDC, and the V_5 valve controlled the direction of the flow. When this valve was OFF, the solution was recycled to the respective flask. When the valve was ON, the NaOH solution was pumped to the PDMC. The sixth valve (V_6) was used to control the emptying of the PDMC. Additionally, a flask containing water was connected to V_6, and this flask was used to ensure that the valve operated correctly when fluids were not carried from the PMDC to the waste.

Using this method, 0.50 mL of the eluted solution and 0.40 mL of 0.025 mol L^{-1} NaOH solution were pumped to the PDMC. The mixture was homogenized by stirring for 10 s. The UV lamp of the spectrofluorometer was then switched ON, and the photodegradation of MSM was performed at 240 nm for 30 s. After this step, the fluorescence signal was recorded (λ_{exc}=276 nm; λ_{emi}=385 nm). Finally, the V_6 valve was switched ON for 50 s, and the contents of the cell were aspirated toward the waste. The PDMC was cleaned between measurements. The PDMC cleaning procedure was performed by switching the V_1 and V_4 valves to the ON position and stirring for 60 s. As the PDMC was cleaned, the conditioning of the minicolumn started because V_1 was switched ON. The complete emptying of the PMDC was assured by switching the V_6 valve to the ON position for 65 s.

2.4. Sampling and sample preparation

Diverse waters that are used for irrigation and consumption by humans and animals were sampled from the region surrounding the city of Bahia Blanca located in the Buenos Aires province in southwest, Argentina. Two samples were collected from different locations in the Napostá Grande Creek, one sample was obtained from an artesian well used for human consumption located in Mayo Park, and tap water was collected from the laboratory. In addition, one of the samples was obtained from a well located in a field close to San Martin city, La Pampa province that is used for human and animal consumption. The samples were stored in the dark at 4 °C until the analysis was performed.

The water samples were pre-filtered using 80 μm Whatman™ filter paper to remove any sand and other possible major particles. The samples were then filtered on-line with a 0.45-μm Whatman™

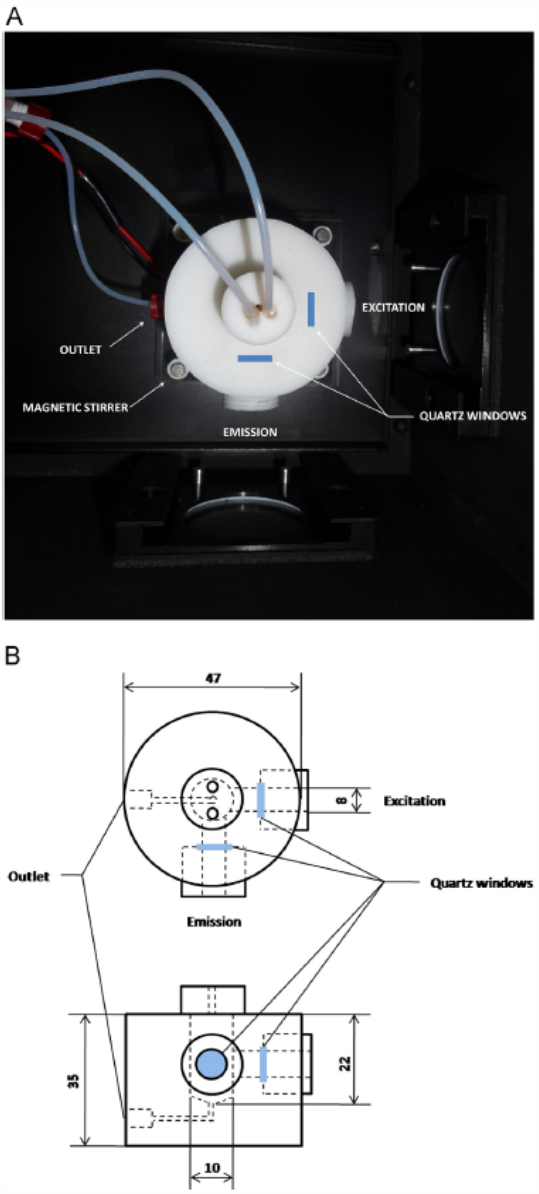

Fig. 2. (A) Top view of the PDMC placed instead of the cell holder in the spectrofluorometer. (B) Schematic diagram of the top and lateral views of the PDMC. Dimensions are expressed in mm.

syringe filter. The pH of the samples was between 6.0 and 7.0. Therefore, the pH of these samples was adjusted to 5.0 with 0.01 mol L^{-1} HCl before the samples were introduced into the SPE column. The samples were analyzed in triplicate.

For the recovery study, the samples were spiked at two concentration levels in the calibration range of 2.75–5.75 μg L^{-1} following an identical protocol. The recoveries were calculated according to the AOAC definition [27].

3. Results and discussion

3.1. Optimization of experimental conditions

The extraction and FBA variables were optimized based on a higher fluorescence signal and the repeatability of the measurements.

3.1.1. Instrumental conditions and photodegradation time

The instrumental conditions for the photodegradation step were optimized. To irradiate the samples, the slit width on the excitation monochromator was fixed to 20 nm, which is the maximum width in the employed instrument.

Because the degradation of MSM can be achieved with a germicide lamp (254 nm) [23], excitation wavelengths between 220 and 260 were tested, and the highest signal was obtained with an excitation wavelength of 240 nm. Because emission is not involved in this step, the emission wavelength was fixed to 385 nm to monitor the process. The photomultiplier tube (PMT) voltage was fixed to 475 V.

The irradiation time was optimized over the range of 20–60 s. As the photodegradation time increased, an increase in the signal was observed. The largest increase occurred between 20 and 30 s, and, a tendency to form a plateau has being noticed at higher irradiation times. As a compromise between the highest signal acquired and the analysis duration, 30 s was chosen as the optimum value.

After photodegradation was achieved, measurements of the photoproduct were performed by exciting the sample at 276 nm and recording the signal at 385 nm using a slit width of 10 nm for excitation and a slit width of 20 nm for emission.

3.1.2. Effect of chemical variables on fluorescence signal

According to previous studies [23], MSM photoproducts can be obtained in aqueous alkaline media (pH 12.0–13.0) in the presence of micellar solutions of SDS. As was mentioned in introduction part, MSM is presented in low levels in environmental samples and SPE was selected as preconcentration technique considering its intrinsic characteristics. In order to couple both, preconcentration and photodegradation, the eluate must be in the appropriate medium to form the photoproduct. Because MSM solubility at pH above 9.0 is high [28], an aqueous alkaline solution was considered to be used as eluent. However, silica is stable at a pH range between 2.0 and 8.0. Thus, considering the solubility of MSM in different organic solvents, the effect of two possible eluents, methanol and acetonitrile, on the fluorescent signal was studied.

Therefore, 1.25 mg L^{-1} MSM solutions were prepared in different aqueous:organic solvent mixtures and irradiated for 30 s. After irradiation, the fluorescence signal of the photoproduct was measured at λ_{em}=385 nm (λ_{ex}=276 nm). These signals were compared to the signal of a MSM solution that was prepared in 0.01 mol L^{-1} SDS (pH 12.0, adjusted with 0.1 mol L^{-1} NaOH) and irradiated for an identical duration. As shown in Fig. 3, similar signals were obtained using ACN as the organic solvent. Of the two solutions containing ACN, the solution prepared with SDS exhibited a higher signal than that prepared without SDS. Additionally, the inclusion of SDS generated bubbles in the system, which resulted in the need of an additional cleaning step. Therefore, a mixture of 0.01 mol L^{-1} NaOH in ACN was selected as the adequate media to achieve a compromise between the highest signal and the lowest cleaning time.

3.1.3. pH of the photodegradation medium

The pH of the photodegradation medium was optimized between 8.0 and 13.0. The pH was varied by increments of 0.5 units. To select the optimal value, the main criterion was to

Table 1
Valve switching time intervals and delivered volumes.

Valve switching time intervals, *s* (volumes, mL)	V_1	V_2	V_3	V_4	V_5	V_6
Filling channels	**20 (0.40)**	**20 (0.40)**	**20 (0.46)**	OFF	**20 (0.40)**	OFF
Column conditioning						
ACN	**120 (2.4)**	OFF	OFF	OFF	OFF	OFF
Water	OFF	**180 (3.7)**	OFF	OFF	OFF	OFF
Column loading – sample/standard	OFF	OFF	**600 (13.7)**	OFF	OFF	OFF
Column washing	OFF	**120 (2.4)**	OFF	OFF	OFF	OFF
Elution and transport to PDMC						
Pre-elution	**21 (0.43)**	OFF	OFF	OFF	OFF	OFF
Elution and filling PDMC	**25 (0.50)**	OFF	OFF	**25**	**20 (0.40)**	OFF
PDMC cleaning						
Emptying PDMC	OFF	OFF	OFF	OFF	OFF	**50**
Cleaning PDMC	**60 (1.2)**	OFF	OFF	**60**	OFF	OFF
Emptying PDMC	OFF	OFF	OFF	OFF	OFF	**65**
Column conditioning between samples						
ACN	**60 (1.2)**	OFF	OFF	OFF	OFF	OFF
Water	OFF	**180 (3.7)**	OFF	OFF	OFF	OFF

Delivered volumes of each solution, expressed in mL, are indicated between brackets.
ACN: acetonitrile; *V*: solenoid valves; and PDMC: photodegradation-detection mixing chamber.

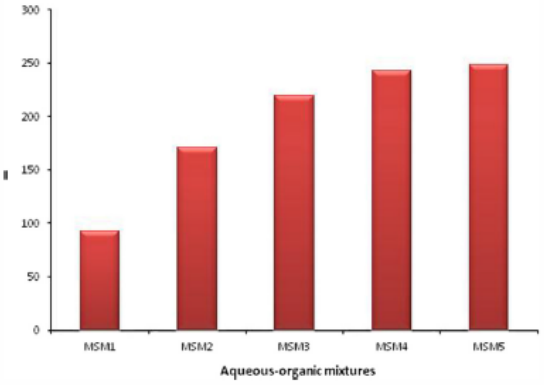

Fig. 3. Effect of different aqueous: organic mixtures used as irradiation-detection media. **MSM1**: 50:50 MeOH: 0.01 mol L^{-1}NaOH; **MSM2**: 50:50 MeOH: 0.02 mol L^{-1} SDS and adjusted to pH 12.0 with 0.1 mol L^{-1}NaOH, **MSM3**: 50:50 ACN: 0.01 mol L^{-1}NaOH, **MSM4**: 50:50 ACN: 0.02 mol L^{-1} SDS and adjusted to pH 12.0 with 0.1 mol L^{-1}NaOH, **MSM5**: 0.01 mol L^{-1} SDS adjusted to pH 12.0 with 0.1 mol L^{-1}NaOH. I: Fluorescence intensity.

achieve the highest signal of the MSM photoproduct with the best repeatability of the measurements. The optimum value was 12.5. At higher pH values, no signal increase was observed.

