CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF ANALYTICAL CHEMISTRY

SEQUENTIAL INJECTION ANALYSIS CAPABILITY IN AUTOMATION OF ANALYTICAL PROCESSES

Dissertation thesis

Candidate: Mgr. Lucie Novosvětská

Supervisor: assoc. prof. PharmDr. Hana Sklenářová, Ph.D.

Consultant: PharmDr. Petr Chocholouš, Ph.D.

Hradec Králové 2019

UNIVERZITA KARLOVA

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

KATEDRA ANALYTICKÉ CHEMIE

POTENCIÁL SEKVENČNÍ INJEKČNÍ ANALÝZY V AUTOMATIZACI ANALYTICKÝCH POSTUPŮ

Dizertační práce

Kandidát: Mgr. Lucie Novosvětská

Školitel: doc. PharmDr. Hana Sklenářová, Ph.D.

Konzultant: PharmDr. Petr Chocholouš, Ph.D.

Hradec Králové 2019

STATEMENT OF ORIGINALITY

I declare that this thesis is my original work, which I developed independently under the supervision of my supervisor and consultant. All literature and other sources, used during the work processing, are listed in the list of references and cited properly in the thesis. This work has not been used to obtain another or the same title.

In Hradec Králové to the date of 22.04.2019 Lucie Novosvětská

PROHLÁŠENÍ O DÍLE

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně pod vedením svého školitele a konzultanta. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

V Hradci Králové dne 22.04.2019 Lucie Novosvětská

ACKNOWLEDGEMENT

I would like to thank prof. RNDr. Petr Solich, CSc., for the opportunity to work at the Department of Analytical Chemistry, to participate in various grant projects, and to visit other research groups abroad during my PhD course.

I would like to thank my supervisor assoc. prof. PharmDr. Hana Sklenářová, Ph.D., for her beneficial advice, discussions, motivation, and friendly leading during my studies.

I would like to thank my consultant PharmDr. Petr Chocholouš, Ph.D., for his valuable advice and discussions when dealing with new experimental work.

I would like to thank my co-workers Sara Marques, prof. Marcela Segundo, prof. Manuel Miró, Jakub Fibigr, Lucie Hyršová, Pavel Svoboda, Josef Řezníček, Ivona Lhotská, assoc. prof. Martina Čečková, and prof. František Švec for their cooperation when dealing with research projects and manuscript publications.

I would like to thank Michaela Kohlová, Ivana Šrámková, and Burkhard Horstkotte for friendly environment at the Laboratory of Flow Methods.

I would like to thank dr. Carolina Acebal, dr. Marcus Grünhut, and prof. Beatriz Fernandez Band from the University of South, Bahía Blanca, Argentina, and to prof. Spas Kolev and dr. Inés Almeida from the University of Melbourne, Australia, for pleasant stays at their research laboratories and working groups.

I would like to acknowledge the financial support of various grant agencies, listed below, which enabled me to accomplish research tasks, to present research data at national and international conferences, and to visit research laboratories abroad.

- the Project of Specific Research, SVV 260412
- the Grant Agency of the Charles University, project 159415
- the Grant Agency of the Charles University, project 274216
- the Grant Agency of the Charles University, project 860216
- the Grant Agency of the Czech Republic, project GA15-10781S
- the TEAB project CZ.1.07/2.3.00/20.0235
- the Czech Ministry of Education, Youth, and Sports, project MOBILITY 7AMB14AR029
- the STARSS project CZ.02.1.01/0.0/0.0/15_003/0000465, co-funded by ERDF
- the Mobility fund of the Charles University

I would like to especially thank my husband, family, and friends for their support, help, and patience during my studies.

ABSTRACT

Charles University, Faculty of Pharmacy in Hradec Králové

Department of Analytical Chemistry

Candidate (married name/maiden name): Mgr. Lucie Novosvětská / Mgr. Lucie Zelená

Supervisor: assoc. prof. PharmDr. Hana Sklenářová, Ph.D.

Consultant: PharmDr. Petr Chocholouš, Ph.D.

Title of dissertation thesis:
Sequential injection analysis capability in automation of analytical processes

This dissertation, composed as a commented collection of four scientific articles and one undergoing project, introduces four novel applications in the field of automated flow methods and one application in the field of chromatographic methods used in analytical part of a complex pharmacological study.

It the field of pharmaceutical analysis, three works were successfully optimized, validated, and published in scientific journals. Those works include:

a) Automation of permeation studies within a sequential injection analysis system connected to a liberation unit and carrying out experiments with living cells.
b) Pharmacological study of an antiretroviral drug efavirenz including its determination by fast chromatographic method in three types of sample matrices – in a medium from permeation studies with cellular models, in a Krebs solution from rat placenta perfusions, and in placenta tissue lysates.
c) Determination of lovastatin, a blood cholesterol decreasing drug, in dietary supplements using an automated solid phase extraction with a selective molecularly imprinted polymer sorbent in a sequential injection chromatography system.

In the field of environmental analysis, one work was published in a scientific journal and one work is still the undergoing project. Those works include:

d) Determination of herbicides metsulfuron methyl and chlorsulfuron in a flow-batch system using automated extraction on a carbon nanotubes sorbent followed by fluorometric detection after UV irradiation of herbicides.
e) Automated determination of herbicide 2,4-dichlorophenoxyacetic acid using a sequential injection system with an extraction cell and a home-made polymer inclusion membrane.

ABSTRAKT

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Katedra analytické chemie

Kandidát (příjmení/rodné příjmení): Mgr. Lucie Novosvětská / Mgr. Lucie Zelená

Školitel: doc. PharmDr. Hana Sklenářová, Ph.D.

Konzultant: PharmDr. Petr Chocholouš, Ph.D.

Název dizertační práce:
Potenciál sekvenční injekční analýzy v automatizaci analytických postupů

Tato dizertační práce, předkládaná jako komentovaný soubor čtyř vědeckých publikací a jednoho probíhající projektu, představuje čtyři nové aplikace v oblasti průtokových metod a jednu aplikaci v oblasti separačních metod, použitou pro analytickou část komplexní farmakologické studie.

Tři práce z oblasti farmaceutické analýzy byly optimalizovány, validovány a publikovány v odborných časopisech. Tyto práce zahrnovaly:

a) Automatizaci permeačních studií v systému sekvenční injekční analýzy s připojenou liberační jednotkou a provedení experimentů s živými buňkami.
b) Farmakologickou studii antiretrovirotika efavirenzu zahrnující jeho stanovení pomocí rychlé chromatografické metody ve třech typech matricových vzorků – v médiu z permeačních studií na buněčných modelech, v Krebsově roztoku použitém při perfuzích placenty potkanů, a v lyzátech z tkáně placenty.
c) Stanovení lovastatinu, látky snižující hladinu cholesterolu v krvi, v doplňcích stravy pomocí automatizované extrakce na tuhou fázi s použitím selektivního molekulárně vtištěného polymerního sorbentu v systému sekvenční injekční analýzy.

V oblasti analýzy životního prostředí byla jedna práce publikována v odborném časopise a jedna práce stále probíhá. Tyto práce zahrnovaly:

d) Stanovení herbicidů metsulfuron methylu a chlorsulfuronu ve „flow-batch“ systému s využitím automatizované extrakce na sorbentu z uhlíkových nanotrubic s fluorimetrickou detekcí po ozáření herbicidů UV zářením.
e) Automatizované stanovení herbicidu 2,4-dichlorofenoxyoctové kyseliny v systému sekvenční injekční analýzy s extrakční celou a laboratorně připravenou polymerní membránou.