3.1.4. Extraction procedure

Sample preparation remains a bottleneck in most analyses because it is time consuming and because successive extractions with large amounts of solvents, several of which are toxic, are required in some cases. Additionally, evaporation to dryness and reconstitution is a common resource because of incompatibilities between the pretreatment and the separation or detection techniques that are intended to be employed.

In our method, a single SPE extraction was performed, allowing both sample preconcentration and cleanup without additional steps. Moreover, the extraction solvent was compatible with the photodegradation and detection steps.

Based on previous studies [15], C18 was selected as the sorbent material to perform the sample cleanup and preconcentration of the MSM. The SPE minicolumn was packed with 200 mg of the sorbent. The minicolumn was conditioned with 2.50 mL of ACN (optimized in the range of 1.20–3.70 mL) followed by 3.70 mL of water (1.20–6.10 mL) at a flow rate of 1.22 mL min^{-1}.

The parameters that affected the online preconcentration and elution of MSM were studied. The behavior of the SPE minicolumn was evaluated in terms of sample volume and loading time. The main criterion was to achieve the detection of low concentrations of the analyte with a short analysis time. Thus, 13.7 mL of the sample (1.0–20.0 mL) was passed through the minicolumn at a flow rate of 1.37 mL min^{-1}. Above this volume, the recoveries of the analytes slightly decreased.

As was described in Section 3.1.2, ACN was the solvent with the smallest effect on the photodegradation of the analyte; thus, ACN was selected as the eluting solvent. The elution volume was also studied, and because quantitative recoveries were obtained using 500 μL, this volume was used in the following experiments. Regarding the ratio ACN: NaOH studied in Section 3.1.2, it was noted that the volume of the alkaline solution could be reduced without any change in the optimum pH. Therefore, the dilution of the eluate could be diminished and a 55:45 ACN: NaOH ratio was selected for further experiments. Considering the elution volume, an enrichment factor of 27 was achieved using this method.

3.1.5. Stirring time

When the solutions were delivered to the PDMC, it was necessary to stir the mixtures to obtain good signal reproducibility. Therefore, different stirring times between 3 and 15 s were tested. The best results with respect to signal height and repeatability were obtained when the solutions in the PDMC were stirred for 10 s. The analytical signal did not improve with longer stirring times.

3.2. Analytical performance

The analytical performance of the proposed FBA method was evaluated in terms of the calibration range, sensitivity (evaluated as limit of detection (LOD) and limit of quantitation (LOQ)), sample throughput, reproducibility (expressed as relative standard deviation) and accuracy (expressed as the recovery percentages of spiked samples).

3.2.1. Analytical curve, LOD, LOQ, RSD and sample throughput

Using the proposed FBA method and the optimized values for the physical and chemical parameters, the calibration curve for the MSM determination was constructed over the range of 1.00–7.20 μg L^{-1} (five points). The regression equation was $A = (72.5 \pm 0.7)$ [MSM μg L^{-1}] + (-61.3 ± 3.1) with a correlation coefficient of 0.9995.

LOD was 0.28 μg L^{-1} and was calculated as $3.3S_0$, where S_0 represents the standard deviation corresponding to the predicted concentration of a blank sample [27]. LOQ was 0.85 μg L^{-1} and was calculated as the concentration corresponding to 10 times S_0. The relative standard deviation (RSD %) was 2.0, and this value was obtained from six replicate runs with a 4.25 μg L^{-1} MSM solution.

The sample throughput of the photodegradation and detection steps was 72 h^{-1}, and the sample throughput for the entire procedure, including the SPE procedure and washing steps, was 3 h^{-1}.

It is important to highlight that the linear range could be extended by linear regression, but considering that MSM is presented in low concentrations in water sources, samples could be easily diluted to employ the constructed analytical curve.

3.2.2. Selectivity

The effect of the presence of other pesticides in water samples on the analysis of MSM was examined. For this study, other sulfonylurea herbicides and pesticides with other structures that are employed in Bahía Blanca's agriculture region were tested (Table 2) to include different chemical groups of pesticides.

The entire procedure, including the pre-concentration step, was performed. To reduce the analysis time, the MSM solution and the standard solutions of each possible interfering compound were prepared at a higher concentration (0.01 μg mL^{-1}), loaded onto the minicolumn for a shorter time (5 min), and then subjected to the procedure described in Section 2.3.1. No signal was observed for the standards of the possibly interfering pesticides, indicating that these were not retained on the minicolumn or eluted with ACN or that no fluorescent product was produced or detected after irradiation under the conditions described above.

Concerning sulfonylurea herbicides, photodegradation varying pH and irradiation time was studied, and the kinetics of degradation was different for each tested analyte. Therefore, the optimal conditions for MSM photodegradation were inappropriate for the tested sulfonylurea herbicides.

The identical procedure was repeated with mixed solutions of MSM and each of the examined substances. The ratios of interferent:MSM were 1:1, 10:1 and 100:1, and the MSM concentration was maintained constant. The results are given in Table 2.

Table 2
Pesticides studied as possible interferences for MSM determination.

Pesticide family	Interferent	Tolerance ratio (interferent:MSM)
Sulfonylureas	Ethoxysulfuron methyl	100:1
	Sulfometuron methyl	30:1
	Chlorsulfuron methyl	3:1
	Nicosulfuron	100:1
Pyretroids	Deltametrin	100:1
	Cypermetrin	100:1
Organophosphates	Malathion	100:1
	Fenitrothion	100:1
Organochlorines	α-Endosulfan	100:1
	β-Endosulfan	100:1

The change in the signal due to the presence of the tested pesticide did not exceed the RSD value (2.0%), indicating measurement reproducibility. Two exceptions were observed: the signal of MSM recorded in the presence of higher concentrations of SMM or CSM was significantly higher than that obtained for MSM alone. SMM did not appear to interfere with the MSM signal for SMM:MSM mixtures at ratios below 30:1; however, at a ratio of 50:1, the signal was approximately 2.4-fold higher than the average value measured for MSM. At a ratio of 5:1, the presence of CSM resulted in an approximately 1.5-fold increase in the signal. Because regulations allow the use of sulfonylurea herbicides at similar concentrations to MSM, these pesticides should not be a source of difficulties in real sample analysis.

3.3. Analysis of real samples

The proposed method was tested through the preconcentration and determination of MSM in water samples. Although the samples were collected from different potentially contaminated water sources in which pesticide contamination is expected because of agricultural techniques, MSM residues were not detected. Thus, the samples were spiked as described in 2.4. For both concentration levels, Table 3 shows the added concentrations, the value determined for each sample, and the calculated recovery percentage. The recovery values ranged from 94.7% to 109.8% and were acceptable for determining MSM at μg L^{-1} levels.

The allowable amount of MSM in drinking water is not stipulated by Argentina's regulations; only a maximum concentration level for total pesticides (100 μg L^{-1}) has been established [29]. However, the Drinking Water Directive in the European Union (EU) established a maximum concentration of 0.10 and 0.50 μg L^{-1} for individual and total pesticides, respectively [30]. Because drinking water is derived from a variety of sources, including rivers, reservoirs and groundwater, it can regularly contain trace levels of pesticides in excess of the drinking water standards [31]. Therefore, considering Argentina´s regulations and the fact that pesticides may be present in higher amounts in raw waters, the proposed method is adequate for determining the MSM level in the analyzed samples, with the exception of tap water, which requires higher sensitivity.

3.4. Comparison with previous methods

Table 4 shows different analytical methods that have been proposed for the determination of the MSM concentrations in water samples. The methods are compared by considering the

Table 3
MSM determination in spiked real water samples applying the proposed FBA method.

Sample	Added (μg L^{-1})	Found (μg L^{-1})	Recovery (%)
A[a]	2.75	2.80 ± 0.01	101.8
	5.75	5.55 ± 0.01	96.4
B[b]	2.75	3.02 ± 0.02	109.9
	5.75	5.44 ± 0.01	94.7
C[a]	2.75	2.85 ± 0.01	103.6
	5.75	6.06 ± 0.01	105.4
D[c]	2.75	3.02 ± 0.01	109.8
	5.75	6.25 ± 0.01	108.7
E[d]	2.75	2.69 ± 0.02	97.8
	5.75	5.61 ± 0.01	97.6

[a] A and C, Naposta Grande creek.
[b] B, San Martín, La Pampa, water well.
[c] D, Water upwelling.
[d] E, Tap water.

Table 4
Brief comparison of the analytical methods for MSM determination in water samples.

	GC/ECD [13]	HPLC [15]	UHPLC [17]	FIA-PIF [23]	FIA-PIF [24]	FBA
Automation	No	Yes	Yes	Partial	Yes	Yes
Pretreatment	SPE	SPE	SPE	LLE	SPE	SPE
Sample volume (mL)	500	10.0	1.5	25.0	2.06	13.7
Eluent volume (mL)	–	0.03	–	–	Continuous	0.50
Enrichment factor	100	330	125	5	–	28
Detection technique	GC/ECD	LC–ESI–MS	MS–MS	Fluorescence	Fluorescence	Fluorescence
Multianalyte[a]	Yes(2)	Yes(6)	Yes(16)	No	Yes(2)	No
Complexity of analysis	High	Medium	Medium	High	Low	Low
Linear range ($\mu g\ L^{-1}$)	0.50–50.0	0.01–1.00	0.007–0.300	0.10–39.0	11.0–400	1.00–7.20
LOD ($\mu g\ L^{-1}$)	0.01	0.03	0.002	0.10	3.30	0.28
LOQ ($\mu g\ L^{-1}$)	0.05	0.09	0.007	Not informed	11.0	0.63
RSD (%)	$\leq$ 12	6.0	8.0[c]	3.7	2.4	2.0
Throughput (h^{-1})	8	3	3.75[b]	80	14[b]	3[b]

[a] Between brackets, amount of determined analytes.
[b] Included preconcentration step.
[c] For 0.01 $\mu g\ L^{-1}$.

automation of the entire procedure, the preconcentration step, the complexity of the analysis and the analytical parameters.

Lower detection limits are achieved with chromatographic methods. However, in most chromatographic analyses, time-consuming off-line preconcentration processes that include a higher number of steps are required. In the FIA-PIF method [23], satisfactory analytical parameters are obtained, but a tedious, long off-line preconcentration is performed involving consumption of high amounts of toxic solvents (such as dichloromethane) due to successive liquid–liquid extractions to clean up the sample. On the other hand, an automated FIA system with SPE preconcentration was proposed, but the preconcentration of MSM photoproducts instead of MSM was achieved [24].

The proposed FBA method is a simple and fully automated system with short SPE clean-up and preconcentration steps followed by a photodegradation process and the detection of the MSM fluorescent active photoproduct. Additionally, the entire procedure (including the SPE preconcentration step) was performed on-line. The FBA system required a low sample volume (13.7 mL) and a low ACN volume (6.0 mL, including the volume required for the conditioning of the minicolumn and washing of the PDMC). Compared with chromatographic methods, the FBA method showed a high sample throughput and substantial cost savings because of the minimal consumption of reagents and the low equipment cost. Additionally, the LODs were of the same order of magnitude as the majority of those reported previously in the literature with the exception of the LODs reported with the HPLC method [15] and the UHPLC MS/MS method [17].

4. Conclusions

The developed FBA method successfully determined the MSM concentration in real surface water samples that were spiked with MSM. The novelty of the proposed configuration allowed the photodegradation and detection processes to occur in the same chamber without any loss or dispersion of the sample. Additionally, the inherent advantages of using a FBA system (e.g., low consumption of reagents, decrease of waste generation, and reproducibility) were compounded with the advantages of using UV light as the decomposition agent. The combination of these two processes resulted in a significant decrease in the amount of the chemicals employed in the analysis. This decrease in consumption is one of the most important principles in green chemistry.

Furthermore, a simple SPE procedure was coupled to the FBA system, allowing the realization of the sample clean-up and analyte extraction and preconcentration more rapidly without any additional steps. This process obtained a satisfactory enrichment factor of 27. Moreover, the inclusion of an organic solvent did not significantly affect the formation of the photoproduct or the fluorescent signal and thus facilitates the completely on-line determination of the concentration through the simple fully automated system.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.05.024.

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5.5 PUBLICATION 5

P. Bolinová, ***I. Šrámková,*** H. Sklenářová, C.C. Acebal, B.S. Fernández Band, P. Solich,
A study of the effect of organic solvents on the fluorescence signal in a sequential injection system.
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A study of the effect of organic solvents on the fluorescence signal in a sequential injection analysis system†

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A study of the effect of different organic solvents and their mixtures with water on the fluorescence intensity of two model compounds (quinine sulphate as a naturally fluorescent substance and metsulfuron methyl with fluorescent properties upon UV irradiation) was carried out in a sequential injection analysis system.

Introduction

Fluorometry is one of the most used detection techniques in a number of methods based on flow systems[1–5] due to its higher sensitivity in comparison with spectrophotometric detection, simplicity of instrumentation and lower cost of equipment in comparison with more sophisticated detectors, such as atomic absorption or mass spectrometry, and feasibility of coupling to a sequential injection analysis (SIA) system. Fluorescence depends, among other parameters, on the solvent used not only in terms of emission wavelength but also in terms of intensity of the measured signal.[6] The effect of different solvents on fluorescence shift and intensity in the case of several analytes, *e.g.* metallophthalocyanines,[7] citrinin[8] or carbamate and indol compounds,[9] was studied. The limitation in the wider use of fluorometry is the number of naturally fluorescent substances. However, fluorescent properties can be evoked *e.g.* by chemical reactions or irradiation which enable the use of fluorometric detection for determination of a large number of analytes. Moreover, induced fluorescence can also lead to increased method selectivity.

Recently, the use of organic solvents in flow systems has been increasing, since many novel applications, such as different modes of online sample pre-treatment automated in flow systems, mainly based on sequential injection analysis (SIA) principles,[10–12] require the use of such solvents. Moreover, several ways of handling the combination of organic solvents together with aqueous solutions in one flow system were proposed, such as wetting the Teflon tubes with organic solvents,[13] using a de-bubbling device in front of the detection cell to eliminate bubbles arising from mixing aqueous and organic solutions,[14] or modified flow systems to separate organic and aqueous solution handling units in the case of a dual-valve system (DV-SIA).[11] In this way, possible disturbing phenomena (that could arise from adsorption of organics to lipophilic Teflon tubes and could cause noise in signal readout because of different optical properties of different solvents) are not considered as problems but as advantages of employing organic solvents even in a SIA system. Also, due to the miniaturization related to flow techniques, only small volumes of organic solvents are commonly used, so the principles of green chemistry are adopted. However, the compatibility of the carrier stream with the sample solution must be taken into account in the development of a flow method to exclude any unwanted effects in the system, such as carryover or Schlieren effect,[15] resulting from different chemical and physical (optical) properties.

Flow techniques, especially SIA, are characterized by high reproducibility ensured by computer-control of all steps of the operating protocol. This makes it a convenient tool for automation of solution handling so that the sample treatment prior to or during analysis, including derivatization by a reagent or even by light (UV irradiation), can be performed on-line. Automation of UV irradiation in a flow system prior to fluorescence detection of the irradiation product has been reported elsewhere.[16]

In this work, the effect of several organic solvents on the fluorescence signal of two model compounds in an SIA system was studied. Quinine sulphate is a well-known substance used for treatment of malaria. As a naturally fluorescent compound in acidic pH conditions, it is used as a standard to study the fluorescence quantum yield under different conditions.[17,18] Methods based on SIA were reported for either determination of quinine[19] or using quinine as a sensitizer of photo-oxidation[20] or chemiluminescence[21] in determination of other substances.

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† Electronic supplementary information (ESI) available: Figures showing the effects of different solvents, pH adjustment and UV decomposition on the MSM fluorescence intensity without and after pH adjustment. See DOI: 10.1039/c4ay01977f

Automated flow systems could involve on-line UV irradiation to decompose the original analyte to a fluorescent product that is determined afterwards (*e.g.* thiamine[22]). In such applications the UV irradiation time should be short enough to obtain measurable fluorescence without the need to reach the steady state so that the advantage of a fast flow method is not neglected by a long irradiation time. Metsulfuron methyl, a sulfonylurea selective herbicide, shows fluorescence upon UV irradiation and this property was used in several analytical methods for MSM determination, even in flow systems.[16,23] Here, it was chosen as a model compound representing molecules which are not naturally fluorescent but can be converted into a fluorescent photoproduct. The fluorescence of such photoproducts can vary in different solvents.

A thorough study of different conditions influencing fluorescence of the model compounds was performed in this work. The studied parameters such as organic solvent, pH adjustment, irradiation time, and wavelength of UV light were tested off-line to get information concerning the effect of the mentioned parameters on the decomposition pathway and the possibility to transfer such decomposition to an on-line system (the key parameter is the time of the UV irradiation step) in order to obtain expected benefits in terms of determination sensitivity in compromise with quick analysis in the flow system. The compatibility of the carrier stream with the solvent used for sample preparation was studied with respect to the flow conditions and compared in terms of fluorescence signal intensity and repeatability of measurements. The study intends to give a good starting point when choosing suitable conditions for development of analytical methodologies based on fluorescence measurements in an SIA system, including the use of organic solvents for sample pre-treatment or other applications.

Experimental

Reagents

Standards of quinine sulphate (QS) and metsulfuron methyl (MSM) were purchased from Sigma-Aldrich (Prague, Czech Republic) and the different solvents tested were: acetone and isopropanol (Penta, Czech Republic); acetonitrile, methanol, ethanol and toluene (Sigma-Aldrich, Czech Republic); dichloromethane (Fluka, Switzerland), and trichloromethane (Lachema, Czech Republic). Demineralized water (Merck-Millipore, Czech Republic) was used as the main carrier stream if not specified otherwise, and was also tested as a solvent for fluorescent substances. Phosphoric acid and sulfuric acid were purchased from Penta (Pardubice, Czech Republic).

Apparatus

Fluorescence detection was studied in the flow system based on sequential injection analysis (SIA) principles. The setup corresponded to a commercially available FIAlab® 3500 system (FIAlab® Instrument Systems Inc., USA) with a syringe pump (syringe reservoir of 5 mL), a central eight-port Cheminert selection valve and 0.50 mm i.d. PTFE tubing used for all connections (Fig. 1). Fluorescence detection was scanned in the flow cell (3.0 mm optical length) of the PMT detector (FIAlab® Instrument Systems Inc., USA), scanning the whole spectrum without using excitation or emission filters. A UV lamp (D1000 CE, Analytical Instrument Systems, Inc.) was used for excitation. The latest version of the FIAlab® software (version 5.9.312) was employed for system control and data acquisition.

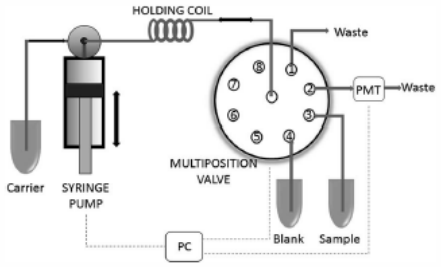

Fig. 1 Set-up of the sequential injection analysis system.

UV decomposition was carried out off-line using two wavelengths – 254 and 366 nm (UV lamp, Camag, Switzerland, 50 Hz, 220 V).

Measurement procedure

QS and MSM solutions in the respective solvent or a mixture of solvents were prepared and aspirated into the SIA system using a 50 μL sample zone that was transported to the detection flow cell at a flow rate of 80 μL s^{-1} and the fluorescence signal was scanned using 100 ms for integration time and 8 Hz as a detector frequency. To obtain statistically relevant data, all samples were measured in triplicate and the average value of peak heights was used for the final evaluation.

QS at a concentration of 10 mg L^{-1} was tested using water as a carrier stream for fluorescence detection of solutions prepared in methanol, ethanol and isopropanol. Then the carrier stream was changed to pure organic solvents to avoid the effect of mixing the aqueous and organic phases. These tests were carried out with methanol, ethanol, isopropanol and dichloromethane as the carriers. Dichloromethane was not tested in combination with the aqueous carrier because of the immiscibility of these two solvents.

MSM fluorescent properties were tested with respect to different solvents following the study published by Caselli[24] where UV decomposition and an increased fluorescence of degradation products were discussed. The tested concentration of MSM solutions was 25 mg L^{-1}. The solutions were prepared in: water, acetone, mixtures of water and acetonitrile and trichloromethane. Two different pH values were tested for each solvent, pH 2 and pH 7, adjusted by concentrated phosphoric acid. UV decomposition was carried out off-line using a low-pressure mercury arc lamp and 15 mL of each solution were placed in a beaker at a distance of 15 cm from the mercury lamp. The effect of the UV radiation on the fluorescence (detection) intensity of the obtained decomposition products was observed at intervals of 15 min for periods of 60–120 min.

Results and discussion

Effect of organic solvents on fluorescence detection of QS

At first a calibration of QS determination in the SIA system under the described conditions was performed using aqueous solutions in the concentration range of 1–100 mg L^{-1} prepared in 0.05 M sulfuric acid with water as a carrier stream. The linear part of the calibration was found in the range from 1 to 10 mg L^{-1} with a correlation coefficient of 0.9966 using baseline subtraction. The effect of dilution of the acidified solutions in water without pH adjustment used for transport of the QS zone to the detection flow cell was not observed. The calibration curve for higher concentrations of QS (10–100 mg L^{-1}) showed logarithmic shape because of concentration quenching of the fluorescence signal. Thus the highest concentration of the linear range was chosen for further experiments to evaluate the effect of organic solvents expressed as the difference in the fluorescence intensity obtained under different conditions.

The effect of organic solvents was studied for combinations of different solvents used for preparation of acidified QS solutions at the 10 mg L^{-1} level and water was used as a carrier stream. The evaluation was based on the difference of fluorescence intensity between aqueous and organic solutions together with comparison of repeatability of these measurements expressed as standard deviation (SD) values.