CONTENT

STATEMENT OF ORIGINALITY 5
PROHLÁŠENÍ O DÍLE 5
ACKNOWLEDGEMENT 7
ABSTRACT 9
ABSTRAKT 11
CONTENT 13
INDEX OF ABBREVIATIONS 15
INDEX OF FIGURES 17
INDEX OF TABLES 19
1. INTRODUCTION 21
2. OBJECTIVES 23
3. THEORETICAL PART 25
3.1. Automation of analytical processes 25
3.2. Flow techniques 27
3.2.1. Sequential injection analysis 28
3.2.2. Sequential injection chromatography 31
3.2.3. Flow batch analysis 34
3.3. Automation of kinetic/time-based procedures in flow systems 36
3.3.1. Dissolution studies 36
3.3.2. Drug release studies 39
3.3.3. Pharmacokinetic studies 40
3.4. Sample preparation as a part of analytical process 44
3.4.1. Direct extraction into solvent 46
3.4.2. Solid phase extraction 46
3.4.3. Application of nanoparticles in solid phase extraction 49
3.4.4. Selective SPE approaches 51
3.4.5. Membrane-based techniques 53
3.4.6. Membrane-based techniques in flow systems 57
4. WORKS INCLUDED IN DISSERTATION THESIS 61

4.1. Fully automatic flow-based device for monitoring of drug permeation across a cell monolayer 63

4.2. Universal efavirenz determination in transport study, rat placenta perfusion and placenta lysate by HPLC-UV 69

4.3. An integrated on-line method for the preconcentration and simultaneous determination of metsulfuron methyl and chlorsulfuron using oxidized carbon nanotubes and second order fluorescent data 73

4.4. Fully automated method based on on-line molecularly imprinted polymer solid-phase extraction for determination of lovastatin in dietary supplements containing red yeast rice 76

4.5. Sequential injection determination of 2,4-dichlorophenoxyacetic acid herbicide using preconcentration with a polymer inclusion membrane 80

5. CONCLUSION 83

6. LIST OF OTHER OUTPUTS OF CANDIDATE 85

6.1. Oral presentations at domestic and international conferences 85

6.2. Poster presentations at domestic and international conferences 85

6.3. Grant projects and scholarships 87

6.4. Foreign experience 87

7. REFERENCES 89

8. SUPPLEMENTARY MATERIAL 97

INDEX OF ABBREVIATIONS

2,4-D – 2,4-dichlorophenoxyacetic acid

ABC transporters – group of human transporters involved in cellular transport

BI – bead-injection technique

CSM – chlorsulfuron

FBA – flow-batch analysis

FDA – U.S. Food and Drug Administration

FDC – Franz diffusion cell

FIA – flow injection analysis

GC – gas chromatography

HPLC-UV – high-pressure liquid chromatography with UV detection

IS – internal standard

LC – liquid chromatography

LLE – liquid-liquid extraction

LM – liquid membranes

LOV – lab-on-valve

LOV-BI – bead-injection technique in lab-on-valve format

MCFA – multicommutated flow analysis

MDCKII-MDR1 – Madin-Darby canine kidney type II cells transfected with human P-glycoprotein

MEPS – microextraction by packed sorbent

MIP – molecularly imprinted polymers

MISPE – solid phase extraction using molecularly imprinted polymer sorbent

MISPE UHPLC-MS/MS – off-line extraction on molecularly imprinted polymer sorbent followed by ultra-high pressure liquid chromatography with mass spectrometry detection

MISPE-SIA-UV – on-line extraction on molecularly imprinted polymer sorbent in sequential injection analysis system with UV detection

MS – mass spectrometry

MSC – multi-syringe chromatography

MSFIA – multi-syringe flow injection analysis

MSM – metsulfuron methyl

MWCNT – multi-walled carbon nanotubes

NP – nanoparticles

ox-MWCNT – oxidized multi-walled carbon nanotubes

PIM – polymer inclusion membrane

PTFE – polytetrafluoroethylene

PVC – poly(vinyl)chloride

PVDF – poly(vinylidene fluoride)

PVDF-HFP – poly(vinylidene fluoride-co-hexafluoropropylene)

Rho123 – Rhodamine 123

r-MWCNT – raw multi-walled carbon nanotubes

SIA – sequential injection analysis

SIA-LOV – sequential injection analysis system with lab-on-valve module

SIA-LOV-BI – bead-injection technique in sequential injection analysis system with lab-on-valve module

SIC – sequential injection chromatography

SLM – supported liquid membrane

SPE – solid phase extraction

UHPLC - ultra-high pressure liquid chromatography

INDEX OF FIGURES

Figure 1: Timeline with the main contributions in flow techniques. 27
Figure 2: Single-line sequential injection analysis assembly [13]. 29
Figure 3: Lab-on-valve (LOV) assembly with spectrophotometric (A) and fluorimetric (B) detection. 30
Figure 4: Principle of bead injection technique [13]. 31
Figure 5: Sequential injection assembly with post-column derivatization [13]. 32
Figure 6: Multi-syringe chromatography system for separation of hydrochlorothiazide and losartan potassium in tablets 33
Figure 7: Multicommutated flow analysis assembly. 34
Figure 8: Flow-through apparatus for dissolution testing of oral for solid dosage forms [38]. 37
Figure 9: Schematic diagram of sequential injection drug-dissolution test system. 38
Figure 10: Schematic view of the sequential injection set-up for liberation study 40
Figure 11: Scheme of the batch-wise configuration of transport study 41
Figure 12: Scheme of the programmable flow system connected to Franz diffusion cell for automated monitoring of permeation test across a cell monolayer [14]. 42
Figure 13: Schematic depiction of the automated apparatus for simultaneous measurement of dissolution and transport of propranolol [13]. 43
Figure 14: Sample preparation techniques overview. 45
Figure 15: Solid phase extraction scheme. 47
Figure 16: Sequential injection chromatography system used for automated extraction of lovastatin [65]. 48
Figure 17: Scheme of SIC setup with on-line SPE for determination of sulfonamides 49
Figure 18: Scheme of molecular imprinting [74]. 51
Figure 19: Schematics of extraction cells using supported liquid membrane (left) and polymer inclusion membrane (right). 54
Figure 20: Structures of chosen substances used in the preparation of polymer inclusion membranes. 55
Figure 21: Membrane separator [13]. 58
Figure 22: Schematic of the flow analysis manifold for phosphate determination. 59
Figure 23: Membrane extraction micro-device based on the PIM sandwiched between two serpentine micro-channels built in Teflon plates. 60
Figure 24: Structure of Rhodamine 123 (left) and verapamil hydrochloride (right). 63
Figure 25: Detailed scheme of the circulation loop integrated in the automatic flow system and Franz diffusion cell [14]. 64
Figure 26: Schemes of protocols used in pharmacokinetic assays with Rhodamine 123. 65
Figure 27: Kinetic profiles of Rhodamine 123 transport using protocol A (●), B (●), and C (●). Comparison with batch-wise results corresponding with protocol A (●) and B (●) ... 65

Figure 28: Detailed photo of three Franz diffusion cells placed in a water bath. 67
Figure 29: MicroSIA system with three Franz diffusion cell units. 67
Figure 30: Scheme of sampling from the system using three Franz diffusion cells. 68
Figure 32: Structure of antiretroviral drug efavirenz. 69
Figure 33: Scheme of filtration in syringe filters accelerated by centrifugation.. 70
Figure 34: Comparison of sample processing for three different matrices at pharmacological study of efavirenz. 72
Figure 35: Structures of metsulfuron methyl (left) and chlorsulfuron (right). 73
Figure 36: Flow-batch analysis system used for determination of metsulfuron methyl and chlorsulfuron in water samples. 74
Figure 37: Structure of lovastatin lactone, a blood cholesterol decreasing drug. 76
Figure 38: Acetonitrile extracts of different matrices naturally containing lovastatin. 77
Figure 39: Scheme of the SIC system, used in 2,4-dichloroacetic acid determination, connected to the extraction cell, peristaltic pump, and LabJack module. 80
Figure 40: Scheme of the home-made extraction cell composition. 81

INDEX OF TABLES

Table 1: Examples of bioprocess monitoring in flow systems. 36
Table 2: Comparison of assemblies of four apparatuses for dissolution testing of oral solid dosage forms described by the European Pharmacopoeia 9.0 [38]. 37
Table 3: Possible modes of nanoparticles (NP) immobilization considering their nature and possibility of packing preconcentration units. 50
Table 4: List of commercially available filter materials. 53
Table 5: Examples of polymer inclusion membrane (PIM) components [91, 93]. 56

1. INTRODUCTION

Flow techniques can be characterized as analytical methods based on sample manipulation and detection within one closed analytical instrument. Such instrument is usually consisted of a solution propelling device, tubing connections, and a flow-through detector. Various valve types can be used depending on a flow system origin, for example an injection valve for sample introduction, a multi-position selection valve as connections with solution reservoirs and/or additional system parts, or a 3-way solenoid valve for flow redirections. Depending on particular application, the sample is injected into a carrier stream and subjected to analyte dilution/preconcentration, matrix elimination, and/or analytes separation on its way towards detection.