The results given in Fig. 2 show that in the case of an aqueous carrier stream, the aqueous QS solution was the most fluorescent. What is more, the increased SD values for organic solutions showed problems with mixing of aqueous and organic phases in the flow system. The repeatability of aqueous solution measurements (0.53%) compared to the highest RSD values for methanol solution (11.73%) was the main reason for the next assay comprising modification of the carrier stream. The solvent used as a carrier stream was identical with the solvent used for preparation of the respective QS solution and the sulfuric acid content was kept at the same level as in the aqueous solution. The obtained results are summarized in Fig. 2. Baseline subtraction of the respective blank solution was applied for evaluation of each measurement. In this study, the fluorescence intensity of acidified isopropanol solution was found to be higher than in aqueous solution and also the methanol solution revealed comparable fluorescence. The observed repeatability was in an acceptable range for all solvents (0.21% for isopropanol–2.75% for ethanol).

A very large difference of QS fluorescence in different solvents showed the effect on the fluorescence intensity that could highly influence fluorescent properties even in the flow systems. Compared to the aqueous solution, dichloromethane solution expressed just 8.93% (because of fluorescence quenching caused by chlorine atoms) and ethanol 63.50% of the fluorescence intensity while QS prepared in isopropanol had 116.89% of the original fluorescence of the aqueous solution. In the case of QS fluorescence, some of the tested organic solvents slightly increased the measured intensity. Their consumption, however, could be considerably large, which is a serious disadvantage. From another point of view, the use of an aqueous carrier stream can decrease their consumption. Nevertheless, the combination of aqueous and organic phases led to increased RSD values which affected the measurement repeatability. Thus a compromise should be always found in optimization of flow fluorescent measurements to obtain not only the desired sensitivity but also repeatability and low consumption of organic solvents that corresponds to the green chemistry requirements.

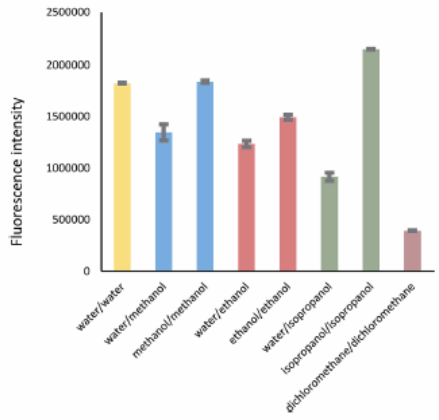

Fig. 2 Fluorescence intensity and standard deviation of QS measured in the combination of different solvents – effect of the carrier stream/acidified QS solution composition.

Effect of UV decomposition of MSM solution in different organic solvents

In the following experiments, MSM was chosen to study the influence of organic solvents on the UV decomposition that is commonly used in the case of light-induced fluorescence determinations. At first, the fluorescence of MSM aqueous solution at a concentration of 25 mg L^{-1} was measured, using water as a carrier stream. Fig. 3 shows the effect of UV (254 nm) decomposition applied to a solution without and with pH adjustment (pH 2) following the literature[24] where mainly the effect of UV decomposition was stressed over the effect of pH. In

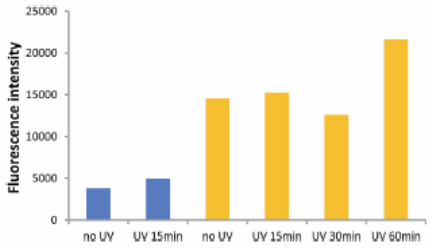

Fig. 3 Fluorescence intensity of MSM in aqueous solution – blue (without pH adjustment) and yellow (pH 2).

the tested solution, UV decomposition increased the fluorescence relatively slowly. This is not advantageous for flow systems where this step could be carried out even on-line using a reaction tube coiled and fixed around an UV lamp. Also, a much higher effect of pH adjustment on the fluorescence intensity was found which was demonstrated by a 3.86-times higher signal for acidified solution compared to a signal of the solution without a pH adjustment.

In the next experiments, organic solvents or mixtures tested for UV decomposition were used as carrier streams. In the case of acetone solution, UV decomposition showed an effect on the solution without pH adjustment and also the effect of prolonged decomposition was visible (2-times higher fluorescence was obtained after 30 min of degradation). The mixture of water and acetonitrile that was described as the most suitable in the literature[24] was tested in different ratios (7 : 3, 1 : 1, 3 : 5 and 1 : 9), with water as a carrier stream including the pH adjustment and UV decomposition. Comparison of the obtained results for the ratio of 7 : 3 is shown in Fig. 4 where a significant difference caused by pH adjustment (4.5-times increased fluorescence) was proved. However, the UV decomposition had only a slight effect on the fluorescence intensity – even after 60 min of UV irradiation at 254 nm, the measured solution showed a comparable signal to the original one.

Then the UV wavelength was changed to 366 nm to test the effect of wavelength on the decomposition step but only a decrease of the fluorescence intensity in the period of 30 min by 18.66% was observed. The effect of UV decomposition that caused a decrease in the measured fluorescence was similar for solutions without pH adjustment using 254 nm and with acidified solutions in the case of 366 nm. The other solutions were not affected by the applied UV degradation. It was found that pH adjustment affected the fluorescence more significantly, since an 8-times higher fluorescence signal was observed. This corresponded to the higher effect of ionization of the MSM molecule which is related to increased solubility but suppressed fluorescent properties. The other previously mentioned ratios of water and acetonitrile did not show any fluorescence either for original solutions or after UV decomposition.

The last solvent tested was trichloromethane in which a higher effect of acidification of MSM solution was also proved.

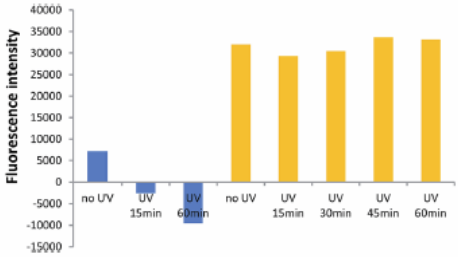

Fig. 4 Fluorescence intensity of MSM in the mixture of water and acetonitrile (ratio of 7 : 3) – blue (without pH adjustment) and yellow (pH 2).

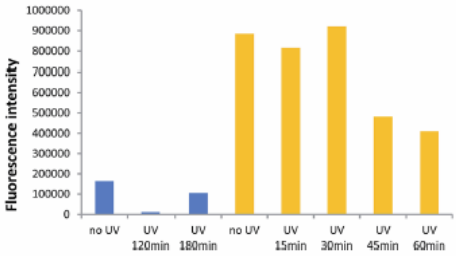

Fig. 5 Fluorescence intensity of MSM in trichloromethane – blue (without pH adjustment) and yellow (pH 2).

Fig. 5 shows that 10-times higher fluorescence could be found for acidified solutions prepared in this solvent and again only a small effect of UV decomposition was observed leading even to lower signals. Comparing the same conditions with aqueous solutions in the case of pH adjustment, 54-times higher and for pH adjustment with UV decomposition using 30 min at 254 min even 66-times higher fluorescence was found. Since the most intense fluorescence was obtained with trichloromethane solution, calibration using this solvent with pH adjustment was also measured with linearity in the range of 5–25 mg L^{-1} and a correlation coefficient of 0.999. Limits of detection and quantitation were calculated based on the 3-times and 10-times baseline noise and were found to be 1.52 and 5.13 mg L^{-1}, respectively.

Transfer of the UV decomposition step to an on-line procedure in the flow system should provide a compromise between benefits of determination sensitivity and analysis time (sample throughput). Although in this tested determination simple pH adjustment showed lower fluorescence signal, the automated determination of MSM is not expected to be prolonged by 30 min decomposition. Thus fast and simple SIA determination was found to be more suitable for practical applications in this example.

The detailed comparison of fluorescence intensity obtained in different organic solvents and after different degradation times is given also in ESI 1–3.†

Conclusions

The effect of organic solvents that are commonly used in flow systems based on sample pre-treatment automation (extraction techniques) was studied in the SIA system. Mainly carrier stream composition was taken into account and a combination of organic solvents used for sample preparation with aqueous carriers was tested and compared with respect to fluorescence intensity and measurement repeatability. Varying fluorescence was found for the respective combinations and decreased repeatability (increased RSD values) was proved in the case of combinations of small volumes of samples in organic solvents aspirated into the aqueous carrier stream.

Testing organic solvents for UV irradiation as preliminary information for transfer of this step into the on-line procedure

in the flow system was carried out, too. In this case, different organic solvents show very big differences that could be expected because of different pathways of decomposition. Thus the choice of solvent could be the key factor to get a highly fluorescent decomposition product for detection. As the other important parameter, the time of irradiation should be optimized to get reasonably high sensitivity together with acceptable analysis time.

Crucial parameters should be studied in detail during optimization of fluorometric measurements in flow systems using organic solvents to take advantage of all benefits of such a methodology, such as sensitivity, speed and precision of analysis.

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5.6 PUBLICATION 6

I. Šrámková, P. Chocholouš, H. Sklenářová, D. Šatínský,

On-line coupling of micro-extraction by packed sorbent with sequential injection chromatography system (MEPS-SIC) for extraction and determination of betaxolol in human urine.

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On-line coupling of Micro-Extraction by Packed Sorbent with Sequential Injection Chromatography system for direct extraction and determination of betaxolol in human urine

(Short communication)

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Abstract

A novel approach for automation of Micro-Extraction by Packed Sorbent (MEPS), a solid phase extraction technique, is presented, enabling precise and repeatable liquid handling due to the employment of sequential injection technique. The developed system was used for human urine sample clean-up and pre-concentration of betaxolol before its separation and determination. A commercial MEPS C-18 cartridge was integrated into a SIChrom™ system. The chromatographic separation was performed on a monolithic High Resolution C18 (50 × 4.6 mm) column which was coupled on-line in the system with micro-extraction using the additional selection valve. A mixture of acetonitrile and aqueous solution of 0.5% triethylamine with acetic acid, pH adjusted to 4.5 in ratio 30:70 was used as a mobile phase for elution of betaxolol from MEPS directly onto the monolithic column where the separation took place. Betaxolol was quantified by fluorescence detector at wavelengths λ_{ex} = 220 nm and λ_{em} = 305 nm. The linear calibration range of 5 – 400 ng mL^{-1}, with limit of detection 1.5 ng mL^{-1} and limit of quantification 5 ng mL^{-1} and correlation r = 0.9998 for both the standard and urine matrix calibration, respectively, were achieved. The system recovery was 105.8 ± 4.8; 100.4 ± 4.1; 107.6 ± 0.9% for three concentration levels of betaxolol in ten times diluted urine - 5, 20 and 200 ng mL^{-1}, respectively.