Flow techniques, as we know them today, originated in the 1950´s from segmented flow analysis [1]. This method used sample introduction into a carrier stream, separated by air bubbles, and mixing downstream with reagents prior detection. Even though such method required to reach a reaction equilibrium within a sample plug, it was possible to automate several assays without the use of a computer.

In 1970´s, the improved approach of flow injection analysis (FIA) was introduced [2]. FIA uses processing of one sample at a time with reproducible sample injection, continuous and controlled flow conditions, and repeatable timing which result into a reproducible kinetic response available prior the reaction equilibrium is reached. FIA together with its further generations do not require to reach the reaction equilibrium to accomplish analytical measurement.

Further generation of FIA, sequential injection analysis (SIA) [3], uses a bi-directional pump and thus works under non-continuous flow. Precise sample introduction and timing of the procedure is under the computer control. An implementation of short analytical column within the SIA system opened a new field for medium-pressure separations offering an alternative to chromatographic separations with the possibility of on-line connection with a sample preparation step [4].

Flow techniques found their applications in automation and miniaturization of colorimetric reactions, fast enzymatic or chemiluminescence reactions, extraction approaches based on membrane, liquid-liquid, and solid phase extractions, monitoring of time-based analytical processes, or connections with modern detection systems in fields of oceanography, environmental, pharmaceutical, and food analysis.

Various applications of flow techniques are presented in this doctoral thesis, except one chromatographic work dealing with pharmacological study, in a form of five commented projects while four of them were published in scientific journals with impact factor and one is still the undergoing project.

2. OBJECTIVES

This dissertation thesis is composed as a commented collection of scientific articles introducing to the reader various applications of automation using versatility of non-separation flow methods, mainly the family of sequential injection analysis, in a field of pharmaceutical and environmental analysis. The thesis is extended with an application of chromatographic method in complex pharmacological study as very close topic.

Theoretical part of the dissertation thesis is aimed at the introduction of flow methods with the emphasis on those used in presented experimental works. The potential of flow-based methodologies in the automation of analytical procedures was described focusing on sample preparation area, mainly solid phase-based and membrane-based extractions, together with monitoring of time-based processes, focusing on the area of pharmaceutical analysis.

Specific aims of experimental part of the dissertation thesis were:

a) Development and optimization of a fully automated flow-based system enabling automation and real-time monitoring of a studied substance across a monolayer of living cells, flow system improvements leading to a parallel monitoring, and addition of separation step prior detection of studied substances.
b) Development, validation, and application of fast chromatographic method for determination of an antiretroviral drug in a pharmacological study including three types of samples – samples from cellular transport experiments, samples from placenta perfusion experiments, and samples of placenta lysates.
c) Development and validation of method analyzing two sulfonylurea herbicides in water samples with the benefit of on-line solid phase extraction on the carbon nanotubes sorbent, UV-initiated decomposition of extracted substances, and their detection and differentiation using chemometric treatment of the second order fluorescent data.
d) Automation of a highly selective solid phase extraction based on the use of a molecularly imprinted polymer sorbent, its validation and application on control of active substance content in dietary supplement formulations.
e) Automation of membrane-based herbicide extraction from environmental water samples using incorporation of an extraction cell with a home-made polymer inclusion membrane within a flow-based system.

3. THEORETICAL PART

3.1. Automation of analytical processes

Requirements of analysis comprising high sample throughput with maintaining high repeatability in routine analytical laboratories led to the necessity of analytical procedures automation [5]. Automated platforms can bring a reduction of workflow and its cost, a decrease of requests on operators, an increase of procedural reproducibility and robustness, and a decrease of errors incidence [5]. In case of flow-based techniques, miniaturization and time efficiency of the procedure as well [6]. Automation started with simpler tasks as pipetting, centrifugation, mixing or on-line detection of chemical species. Nowadays, several automation strategies can be recognized such as flow-based techniques, well plate systems, centrifugal and cartridge analyzers, robotic systems, and complex solutions to total laboratory automation [5].

Flow techniques are briefly described in following chapters with the emphasis on those used during the experimental work.

Well plate systems use plastic plates with number of wells, typically 96. Samples and reagents are pipetted into the respective wells and various chemical reactions can be carried out. Products are usually detected by plate readers using spectrophotometric, fluorometric, or chemiluminescence detections [5]. However, microplates can be used in a wide range of the pre-analytical steps for both storage and preparation of the sample [7]. Depending on microplate material, long-term storage in the freezer, cold storage, or incubation and thermo-cycling can be carried out. Sample centrifugation in microplates is possible by a simple change of a centrifuge rotor adapted for microplates.

Filtration, protein precipitation and solid phase extraction (SPE) as conventional sample preparation techniques can be proceeded in the well plate systems as well using the proper type of plates with vacuum manifolds. Various sorbents for both, filtration and SPE, are commercially available. Sample evaporation and reconstitution in the mobile phase, often required prior chromatographic analysis, can be completed in microplates as well [7]. Moreover, chromatographic systems with autosamplers designed for the use with microplates are commercially available. Microplates enable parallel sample analyses compatible with many standard assays, but on the other hand large volumes of single-use consumables (plates) are necessary [5].

Centrifuge analyzers are rotational platforms with vessels for samples and reagents, which use centrifugal force for mixing. Samples are analyzed by dynamic spectrophotometric absorbance measurements, for example for kinetic determinations in clinical biochemistry. Combination of microfluidic chips with centrifugation opened

a new direction of research. Centrifuge analyzers enable parallel analyses with reduced liquid handling, but are limited only for specific assays [5].

Discrete analyzers are platforms processing samples using various reactions carried out in separate vessels, for example automatic titrators. Robotic systems for chemistry are accessible in configurations of multistep sampling processing devices, autosamplers delivering sample into detection or separation instruments, or combining both, sample preparation and transport into the instrument. Those systems are relatively robust and easy to operate, but involve multiple mechanical operations and number of analytical procedures is limited [5].

Industry-scale total laboratory automation solutions for large laboratories are also available on the market. Robotic systems are designed to perform analyses with sets of standards and defined procedures with high reproducibility of the obtained results. Such automation reduces labor and personnel costs, but its mechanical complexity requires high investments and complex maintenance [5].

3.2. Flow techniques

Flow techniques emerged from the need of automation of simple analytical procedures in the 1950´s when numbers of samples to be analyzed increased, mainly in the clinical research [8, 9]. Flow techniques can be characterized as analytical methods based on sample treatment within the closed manifold usually composed of tubing, solution propelling device (a pump), and a flow-through detection unit. The sample volume is precisely measured, introduced into a carrier stream, preconcentrated and/or modified using derivatization reaction, and the product is detected within one analytical run.

Flow techniques have evolved thanks to the development of a technical equipment and information technology. Timeline of main contributions in the field of flow techniques is presented in Figure 1.