Keywords

Micro-Extraction by Packed Sorbent (MEPS); Automation; Sequential Injection Chromatography (SIC); Betaxolol; Liquid Chromatography; Fluorimetry

1. Introduction

Downscaling the dimensions of solid phase extraction cartridge (SPE) led to the introduction of Micro-Extraction by Packed Sorbent (MEPS) by Abdel-Rehim in 2004 [1]. The achieved

advantage was the reduction of sorbent quantity, solvents consumption, waste production and sample volumes, labour-intensity, time and costs of the procedure, considering also that MEPS can be used repeatedly (up to 100x for urine samples). Typically, MEPS is performed manually with the cartridge mounted on the Hamilton syringe or semi-automated using special engine-driven syringe. It is crucial to keep the parameters such as volumes of the sample/solvents and flow rate constant since a minor change in conditions (different operator, flow rate fluctuation) can affect the recovery and repeatability of the method [2, 3]. However, constant conditions might be tedious to accomplish when performing manually and a well-trained operator is required for this task. Even though the MEPS procedure requires less time than SPE due to smaller volumes to handle and no solvent evaporation and sample reconstitution, manual processing of samples can take significant part of the total analysis time. Another shortcoming of the manual performance is the need of repeated loading when large volume samples are to be handled. Volumes of only up to 500 µL can be loaded at once and multiple repetitions of this step can prolong significantly the total analysis time [4].

Taking into account the advantages of MEPS, several attempts of automation of this technique were recorded lately to overcome the drawbacks of the manual handling. In the works of Abdel-Rehim et al. and Said et al., programmable autosampler of a MEPS-LC-MS system was used for handling the MEPS process and subsequent injection on chromatographic column [5, 6]. Attempts of direct coupling of MEPS with HPLC, GC or MS were reported in the literature [7]. Nevertheless, the automation and on-line coupling of MEPS with analytical instrumentation remains a most critical step in the method development [4].On the other side, the modern and effective approaches of on-line sample clean-up using SPE procedure is presented by on-line SPE-HPLC based either on the column-switching or coupled column technique. This methodology is widely used for the analysis of trace level compounds in complex samples because of its potential for on-line sample extraction and automation [8].

Low pressure flow techniques represent a powerful tool for automation of both liquid and solid phase-based sample pretreatment due to their versatility, as it was discussed in several reviews [9-11]. Sequential Injection Chromatography (SIC) was introduced as a low pressure separation system in 2003 by Šatínský et al. [12]. This technique combines the principles of Sequential Injection Analysis with chromatographic separation and gained popularity due to the possibility of flow speed and direction programming (including stop-flow), and reduced solvents consumption. The SIC developments enabled the use of not only monolithic but short core-shell particle columns, too [13]. On-line coupling of the sample pretreatment with separation was also carried out in a SIC system e.g. for preconcentration and SPE of sulfonamide antibiotics from water solutions [14].

In this work, the advantage of precise and repeatable sample handling inherent to flow techniques was explored for automation of miniaturized sample pretreatment and separation. MEPS cartridge was coupled directly into a SIC system, aiming for a fully automated, highly reproducible, labour reducing on-line sample clean-up – separation procedure. The potential, advantages and limitations of this approach was demonstrated by determination of betaxolol in human urine with fluorescence detection.

Betaxolol is a cardio-selective β-adrenergic blocking agent used in treatment of cardiovascular diseases [15, 16] and intraocular hypertension [17]. Analytical methods for betablockers determination in biological samples include liquid chromatography with UV or fluorescence detection, respectively [18-20], TLC [21] or voltammetry [22]. Due to the complex matrix of biological samples, a pretreatment procedures such as LLE, or SPE are required for β-blockers analysis [23]. Monitoring of betaxolol in bio-fluids is important due to the narrow drug therapeutic range. To the best of our knowledge, this is the first report of coupling MEPS and chromatographic separation in a SIC system.

2. Material and methods

2.1. Equipment (MEPS - SIC system)

SIChrom™ system (FIAlab® Instrument Systems Inc.,USA) comprising a Sapphire™ S17 PDP piston pump (4 mL volume) and an eight-port selection valve (MV 1) together with second Cheminert eight-port selection valve (MV 2) (both Valco Instruments Co., USA) were used to assembly the MEPS-SIC system as shown in Fig. 1. The pump was connected with central port of MV1 with a 1.2 mL holding coil made of 0.75 mm i.d. PEEK tubing. The HyperSep C-18 MEPS, particle size 40-60 µm, pore size 60 Å (Thermo Scientific, MEPS Products, cat. No. 60308-407) cartridge was integrated in the system in such way that its inlet was connected to port 3 of MV 1 and its outlet was connected to the central port of MV 2. Chromolith® HighResolution RP18 column (50 x 4.6 mm) (Merck, Czech Republic) was mounted to port 2´ of MV 2 by its inlet and to the detector by its outlet. PEEK tubes (0.25, 0.5 and 0.75 mm i.d.) were used for connections in the system. The system was coupled to a RF-10A XL (Shimadzu Corporation, Kyoto, Japan) fluorescence detector. The detection wavelengths were set to 220 nm for excitation and 305 nm for emission. The SIC operational protocol was written in FIAlab® software (FIAlab® Instrument Systems Inc., USA), version 5.9.312. Data acquisition and treatment were carried out using LC Solution software (Shimadzu Corporation, Kyoto, Japan).

2.2. Reagents and solutions

Ultrapure water acquired by Milli-Q water purifier (Millipore, Bedford, MA, USA) was used throughout the study. Betaxolol standard (98% purity), glacial acetic acid, acetonitrile (ACN; HPLC grade) and triethylamine (TEA; p.a. grade), were purchased from Sigma-Aldrich. The stock solution of betaxolol (1 mg L^{-1}) was prepared by dissolving the substance in water.

Working solutions of betaxolol for calibration were prepared by appropriate dilution of the stock solution in water in the range of 5 – 400 ng mL^{-1}, using eight calibration points. Matrix calibration solutions were prepared in equal concentration range by dilution of the stock solution in ten times diluted and filtered urine. The TEA solution was prepared by dissolution in water and pH was adjusted to 4.5 by addition of glacial acetic acid. For the MEPS procedure, 15% ACN in water was used for sorbent equilibration before sample injection as well as for the sample matrix washout. ACN:0.5%TEA water solution adjusted with acetic acid to pH 4.5 in ratio (30:70, *v/v*) was used as an MEPS elution solution and mobile phase for separation.

Urine samples were obtained from healthy volunteers. They were filtered through a 0.2 μm PTFE filter after ten - times dilution with water. The stock solution and all working solutions and samples were stored at 4°C in dark.

2.3. Operation protocol

The MEPS-SIC measurement protocol for the whole analysis performance is described in Table 1. The system was primed before the first measurement, both the MEPS cartridge and the column were activated and conditioned with the elution solution/mobile phase. The MEPS extraction step used multiposition valve MV 2 switched into port 1′ position, which directed the MEPS outlet to the waste. The MEPS was successively washed with 500 μL of washing solution for the equilibration before sample injecting (steps 1 – 3) and then, 250 μL of urine sample followed by 700 μL of the washing solution for sample loading and matrix washing were propelled through the MEPS (steps 4 – 5). The rest of matrix was eliminated by another 500 μL of the washing solution (steps 7 – 8). The holding coil was then washed by the elution solution before the elution (steps 10 – 11). In the next step, the MV 2 was switched to the position 2′ to link the outlet of the MEPS to the inlet of the chromatographic column. The elution/separation step was performed by propelling 2500 μL of the elution solution/mobile

phase through the MEPS and the column (steps 12 – 13). The retained betaxolol was eluted from the MEPS directly to the chromatographic column where the separation took place and eluted betaxolol was determined in the fluorescence detector.

Table 1: SIC-MEPS operational protocol

Step	Command	Description
1.	MV 1 port 6 MV 2 port 1 PP aspirate 700 µL at 70 µL s^{-1}	MEPS equilibration
2.	MV 1 port 3 PP dispense 500 µL at 15 µL s^{-1}	
3.	MV 1 port 1 PP empty at 50 µL s^{-1}	
4.	MV 1 port 6 PP aspirate 700 µL at 70 µL s^{-1}	Sample loading
5.	MV 1 port 5 PP aspirate 250 µL at 10 µL s^{-1}	
6.	MV 1 port 3 PP dispense 800 µL at 10 µL s^{-1}	
7.	MV 1 port 6 PP aspirate 500 µL at 70 µL s^{-1}	Washing out sample matrix
8.	MV 1 port 3 PP dispense 500 µL at 15 µL s^{-1}	
9.	MV 1 port 1 PP empty at 50 µL s^{-1}	
10.	MV 1 port 7 PP aspirate 500 µL at 70 µL s^{-1}	Cleaning the holding coil
11.	MV 1 port 1 PP empty at 50 µL s^{-1}	
12.	MV 1 port 7 PP aspirate 2500 µL at 70 µL s^{-1}	Elution/Separation
13.	MV 1 port 3 MV 2 port 2 PP empty at 10 µL s^{-1}	

3. Results and discussion

3.1. System assembly and method development

The MEPS-SIC system was assembled as in Fig.1. The pump outlet was connected through holding coil (volume 1.2 mL) to the central port of MV 1, since the ports were used for aspiration of sample, standard solution, washing solution and elution solution and for dispensing of the solutions to the MEPS or waste. The outlet of the chromatographic column was connected with the inlet of the fluorescence detector. All the parts of the system were connected with adequately long tubing to prevent excessive dispersion of zones during the operation.

Both ends of the MEPS cartridge was inserted in the PEEK tubes and fitted by means of two 1/16" ferrules and a joint connector (Figure 2). The MEPS inlet was connected to port 3 of MV 1, using 0.75 mm i.d. tubing (green).The MEPS outlet was connected to the central port of MV 2, using 0.50 mm i.d. tubing (orange). The chromatographic column was connected to port 2´ of MV 2. This setup enabled switching the flow passing the MEPS to the waste - sample loading and matrix washing (Fig. 1a) or towards the column for separation – sample elution (Fig. 1b). Unlike in the manual performance or MEPS coupled to the autosampler of an LC or GC system where the stream flow is bidirectional and only small volumes of the sample can be loaded each time (up to 500 μL), the flow of all solutions (sample, washing solution and elution) through the MEPS cartridge was unidirectional in the described MEPS-SIC system. This arrangement of MEPS in a flow manifold offers the possibility to load a large sample volume (up to the syringe volume) on the MEPS sorbent, if increased sensitivity is required, in a single step. No performance worsening was observed which would indicate clogging of frits, aging or compression of the sorbent in the MEPS cartridge outlet side by the passing stream of solutions even after approximately 100 sample analyses.

The operational protocol for automated MEPS procedure was written with respect to the principles of SIC technique. The volumes of solutions aspirated in the holding coil were approximately 30% larger then volumes injected on the MEPS and the excess volume was discarded to waste to avoid the use of dispersed parts in the zones boundary which could cause deviations. Aspiration of solutions was carried out at higher flow rates (70 μL s^{-1}) in order to keep the analysis time short. Injection flow rates were kept low (10 μL s^{-1} for sample loading and elution, 15 μL s^{-1} for matrix washing), following the usual MEPS protocols, in order to maintain high efficiency of sample loading and prevent the analyte loss by high-speed flow.