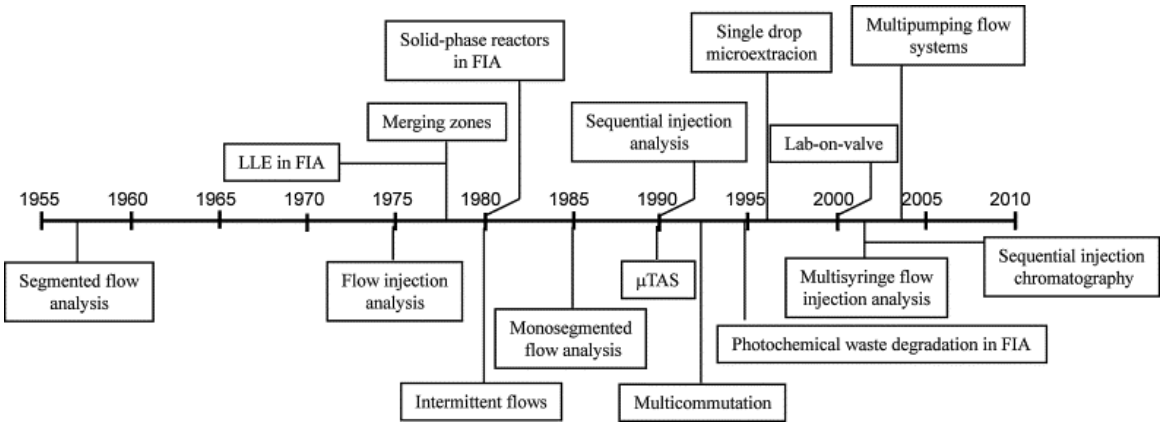

Figure 1: Timeline with the main contributions in flow techniques. LLE: liquid-liquid extraction, FIA: flow injection analysis, µTAS: micro total analysis systems. Reprinted with permission from [10].

Flow techniques offer a wide variety of analytical processes automation due to its easy connection with additional parts of the flow systems. Simple operations such as sample dilution or derivatization can be extended using a gas diffusion cell, a dialysis cell, a reduction column, a heated coil, a liquid-liquid extraction (LLE) chamber, an SPE column, an extraction cell for membrane separation, a Franz diffusion cell for liberation/transport studies etc.

Various detection systems with flow-designed detection cells are compatible with flow techniques, among them the most commonly used UV and VIS spectrophotometry, fluorescence, chemiluminescence, atomic absorption spectrometry as well as

membrane-based ion selective electrodes, amperometric sensors or optosensors. The possibility of sample preparation and detection in one closed on-line system is an indisputable advantage of flow techniques. In addition, stop-flow approach and kinetic measurements can be realized within the flow systems as well.

During the evolution it was proven that flow techniques features, such as versatility of instruments, compatibility with different detectors, low consumption of sample and reagents, low waste production, lower personnel and consumable costs, and reproducible and precise manipulation and timing, meet the requirements of solving analytical problems towards automation and miniaturization of analytical procedures [9].

Since each flow technique has its own features, developing applications where various flow techniques are hyphenated could improve the analytical performance of proposed methodologies [9, 11].

Besides sequential injection chromatography (SIC) and multi-syringe chromatography (MSC) approach, flow techniques are originally low-pressure non-separation techniques. On the other hand, modern separation techniques require sample preparation due to low concentrations of analytes and complex matrix samples. Analytical performance can be extended by flow techniques coupling with separation technique such as liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis. Combination of on-line sample preparation with separation is very attractive because this approach enables: a) to accomplish the whole process within one instrumental assembly, b) to analyze complex mixtures, c) to adapt a procedure to macro and micro size, and d) to accomplish both quantitative and qualitative analyses with wide range of selective and sensitive detectors [9, 12].

Finally, as it was described in a work of Melchert et al. [10], flow techniques development successfully meets green analytical chemistry principles in the terms of minimized toxic reagents consumption and thus waste generation, possibility of solvent recycling, reagents reuse or on-line waste treatment, and reduced risk for the analyst due to sample processing in one closed system minimizing the operator exposition to toxic substances.

3.2.1. Sequential injection analysis

SIA approach was introduced by Růžička and Marshall in 1990 as an alternative to the FIA [3]. SIA equipment, depicted schematically in Figure 2, comprises a multi-position selection valve connected via central port to a bi-directional syringe pump and via individual side ports to samples and reagents reservoirs and flow-through detector. The syringe pump works bi-directionally without a flow pulsation due to precise piston

movement driven by a step electromotor. It operates only during intervals of aspiration and dispensing of solutions which results in solutions savings. The pump uses head with a two position switching valve. In position 1, the syringe pump is connected to the carrier reservoir. In position 2, the syringe pump is connected to the central port of the selection valve. Usually, zones of the carrier, sample, and reagents are aspirated into a holding coil and then the flow is reversed towards the detection cell [9, 13].

SIA system is under full computer control. Syringe pump valve and selection valve positions together with volumes and flow rates of each step are defined in a control program. Sample and reagent consumption is minimized due to aspiration of particular volumes when needed, but sample throughput is lower than in the FIA due to the need of periodical refilling of the syringe pump [6, 9].

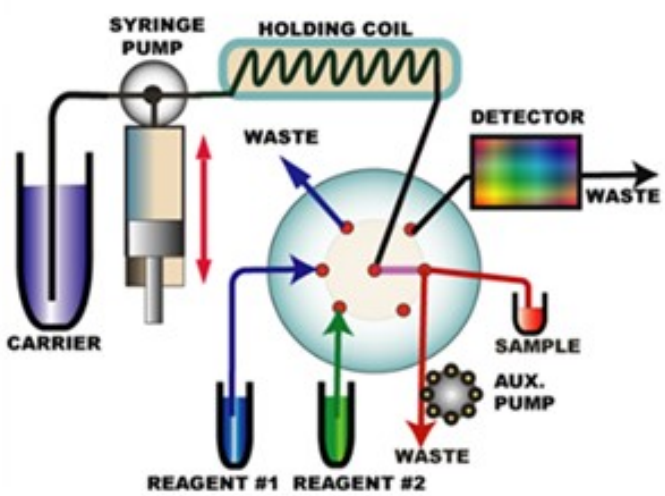

Figure 2: Single-line sequential injection analysis assembly [13].

SIA manifold in combination with the liberation unit was used in the work dealing with automation of *in vitro* transport model evaluation when Rhodamine 123 permeation through cellular monolayer was determined [14]. More details could be found in chapter 4.1. SIA manifold connected to an extraction cell with a polymer membrane used for 2,4-dichlorophenoxyacetic acid herbicide preconcentration from aqueous samples is described in chapter 4.5.

Modification of SIA was introduced by Růžička in 2000 using SIA mode within a microfluidic device so-called lab-on-valve (LOV) [15]. SIA-LOV equipment, schematically shown in Figure 3, uses syringe pump connected via holding coil to the LOV module – a structure with channels mounted atop the 6-position selection valve and connected to sample, reagents, and waste reservoirs. Channel in port 2 of the LOV is connected

with two optical fibers and serves as a UV (Figure 3A) or a fluorometric (Figure 3B) flow-through detection cell according to optical fibers position [13, 15]. SIA-LOV is controlled by the computer program in the same way as the SIA is. LOV approach enabled miniaturization and downscaling of reagent-based assays to microliter level, which is connected to the very low consumption of sample and reagents [9].

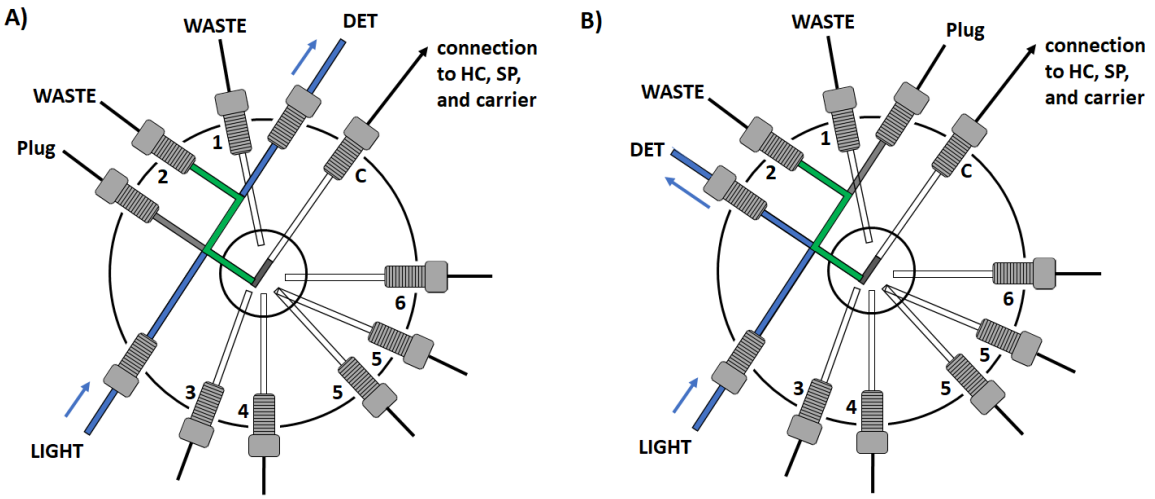

Figure 3: Lab-on-valve (LOV) assembly with spectrophotometric (A) and fluorimetric (B) detection. DET – detector, C – central channel, 1-6 – LOV channels, HC – holding coil, SP – syringe pump. Green line – sample path, blue line – light path.