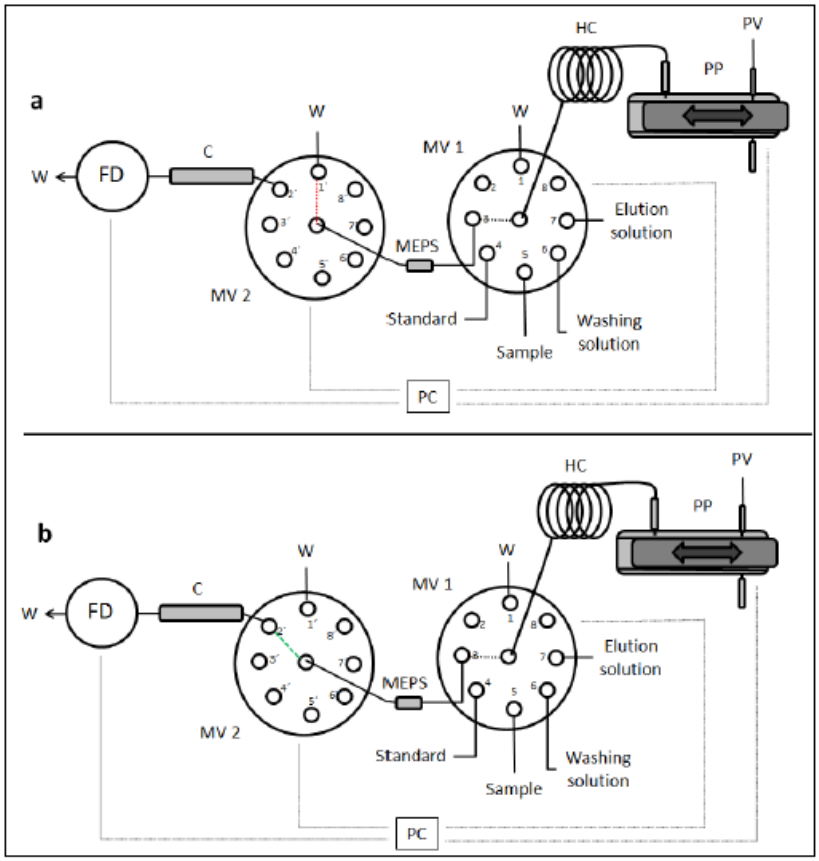

Figure 1. Configuration of the MV:

a - sample loading and washing (steps 1-12), **b -** elution (step 13); C – chromatographic column; FD – fluorescence detector; HC – holding coil; MV 1, MV 2 – multiposition valve 1, 2; PP – piston pump; PC – personal computer; PV – pressure valve; W – waste.

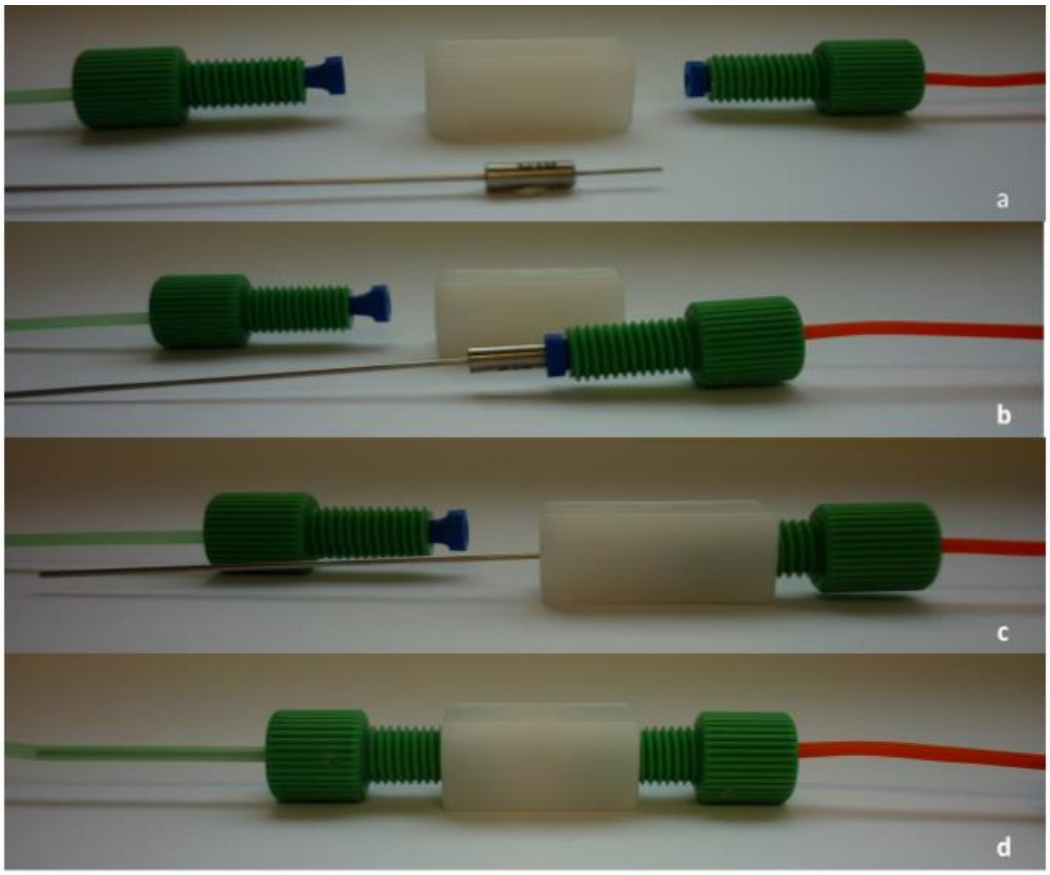

Figure 2. Integration of the MEPS into PEEK tubes holder for SIC system.

3.2. Optimization of on-line MEPS-SIC hyphenation

Compatibility of both parts of on-line hyphenated system is critical for the successful performance of the whole analysis and it must be taken into account since the beginning of optimization of each step. The MEPS working with reduced dead volumes enables fast change of the flow composition with short dispersed zones and fast transfer of analytes on the chromatographic column for appropriate small injection volume to prevent decrease of separation performance. At the same moment the composition of the MEPS elution solution must work as a mobile phase for separation on the chromatography column. The choice of the final conditions of betaxolol analysis in human urine must be done with regards to all these requirements.

3.3. MEPS optimization

The C18 stationary phase of the MEPS sorbent was chosen in order to enable sufficient retention of betaxolol, easy removal of the urine matrix and compatibility with subsequent separation step. In the MEPS procedure, four different concentrations (7, 10, 15 and 20%) of ACN in water were tested as a washing solution. For this study, 5 µL of the standard solution at a concentration of 1 ng mL^{-1} was injected and the signals obtained with different tested washing solutions were compared. The solution of 15% ACN was optimum, since higher ACN concentration led to decreased signal, showing that minor part of betaxolol was also washed out during the matrix washing. The concentration of ACN higher than 15% (*v/v*) resulted in partial elution of betaxolol from MEPS sorbent. The evaluation of peak area of betaxolol vs. ACN concentration in washing solution is depicted in Supplementary material (Fig. 1S). For the optimization of the washing step flow rate, 1 mL of the washing solution (15% ACN in water) was pumped through the MEPS cartridge at flow rates 8, 15 and 25 µL s^{-1} under the same conditions as above. The flow rate of 15 µL s^{-1} was chosen as the compromise ensuring low time of analysis and high recovery.

3.4. Chromatography optimization and elution

The choice of the chromatographic column was based on the properties of the sample and analyzed substances and capabilities of the SIC system. The HighResolution monolithic column (50x4.6 mm) with C18 sorbent was chosen as a good combination of high performance and low working pressure within the used flow rates and low inner volume.

The elution solution/mobile phase was optimized to perform fast elution of the betaxolol from MEPS, trouble-less on-line transfer and subsequent separation on the chromatographic column. The first step was comparison of methanol and acetonitrile as an organic part of the mobile

phase. Methanol was found unsuitable due to the strong peak tailing of betaxolol on the reversed phase column sorbent. The better peak symmetry was achieved with acetonitrile in mobile phase. Addition of triethylamine (TEA) as the competitive amine to the mobile phase enabled sufficient suppression of free silanol groups interactions with betaxolol molecule resulting in good peak symmetry. The final composition of the elution solution/mobile phase was: ACN: 0.5% water solution of TEA, pH adjusted to 4.5 with glacial acetic acid, in ratio 30:70. The volume of 2500 µL of elution solution/mobile phase ensured both the complete elution of betaxolol from MEPS sorbent as well as the entire separation on chromatography column. The elution flow rate of 10 µL s^{-1} and small dead volume of the MEPS ensured fast and effective elution of the analyte from the MEPS sorbent and its fast and undispersed injection on the monolithic column, thus the separation of betaxolol was not affected.

3.5. Sample volume optimization

The choice of the sample volume injected into the system is the key parameter of the detection limit of the developed method. The tested range of injected sample volumes was from 5 µL to 500 µL of ten times diluted urine (tested volumes 5, 50, 100, 150, 200, 250 and 500 µL). Under the optimized washing and elution conditions no interference matrix peaks were observed on the blank urine chromatogram in the whole tested range of sample volumes. Thus, the high selectivity of the MEPS extraction process was confirmed. Although MEPS provides in general a good extraction for broad spectrum of analytes, after injection of 500 µL of betaxolol in ten times diluted urine were observed problems with insufficient recovery of MEPS (decreasing betaxolol signal). The breakthrough volume of MEPS for betaxolol was probably exceeded. Therefore 250 µL of sample injection was found to be optimum for sufficient betaxolol preconcentration without the loss of extraction recovery.

3.6. Study of the fluorescence detection

The SIC instrument was connected with the HPLC fluorescence detector to ensure high sensitivity, fast data collection (20 Hz) and easy and effective data treatment with proprietary software.

The fluorescence wavelengths for betaxolol detection in the elution solution were studied, varying the excitation wavelengths of 210, 220 and 230 nm and the emission wavelengths of 295, 305, 310, 315, 325 and 335 nm. The highest signal response was achieved at λ_{ex} = 220 nm and λ_{em} = 305 nm.

3. 7. Analytical performance and method characteristics

The developed method was validated and the analytical performance characteristics are summarized in Table 2. The retention times were very stable even with the sample pretreatment step included in the procedure (RSD = 0.05%, n = 10).

The chromatograms of two different blank urine samples were compared to the one spiked with betaxolol and no interfering peaks were found at the retention time of betaxolol, confirming the good selectivity of the method.

The linearity of response was confirmed at eight concentration levels (5 – 400 ng mL^{-1}) for both the standards and spiked urine samples. Perfect correlation coefficients were found for both calibration curves, constructed by plotting a relation between concentrations and peak areas (Tab. 2).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the data obtained with the diluted samples. The LOD 1.5 ng mL^{-1} corresponded to the ratio 3 of betaxolol signal to the base line noise. The LOQ was found to be 5.0 ng mL^{-1} (RSD = 2.93% in ten times water diluted urine sample). The accuracy was verified by calculating the recovery of standard solutions at three concentration levels (5, 20 and 200 ng mL^{-1}) in standard solutions and spiked

urine samples, ten times diluted. The results in the range of 100 – 108% were obtained (Tab. 2).

The time of whole analysis including on-line MEPS extraction, interferences removal and chromatography separation was less than 10 min, so the sample throughput was 6 h^{-1} (single injection).

Table 2: Analytical performance/Validation parameters

Parameters	Values
Calibration curve equation (n = 8)	y = (1109928 ± 420)x - (286974 ± 22113) mV
Linear calibration range (ng mL^{-1}, n = 8)	5 - 400
Correlation coefficient (r^2)	0.9996
Matrix calibration curve equation (n = 8)	y = (112744 ± 377)x + (29281 ± 11290) mV
Matrix linear calibration range (ng mL^{-1}, n = 8)	5 - 400
Correlation coefficient (r^2)	0.9997
LOD (ng mL^{-1})	2
LOQ (ng mL^{-1})	5
Intra-day precision* (% RSD)	4.28; 2.47; 0.80
Accuracy – spike recovery* (% + SD)	104.81 ± 4.80; 100.37 ± 4.15; 107.62 ± 0.87
Sample throughput (h^{-1}, single injection)	6

*three concentration levels: 5; 20; 200 ng mL^{-1}

3. 8. Real samples analysis

The developed method with the optimized parameters was applied to the determination of betaxolol in human urine samples. The samples were obtained from a healthy male volunteer. The administrated dose was 20 mg per oral. The urine samples were collected after 3, 4, 5 and 6 h after the administration. The urine sample was necessarily adjusted before injection on the

MEPS. To prevent the system clogging and to enable effective betaxolol retaining the urine sample was diluted ten times with water and filtered through a 0.2 μm PTFE filter.

Each sample was measured in triplicate. The concentration of betaxolol in ten times diluted urine was found to be linearly increasing in time and reached concentrations of 26.6 ± 0.2; 31.9 ± 1.1; 37.8 ± 0.8 and 42.4 ± 0.2 ng mL^{-1}, respectively.

The chromatograms of the blank urine sample and sample obtained after 3 hours from the administration of betaxolol are in Fig. 3.

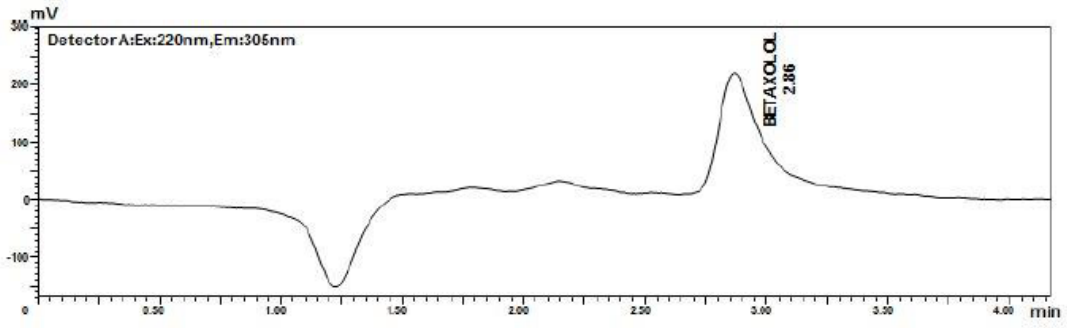

Figure 3. Chromatograms of blank urine (a) and urine sample 3 hours after the oral administration of 20 mg of betaxolol (b).

4. Conclusion

A highly novel mode of automation of MEPS on-line coupled to chromatographic separation manifold using sequential injection technique is presented in this work. The suggested method offers a useful tool in practical performance of MEPS, hence a significant save of the operator´s time and labour is achieved. Moreover highly repeatable results are assured by programmable solutions handling in terms of volumes and flow rates. In the developed system, the extraction is coupled directly to chromatographic separation in one robust, compact and closed SIC system. The concept presents a sensitivity-increasing and selective method thanks to the sample clean-up in the MEPS, chromatography separation and fluorescence detection. The method was successfully applied to determination of betaxolol in human urine, treated simply by filtration

and dilution before analysis. The extension of the application scope of flow techniques in automation of MEPS was presented for the first time. Moreover, the presented approach can be used as an alternative effective tool in on-line MEPS sample pretreatment directly in chromatographic systems with using additional selection valve and MEPS holder as presented in Fig 2. This new methodological concept overcomes the idea that automation and on-line coupling of MEPS with analytical instrumentation remains a most critical step in the method development.

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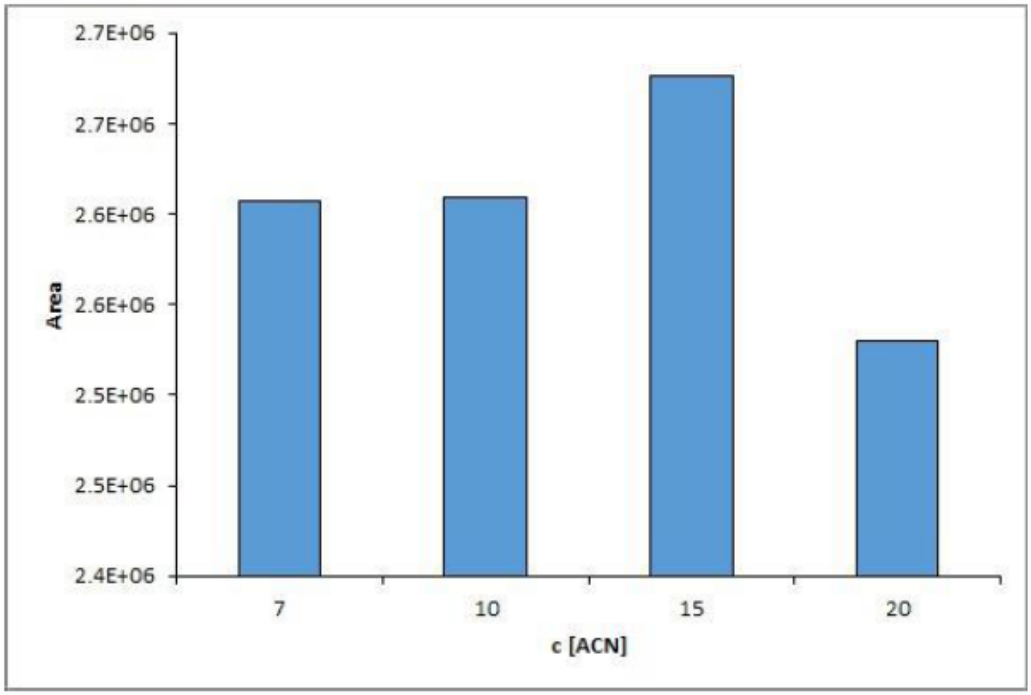
Supplement 1: Optimization of ACN content in the washing solution.



5.7 LIST OF ORAL PRESENTATIONS AT DOMESTIC AND INTERNATIONAL CONFERENCES

1. ***I. Šrámková***, C.G. Amorim, A.N. Araújo, M.C.B.S.M. Montenegro, H. Sklenářová, P. Solich, Spectrophotometric detection of propofol using horseradish peroxidase; application in pharmaceutical analysis, XII Encontro Luso - Galego, 9th - 11th November 2011, Pontevedra, Spain.

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3. ***Šrámková I.***, Acebal C.C., Horstkotte B., Fernández Band B. S., Sklenářová H. Automation of microextraction procedures using flow system; application in determination of metsulfuron methyl, 3rd PhD and 1st PD conference, 29th - 30th January 2013, FaF UK, Hradec Králové.

4. ***I. Šrámková***, B. Horstkotte, H. Sklenářová, P. Solich, Automated In-Syringe Single-Drop Head-Space Microextraction applied to the Determination of Ethanol, 4th PhD and 2nd PD conference, 28th - 29th January 2014, FaF UK, Hradec Králové, pp. 68-69.

5. ***I. Šrámková***, L. Zahálka, D. Šatínský, B. Horstkotte, H. Sklenářová, P. Solich, Sequential injection technique as a tool for sample pretreatment in pharmaceutical analysis, 4th International Meeting on Pharmacy and Pharmaceutical Science, 18th - 21th September 2014, Istanbul, Turkey, p. 65.

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2. ***I. Šrámková***, C.G. Amorim, A.N. Araújo, M.C.B.S.M. Montenegro, H. Sklenářová, P. Solich, Sequential injection analysis of propofol using immobilized peroxidase, P 53, 17th International Conference on Flow Injection Analysis, 3rd - 8th July 2011, Krakow, Poland.

3. ***I. Šrámková***, M. Kořínková, Z. Bártová, H. Sklenářová, I. Voráčová, P. Solich, Study of Quantum Dots nanoparticles effect on luminol chemiluminescence reaction, P. 32, 15th International Symposium On Luminescence Spectrometry, 19th - 22nd June 2012, Barcelona, Spain.

4. ***I. Šrámková.***, C.C. Acebal, H. Sklenářová, P. Chocholouš, L. Zahálka, B.S. Fernandez Band, P. Solich, Comparison of flow cell position in DV-SIA system; alternative for concentration factor enhancement, PA 24, 12th International Conference on Flow Analysis, 23rd - 28th September 2012, Thessaloniki, Greece.

5. ***I. Šrámková***, C.G. Amorim, H. Sklenářová, A.N. Araújo, M.C.B.S.M. Montenegro, P. Solich, Spectrophotometric and fluorimetric detections of propofol: two analytical strategies for pharmaceutical formulation control, PB 26, 12th International Conference on Flow Analysis, 23rd - 28th September 2012, Thessaloniki, Greece.

6. ***I. Šrámková***, H. Sklenářová, B. Horstkotte, P. Solich, Sekvenční injekční analýza jako nástroj pro automatizaci disperzní mikroextrakce z kapaliny do kapaliny, P 55, 2nd - 5th September 2013, 42nd conference Syntéza a analýza léčiv, Velké Karlovice, Czech Republic.

7. ***I. Šrámková***, H. Sklenářová, B. Horstkotte, C.G. Amorim, A.N. Araújo, M.C.B.S.M. Montenegro, P. Solich, Automation of DLLME procedure for propofol determination in serum using DV-SIA system, PB 36, 18th International conference on Flow Injection Analysis, 15th - 20th September 2013, Porto, Portugal.

8. B. Horstkotte, ***I. Šrámková***, H. Sklenářová, P. Solich, In-Syringe head-space single-drop extraction of ethanol, PA 10, 18th International conference on Flow Injection Analysis, 15th -20th September 2013, Porto, Portugal.

9. C.C. Acebal, M. Grünhut, ***I. Šrámková***, P. Chocholouš, H. Sklenářová, A.G. Lista, B.S. Fernández Band, Solid-phase extraction and photo-induced fluorescence detection of metsulfuron methyl in flow-batch system PC 10, 18th International conference on Flow Injection Analysis, 15th - 20th September 2013 Porto, Portugal.

10. ***I. Šrámková***, L. Hanusová, P. Chocholouš, D. Šatínský, H. Sklenářová, P. Solich, Determination of propranolol in urine using automated microextraction by packed sorbent in SIA system with fluorimetric detection, P 6, 16th International Symposium on Luminescence Spectrometry, 24th - 27th September 2014, Rhodes, Greece.