LOV module enabled accommodation of not only solution-based assays, but also a manipulation with microbeads suspension within a fluid stream, so-called bead-injection (BI) technique [16]. SIA-LOV-BI facilitated on-line preconcentration and separation of various analytes. Suspension of beads is injected into the LOV and trapped in the channel creating a microcolumn of beads (Figure 4A). Then, the sample is loaded on the column, the analyte is retained on the beads surface, and matrix is washed out (Figure 4B, C). Additionally, the colorimetric or fluorometric reagent can be applied for analyte derivatization prior to its detection (Figure 4D). When the column is created in the LOV side channel, the analyte is eluted for detection downstream (Figure 4E). When the column is created in the flow cell, the analyte can be detected directly on the column using BI spectroscopy (Figure 4F). After the detection, beads can be kept for next analytical cycle or more often beads are discarded by the flow reversal (Figure 4G), and a new column is created with a fresh portion of beads [13, 16]. Physical properties of beads play an important role in their manipulation within the system (moving, metering, packing, removal).

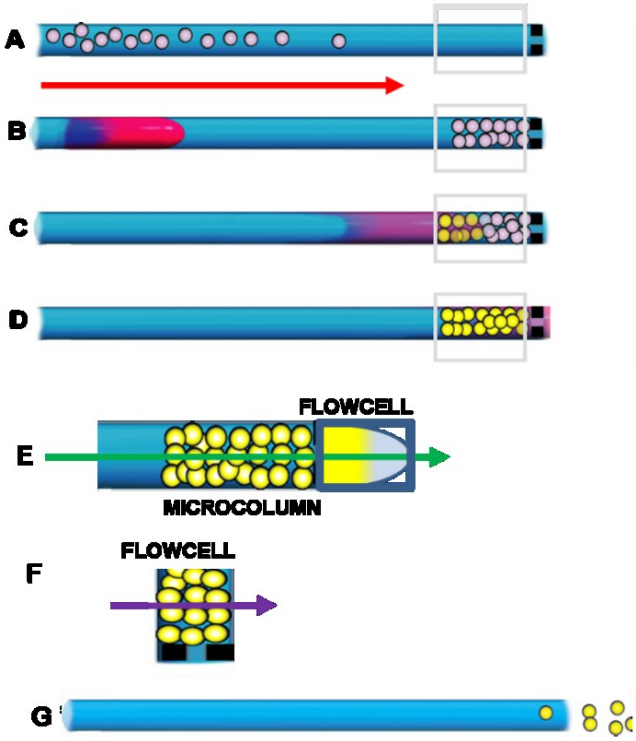

Figure 4: Principle of bead injection technique [13]. A) beads aspiration, B) column creation, C) sample/matrix washing solution/reagent loading, D) analyte capture on beads surface, E) analyte elution and detection down-stream or F) analyte detection on beads surface, and G) beads removal prior to next analysis cycle.

3.2.2. Sequential injection chromatography

In 2003, a novel concept of separation in low-pressure SIA system called SIC was introduced by Šatínský and co-workers [17]. SIC combines SIA manipulation with sample together with a low-pressure LC separation of analytes. SIC equipment is similar to those of SIA. It implements a short separation column and a medium-pressure pump. The column is usually placed between the selection valve and the detection flow-cell, as shown in Figure 5.

Two types of short columns, monolithic and core-shell, can be used [4, 13]. Monolithic columns, manufactured by MERCK (Chromolith®) or Phenomenex (Onyx™), are produced as a single piece of material with macropores reducing a column back-pressure and mesopores forming a surface for separation [4]. Core-shell columns, manufactured by Supelco (Ascentis® Express) or Agilent (Poroshell), consisted of particles formed of a solid core and a porous shell providing high separation efficiency with low back-pressure [18].

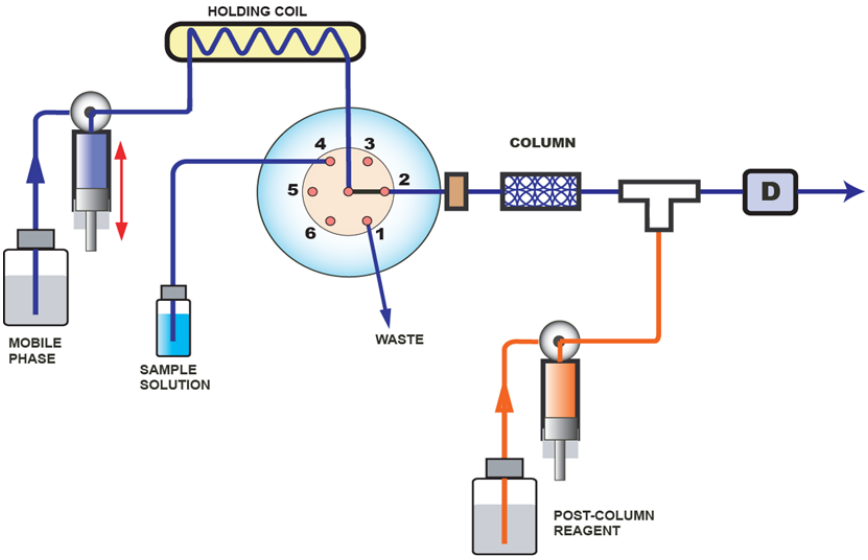

Figure 5: Sequential injection assembly with post-column derivatization [13].

SIC opened a possibility to analyze simple mixtures without the need of LC instrumentation that reduces cost per analysis and organic solvent consumption. The most probable advantage is the on-line handling with the sample comprising its preparation followed directly by its separation and detection [17]. Such approach was used in works of Šrámková and co-workers [19] and Batista and co-workers [20] where SPE extraction of analyte/analytes was followed by separation using short analytical column within a SIC system with additional 8-port selection valve. Limitations of the system are related to syringe reservoir volume and syringe pump flow rates. Complex sample matrices containing high number of analytes may cross the limitations of the SIC separation as well.

Similar approach comprising on-line SPE followed by separation can be performed in a column-switching LC instrument [21]. After on-line SPE, the eluate flows towards the analytical column and separation is completed. The column-switching LC system is a complex instrument working under continuous flow of mobile phases. Thus, parameters such as column characteristics and flow rates together with programming of the switching valve have to be carefully chosen. Compatibility of mobile phases used for elution in the first dimension and for separation in the second dimension is also a critical parameter. More complex optimization of such system is required. This is similar for both approaches, on-line SPE/separation in SIC and column-switching LC.

SIC system was used for automation of SPE for determination of drug lovastatin in dietary supplements. More details can be found in chapter 4.4.

In 2008, Cerdá and co-workers introduced MSC approach that follows the concept of SIC [22]. Again, the short monolithic column was incorporated in the flow system to carry out low-pressure separations, this time within a multi-syringe flow injection analysis (MSFIA) instrument [23], shown in Figure 6. Propelling device in the MSFIA equipment is a titration burette with four syringes in one block moved by a stepper motor. Each syringe has a fast-switching three-way solenoid valve with two positions, "OFF" connected to the solution reservoir and "ON" leading to the flow system. Due to that, solutions are aspirated from/dispensed to reservoir or flow system according to bi-directional syringe movement. All four syringes move simultaneously and flow rates of particular channels can be adapted by the syringe volumes and diameters [9].

Similarly as SIC, MSC is an alternative for LC separations in terms of lower sample/solvent consumption and operational costs, but suffering from limited flow rates of the burette module with adjustable burette volumes [22]. In comparison with MSFIA/MSC complex apparatus, a single-line SIA/SIC apparatus is more universal and user-friendly for handling.

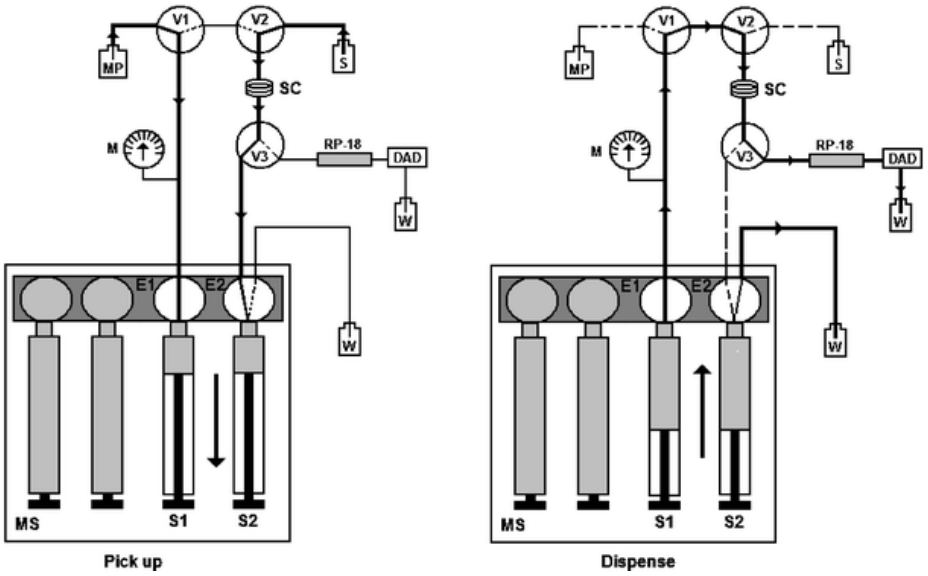

Figure 6: Multi-syringe chromatography system for separation of hydrochlorothiazide and losartan potassium in tablets. MS: multi-syringe burette, S_1, S_2: syringes, M: manometer, V_1–V_3: solenoid valves, E_1: Delrin two-way connector, E_2: three-way solenoid valve, MP: mobile phase, S: sample, W: waste, RP-18: monolithic column, DAD: diode array detector, SC: sample coil. Reprinted with permission from [22].

3.2.3. Flow batch analysis

Reis and co-workers introduced a concept of a multicommutated flow analysis (MCFA) in 1994 [24]. As shown in Figure 7, MCFA equipment comprises a propulsion device (peristaltic pump, syringe pump), a tubing, fast-switching three-way solenoid valves, and a detector. Three-way solenoid valves operate in two directions. While the valve is "OFF", the solution is pumped back into its reservoir. While the valve is "ON", the solution is pumped into the system during a defined time period [9].

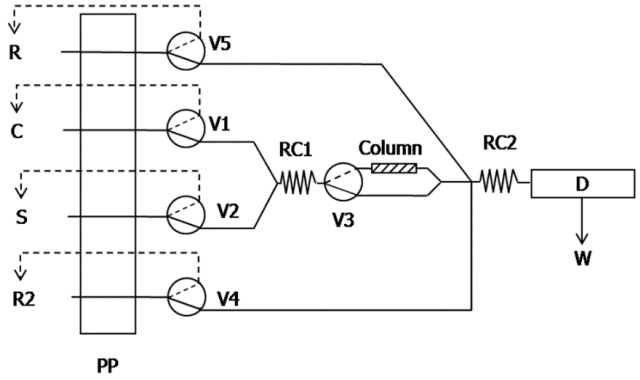

Figure 7: Multicommutated flow analysis assembly. R: reagent, C: carrier, S: sample, PP: peristaltic pump, V: solenoid valve, RC: reaction coil, D: detector, W: waste. Reprinted with permission from [9].

When mixing or reaction chambers are used for subsequent sample processing, the approach is called flow-batch analysis (FBA) [25]. Multiple applications were demonstrated using additional mixing chambers in flow systems, as summarized by Zagatto and co-workers [26]. Standard addition method, flow titrations, single standard calibration, or time-based procedures were carried out. Mixing chamber was used for in-line sample preparation such as LLE and solid-liquid extraction or sample dissolution. Also, mixing chamber was used as a detection cell or "mini laboratory" in FBA system where various steps including subsequent addition/removal of solutions for sample dilution, conditioning, or derivatization were applied [26].

MCFA enables higher sample throughput as well as sample and reagents savings due to its recirculation in reservoirs. Main drawbacks could be associated with heat released when operating solenoid valves for longer time periods and worse peristaltic tubing tolerance to solvents and aggressive reagents [9, 25]. Analyte detection can be carried out under both, continuous and stop-flow mode in the flow-through detector or directly in the mixing chamber in FBA [25]. The control program operates mainly

with flow rate parameters and with the timing of switching the solenoid valves positions. When compared with single-line SIA/SIC instrument, MCFA/FBA is more complex system.

FBA system was used in the work dealing with preconcentration of herbicides metsulfuron methyl and chlorsulfuron from environmental water samples [27]. More details can be found in chapter 4.3.

3.3. Automation of kinetic/time-based procedures in flow systems

This chapter describes the application of flow methods in the pharmaceutical field focused on automation of procedures based on kinetics of processes. Determination of analytes within a fixed time period is important for bioprocess monitoring, evaluation of dissolution studies, and pharmacokinetic studies exploiting transport studies [28, 29].

Bioprocess monitoring found its application mostly in pharmaceutical production. Some examples are listed in Table 1. This application has one main goal – to report the state of the process and its possible changes in time. Such information is evaluated in a real-time and can improve the consistency, quality, and recovery of the process [29]. Depending on the sampling rate, it is possible to change conditions of bioprocesses according to the results of real-time monitoring.

Table 1: Examples of bioprocess monitoring in flow systems. Adapted from [28, 29].

ANALYTE	MATRIX ORIGIN	FLOW SYSTEM	DETECTION	REF.
Glucose, penicillin	*Penicillium chrysogenum* cultures	SIA	CL, SPM	[30]
Glucose	broth fermentation process	SIA	CL,	[31]
Glucose	yeast fermentation	SIA	SPM	[32]
Penicillin, glucose, lactic acid	*Penicillium chrysogenum* cultures	SIA	CL	[33]
Morphine	water streams of *Papaver somniferum*	SIA	CL	[34, 35]

CL – chemiluminescence detection

SPM – spectrophotometric detection

3.3.1. Dissolution studies

Dissolution studies are one of the required tests by pharmacopeias for quality control of solid dosage forms such as tablets and capsules. In case of semisolid dosage forms such as ointments, creams, and gels, there is no compulsory method and dissolution is referred as a drug release. The rate of an active compound release is not only important for proper drug dosing in patients, but also in the development of new drug formulations, in manufacturing process monitoring, and in a drug quality control. Dissolution testing and comparison with original drugs are important for generic drugs approval by authorities

during drug registration on the market. Dissolution testing can be also used for predictions of *in vivo* performance [36, 37].

European Pharmacopoeia 9.0 describes four apparatuses for dissolution testing of oral solid dosage forms: 1 – basket apparatus, 2 – paddle apparatus, 3 – reciprocating apparatus, and 4 – flow-through cell [38]. Assemblies are summarized in Table 2.

Table 2: Comparison of assemblies of four apparatuses for dissolution testing of oral solid dosage forms described by the European Pharmacopoeia 9.0 [38].