6 SUMMARY

This dissertation was focused on new applications of the flow techniques, especially Sequential Injection Analysis (SIA) and Lab-In-Syringe (LIS). It demonstrates the potentials of these techniques not only in analysis of pharmaceuticals in their formulation or in biological samples but also of other biologically active substances present in the environment or foodstuff. The thesis comprised a theoretical introduction and six experimental works, five published in international scientific journals and one submitted for publication. These works dealt with different sample pretreatment techniques carried out in a flow manifold, followed by other automated processes in the same system including UV decomposition or separation.

After the introduction and objective, the background for the experimental works was given in the theoretical part of this thesis. As five of the six works dealt with automation of sample pretreatment, the most common techniques were described in the first section of the theoretical part, including both liquid phase-based and the solid phase-based extraction techniques (chapter 3.1.2 and 3.1.3). The requirements on modern sample pretreatment methodologies and their common features were discussed, and the most frequent techniques were described in more details.

The second section of the theoretical part gave a short overview about the fundamentals of flow techniques. It described the basic instrumentation typically used when assembling a flow manifold (chapter 3.2.3). The principles of flow techniques employed in the experimental works were given and some advantages and disadvantages were briefly discussed (chapter 3.2.4 - 3.2.8).

The third section of the theoretical part (chapter 3.3) focused on the selected sample handling possibilities in a flow manifold. The specific characteristics of enzymatic reaction or use of electromagnetic radiation in flow system were presented. Automation of sample pretreatment by flow techniques was reviewed in the following chapters. Examples of non-dispersive and dispersive liquid - liquid microextraction and head-space single drop microextraction were given in chapter 3.3.4 and automation of solid phase extraction in flow was presented in chapter 0.

The next part of the thesis presented the experimental work. It listed the articles published or submitted for publication, and a comment was given to each of the work included in the dissertation.

The first work (chapter 5.1) presented the use of an enzymatic reaction for the determination of the anaesthetic propofol in its pharmaceutical formulation (emulsion) in a SIA system. Special features of this work was the comparison of a spectrophotometric and fluorimetric determinations, and further the use of a de-bubbling device to overcome the problem of bubbles arising in the system from mixing of water and ethanol.

The following work (chapter 5.2) dealt with automation of two different liquid phase microextractions. In the first one, the DV-SIA manifold was used to perform LLME of thiocyanates. The development of the system, optimization of chemical parameters and physical

assembly was described. The same system was used for DLLME, reducing the consumption of the extraction solvent. Two different positions of the detection cell were compared in order to evaluate the effect of the cell position on the dispersion and thus, method sensitivity.

The next work (chapter 5.3) showed a novel approach for automation of head-space single drop microextraction by LIS technique. The method gave a simple and elegant solution for the potential shortcoming of this pretreatment method, which was the formation of a stable drop. The sample handling took place in a closed syringe of the syringe pump. Vacuum formation was used for the first time to enhance the mass transfer into the head-space over the sample. The application to ethanol determination was described.

The fourth work (chapter 5.4) described the automation of solid phase-based microextraction in a flow system. A flow-batch system with a lab-made minicolumn was used for the preconcentration and determination of metsulfuron methyl in natural waters. The system comprised a mixing chamber placed in the measuring chamber of a fluorimeter. The determination was based on UV-irradiation of the substance of interest.

The fifths work (chapter 5.5) dealt with the study of the effects of different solvents used for fluorescence measurement in a flow system. A naturally fluorescent substance and a substance fluorescent upon irradiation was included in the study.

In the last work (chapter 5.6), a novel, simple automated method for MEPS coupled directly to a chromatographic system was described. A sequential injection chromatography system and an additional multiposition valve were used in this case. The coupling of MEPS and a chromatographic column in a SIC was presented for the first time and it was applied to the determination of beta-blockers.

This thesis showed the potential of flow techniques not only in performing of simple reaction but also novel methods for sample pretreatment of different matrices. Coupling of several analytical processes in one method was also presented.

7 SHRNUTÍ

Táto dizertačná práca je zameraná na nové aplikácie prietokových techník, predovšetkým sekvenčnej injekčnej analýzy a "Lab-In-Syringe" (LIS) techniky. Dizertácia predstavuje potenciál využitia týchto techník nielen v analýze farmaceutík v ich prípravkoch alebo v biologických vzorkách, ale takisto aj v analýze ďalších biologicky aktívnych látok prítomných v zložkách životného prostredia alebo v potravinách.

Predložená práca obsahuje teoretický úvod a šesť experimentálnych prác, päť publikovaných v medzinárodných odborných časopisoch a jeden manuskript odoslaný na publikáciu. Tieto práce sa prevažne zaoberajú rôznymi technikami úpravy vzoriek v prietokovom systéme. Na úpravu vzoriek nadväzujú ďalšie automatizované procesy, ako napríklad UV-dekompozícia alebo separácia.

Po úvode a definícii cieľov tejto práce nasleduje teoretický úvod k experimentálnym prácam uvedeným v dizertácii. Keďže väčšina (5 zo 6) experimentálnych prác sa zaoberá úpravou vzoriek, najčastejšie používané metódy úprav sú popísané v prvej sekcii teoretickej časti, kde sú zahrnuté kvapalinové extrakčné metódy ako aj metódy založené na pevných sorbentoch (3.1.2 a 3.1.3).

Ďalšia kapitola teoretickej časti zahŕňa krátky prehľad základoch prietokových techník. Sú popísané súčasti bežne používané pri zostavovaní prietokových systémov (3.2.3). Sú popísané princípy prietokových techník použitých pri vývoji metód zahrnutých v dizertácii, a stručne sú diskutované výhody a nevýhody týchto techník (3.2.4 - 3.2.8).

Tretia kapitola teoretickej časti (3.3) sa zameriava na vybrané prístupy v manipulácii so vzorkou v prietokovom systéme. Sú zahrnuté špecifiká enzymatických reakcií alebo použitie elektromagnetického žiarenia v prietokových systémoch. V ďalších kapitolách sú uvedené krátke prehľady automatizácie vybraných metód úpravy vzoriek. Príklady realizácie disperznej mikroextrakcie z kvapaliny do kvapaliny alebo mikroextrakcie do jedinej kvapky sú uvedené v kapitolách 3.3.4. Príklady automatizácie extrakcií tuhou fázou je v kapitole 0.

V ďalšej časti tejto dizertačnej práce bol uvedený prehľad experimentálnych prác – publikácií v medzinárodných časopisoch a manuskriptu odoslanému k publikácii. Ku každej práci je priložený komentár.

Prvá práca (5.1) sa zaoberala využitím enzymatickej reakcie pri stanovení anestetika propofolu vo farmaceutickom prípravku (emulzii) v SIA systéme so spektrofotometrickou detekciou. Bolo popísané zariadenie na odstránenie bublín v systéme. Táto metóda bola v danej práci porovnaná s jednoduchou metódou s využitím fluorescenčného detektoru.

Nasledujúca práca (5.2) popisovala automatizáciu mikroextrakcie z kvapaliny do kvapaliny. Bolo predstavené použitie sekvenčného injekčného systému s dvomi ventilmi na mikroextrakciu tiokyanatanov, vývoj metódy, optimalizácia chemických parametrov a usporiadania systému. Podobný systém bol použitý na automatizáciu disperznej mikroextrakcie, pričom bolo možné

redukovať objem extrakčného rozpúšťadla. Boli porovnané dve rôzne pozície detekčnej cely v systéme z hľadiska vplyvu pozície cely na disperziu, a teda citlivosť metódy.

Tretia experimentálna práca (5.3) predstavuje nový prístup v automatizácii mikroextrakcie do kvapky s použitím "Lab-In-Syringe" techniky. Vyvinutá automatizovaná metóda prekonala možný nedostatok tohto spôsobu úpravy vzoriek, ktorým je vytvorenie stabilnej kvapky. Manipulácia so vzorkou je umiestnená v striekačke piestovej pumpy. Po prvýkrát bolo v tejto metóde vytvorené čiastočné vákuum, čo malo za následok zvýšenie prenosu hmoty zo vzorky do priestoru nad ňou. Bola popísaná aplikácia metódy na stanovenie alkoholu vo vzorkách vína.

V nasledujúcej práci (5.4) bola popísaná automatizácia mikroextrakcie s použitím sorbentu, a to s využitím "flow-batch" techniky. V systéme bola zapojená kolóna plnená sorbentom, ktorá slúžila na prečistenie vzorky a zakoncentrovanie analytu. Súčasťou "flow-batch" systému bola komôrka na zmiešanie vzorku s činidlom. Zároveň v nej prebehla UV-dekompozícia analytu a jeho fluorimetrické stanovenie.

Piata experimentálna práca (5.5) sa venovala štúdiu vplyvu rôznych faktorov na fluorescenciu látok v prietokovom systéme. Daný experiment zahŕňal prirodzene fluoreskujúcu látku ako aj látku, ktorá získava fluorescenčné vlastnosti po vystavení UV žiareniu.

V poslednej práci (5.6) bola popísaná nová jednoduchá metóda automatizácie mikroextrakcie na tuhý sorbent, ktorá bola priamo spojená s chromatografickou separáciou. Metóda bola realizovaná pomocou systému sekvenčnej injekčnej chromatografie s prídavným ventilom. Takéto spojenie MEPS a chromatografickej kolóny bolo predstavené po prvýkrát. Metóda bola použitá na stanovenie betablokátorov.

Predložená dizertačná práca predstavila potenciál prietokových techník nielen pri automatizácii jednoduchých reakcií, ale ukázala aj nové metódy úpravy vzorku s rôznymi typmi matríc. Zároveň boli prezentované možnosti spojenia niekoľkých analytických procesov v jednej metóde.

8 CONCLUSION

Since their invention, flow techniques found a lot of users and followers from different analytical areas. The applications reach from simple sample delivering to the detector to the more complex ones, including derivatisation reactions, kinetic measurements, sample pretreatment, process monitoring and hyphenation with the latest analytical instrumentation. Flow techniques are employed in the field of environmental analysis, bioassays, and agricultural applications and last but not least, pharmaceutical analysis. The contribution of flow techniques to the solution of complex analytical problems lays in the process rationalization: automation, decrease of analysis time and reagents consumption, esuring the repeatability of the results, prevention of sample contamination and protection of the operator by keeping the sample and reagents in a closed manifold, minimization and simplification of instrumentation.

This thesis presents new contribution to the spectrum of applications of flow techniques in the determination of pharmaceuticals and other biologically active substances in different matrices. It focused on the exploitation of various flow assemblies in multiple sample pretreatment techniques. The flow manifolds were used also in automation of other analytical procedures, e.g. enzymatic reaction or using alternative ("green)" derivatisation agents, or coupling to a chromatographic separation. The developed methods employed flow manifolds with technically novel solutions of different analytical tasks. The methods were validated and proved to be suitable for the determination of the analytes of interest and as such, they were published in scientific journals.

Methods based on flow techniques are therefore in concordance with modern analytical chemistry, demanding precision, selectivity, sensitivity, short analysis time, cost effectiveness, and minimal effect on the environment.

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