APPARATUS TYPE:	**APPARATUS ASSEMBLY:**
1 – basket apparatus	a vessel, a motor, a metallic drive shaft, a cylindrical basket, a water bath with heating device
2 – paddle apparatus	a vessel, a motor, a metallic shaft, a paddle, a water bath with heating device
3 – reciprocating apparatus	a set of vessels, a set of glass reciprocating cylinders, inert fittings and screens for tops of cylinders, a motor and drive assembly for cylinders movement, a water bath with heating device
4 – flow-through cell	a reservoir and a pump for dissolution medium, a flow-through cell, a water bath with heating device

Typically, dissolution testing is carried out in a glass vessels with given size and parameters for dissolution experiment such as dissolution medium composition, volume and temperature (37 ± 0.5 °C) [39]. In defined time intervals, samples are collected from the dissolution medium manually and replaced by the dissolution medium to maintain dissolution medium volume. Subsequently, the samples are analyzed off-line. Flow-through apparatus, described in the European Pharmacopoeia, is schematically shown in Figure 8.

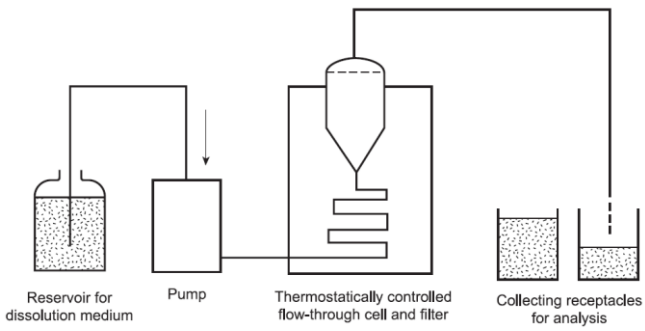

Figure 8: Flow-through apparatus for dissolution testing of oral for solid dosage forms [38].

In the flow system arrangement, schematically shown in Figure 9, the dissolution medium continuously circulates in a loop attached to the system. Sampling, analyte detection, and dissolution medium replacement is carried out in one closed system. Those conditions mimic the real-time monitoring [28]. In continuous monitoring, fast and on-line sample preparation is carried out with the high sampling frequency, low sample consumption, and minimal disturbance in the dissolution medium volume. Parallel testing using more vessels, typically 3 or 6, is possible by the connection of vessels with respective ports of selection valve of the flow system. In formulations containing more active substances, multicomponent dissolution monitoring can be completed. When necessary, long-term monitoring is possible within a range of hours or days [29].

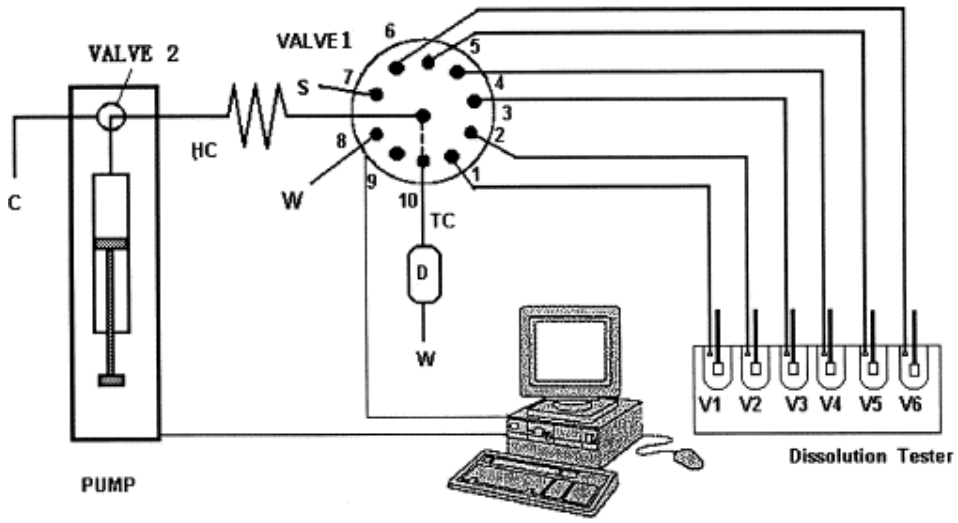

Figure 9: Schematic diagram of sequential injection drug dissolution test system. C: carrier, HC: holding coil, TC: transferring conduit, valve 1: 10-position selection valve, valve 2: syringe valve, S: standard, W: waste, D: detector, V1–V6: dissolution vessels. Reprinted with permission from [40].

Some examples follow. The acetylsalicylic acid determination in tablets was carried out using chemical hydrolysis of acetylsalicylic acid into salicylate followed by its detection by tubular salicylate-selective electrode in SIA system [41]. Simultaneous dissolution profiles of aspirin, phenacetin, and caffeine from tablets were measured within the SIA system using a partial least-squares calibration technique for analytes resolution after UV spectra measurement in a range of 220-310 nm when the sample zone was stopped in the detection cell [42]. Ascorbic acid and rutin were determined using SPE microcolumn in SIA system which enabled separation of both analytes [43]. Chlorogenic acid in dietary supplements was determined after dissolution and filtration in two dialysis filtration units in FIA-LC system [44].

3.3.2. Drug release studies

As it was already mentioned, there is no official method for drug release testing for semisolid formulations such as ointments, gels, and creams. FDA guidelines recommend the drug release investigation using Franz diffusion cell (FDC) with a membrane [36, 45]. Release test evaluates the release rate through the membrane or porcine skin in a specified time interval, similarly to the dissolution testing.

FDC is a glass vessel composed of an acceptor part with a side sampling port and a donor part placed atop while the membrane is usually sandwiched between them. In drug release testing, various membrane types such as synthetic ones (polycarbonate, mixed cellulose esters, Teflon, silicon, or PVDF) or natural ones (pig skin or human skin) can be used [28, 29]. It is necessary to select properly: a) diffusion cell type and volume, b) a receiving medium composition including active substance properties and physiological skin conditions, and c) system parameters such as stirring speed, temperature (32°C to mimic skin), number of FDC, sampling intervals and volumes [46].

In the flow systems, FDC can be connected to the flow system via a circulation loop, as shown in Figure 10. Automated drug liberation apparatus using SIA with fluorometric detection was described for the determination of indomethacin in gel [28] and salicylic acid in gels and ointment [47]. Later, the work was extended using three cells in parallel connected to SIA system [48]. Other work applied SIC separation on a short monolithic column for simultaneous release testing of lidocaine and prilocaine [49]. Alves and co-workers used a similar system for evaluation of caffeine-loaded lipid nanoparticles release through a pig skin placed in FDC connected to the FIA system with a short monolithic column for matrix and caffeine separation prior detection [50].

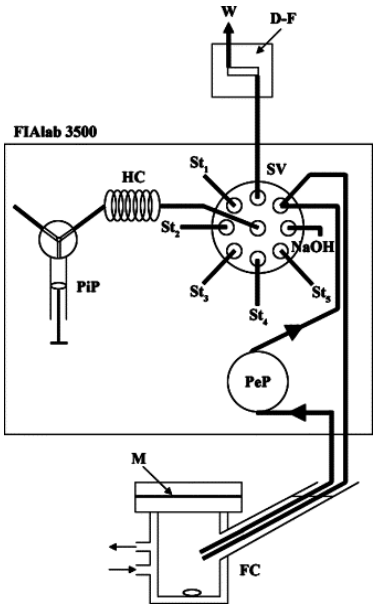

Figure 10: Schematic view of the sequential injection set-up for liberation study. PiP: piston pump, PeP: peristaltic pump, SV: selection valve, St_1-St_5: standard solutions for calibration, HC: holding coil, D-F: fluorometric detector, W: waste, FC: Franz cell, M: membrane. Reprinted with permission from [45].

3.3.3. Pharmacokinetic studies

Pharmacokinetic studies are based on experiments studying interactions of drugs with drug transporters expressed in cells/tissues, and/or interactions with other drugs. Various models can be used to examine drug accumulation, efflux, or transport involving both *in vitro* and *in vivo* tests.

Transport study is one of *in vitro* assays applying cellular models for evaluation of drug transport and/or drug interactions using various types of cells. Transport study is usually carried out in batch-wise configuration, schematically shown in Figure 11. Cells are cultivated onto a porous membrane of plastic insert with specified material, diameter, and pore size. Insert is placed in a well of plate with specified diameter and volume. The plates are typically 6/12/24-well with volumes of 0.6/1.5/2.6 mL and insert diameters of 6.5/12/24 mm.

When carrying out the transport experiment, cells are first washed in the medium/buffer. Then solution with tested substance, e.g. transport marker alone or in a combination with other substance(s) under the study in the medium or buffer, is added. Cells are incubated with tested substances for 2-6 hours and samples are taken manually in predefined time intervals, e.g. 100 µL of sample in 30 min interval. When the transport experiment is finished, samples are analyzed by fluorescent plate readers, radioactivity detection, or LC methods [14].

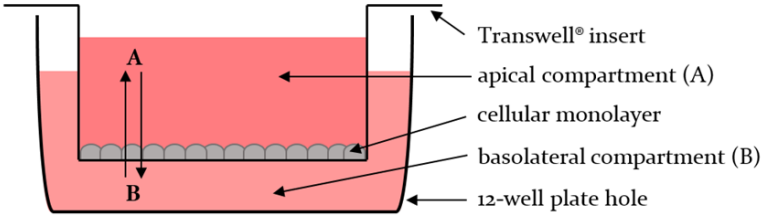

Figure 11: Scheme of the batch-wise configuration of transport study.

To investigate apical to basolateral transport, the tested substance is placed into the insert. To investigate basolateral to apical transport, the tested substance is placed into the plate well. To investigate a bidirectional transport, the tested substance is placed into both the insert and the plate well.

Batch-wise transport study experiments were used in a pharmacological study of a drug efavirenz [51]. This work is introduced in this thesis in chapter 4.2.

Example of *in vitro* model automation using SIA system connected to the FDC is evaluation of Rhodamine 123 transport through cellular monolayer [14]. This work is introduced in this thesis in chapter 4.1, but the flow assembly is briefly described here. Similarly to the drug release testing in the flow systems described above, FDC serves as a liberation unit. This time, the FDC donor compartment is replaced by the insert with cultivated cellular monolayer and covered by a plastic lid. FDC is connected to the SIA system via circulation loop and T-piece which enables on-line sampling from the loop prior fluorometric detection of Rhodamine 123. SIA-FDC system is shown in Figure 12.

On-line system enabled automated sampling and re-filling the medium volume in the FDC, to keep the contact of medium with the cell monolayer, in shorter time intervals than in batch-wise permeation. Procedure was operated by the control program in the closed circuit and thus the risk of the sample contamination during manual sampling was minimized. Due to on-line detection after sampling, more detailed kinetic profile was obtained and possible decomposition of analytes during storage between manual sampling and off-line detection was minimized.

In the automated system, the measured concentrations had to be recalculated in each individual measuring cycle, because the sampled volume was refilled with fresh portion of acceptor solution. Verification of concentration profiles with batch-wise tests is recommended due to possibility of transport rate influencing by the slight differences in concentrations during the time of experiment. Calibration was carried out after each

transport experiment in the flow system which prevented variations in measured analytical signals caused by inter-day variations.

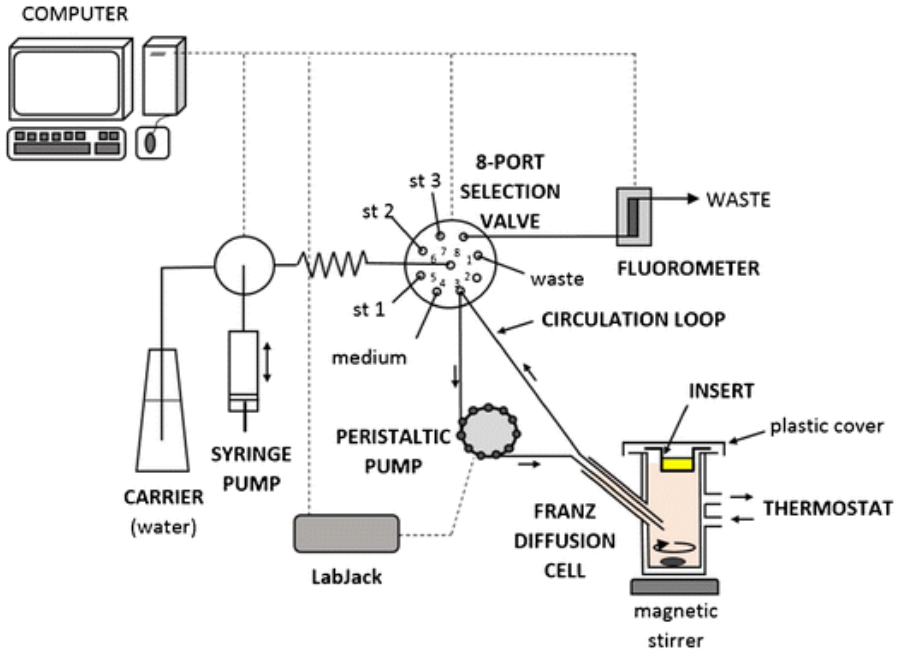

Figure 12: Scheme of the programmable flow system connected to Franz diffusion cell for automated monitoring of permeation test across a cell monolayer [14]. St: standard.

When using more FDC units, a time shift of sampling had to be involved. One possibility is to start at time zero with all units and use the measured points in shifted time intervals from respective units to obtain more detailed profile. The second possibility is to start with the first unit at time zero and to place the other units consequently with time delay. When using more units, the kinetic profiles have wider intervals between points, but the respective experiments are carried out under the same conditions.

When applying more substances in one permeation study, it can be beneficial to determine each substance alone. During experiments with Rhodamine 123 and inhibitors verapamil, quinine, and quinidine, we decided to implement a separation step prior detection. Some requirements, described more in chapter 4.1, had to be followed to carry out the separation on a short analytical column, considering matrix origin, sample volume, and system capabilities.

In work of Motz and co-workers, automated SIA system for propranolol dissolution from tablets and transport through Caco-2 cells was proposed [52]. Flow system, shown in Figure 13, consisted of two parts. Dissolution module incorporating USP apparatus 4 (D) and transport module incorporating flow-through cell with Transwell® insert (A) were

connected by a stream splitter and both attached to respective ports of 8-position valve of the SIA system. Transport module was subdivided into open apical (A) and closed basolateral (B) circulation compartments. Such apparatus enabled sampling from three ports – D, corresponding to propranolol dissolution, A, corresponding to propranolol concentration in contact with cells (apical compartment), and B, corresponding to propranolol apical to basolateral transport.

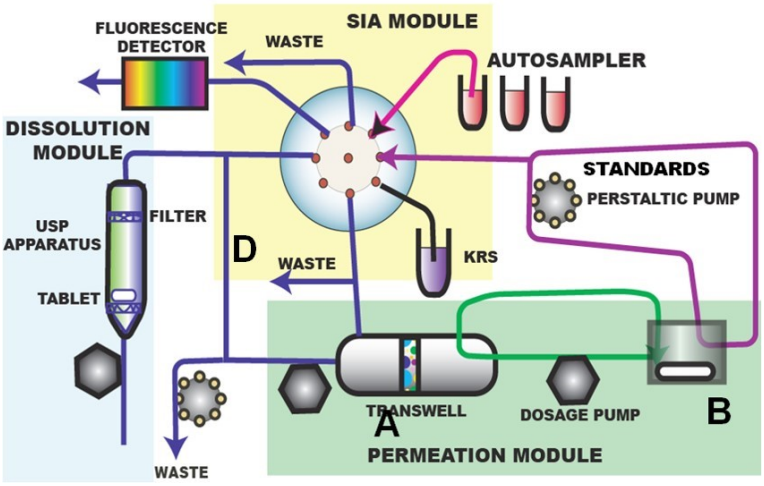

Figure 13: Schematic depiction of the automated apparatus for simultaneous measurement of dissolution and transport of propranolol [13].

Example of *in vivo* pharmacological model is transport through dually perfused rat placenta. Such experiment investigates the functional expression of placental drug transporters. Briefly, a catheter, inserted into the maternal and fetal arteries, is connected to the maternal and fetal solution reservoir, which are pumped through the catheters via a peristaltic pump.

Experiments including evaluation of drug efavirenz transport through rat placenta and its possible accumulation in placental tissue were part of the work described in chapter 4.2.

On-line Rhodamine 123 determination in perfusion experiments evaluating maternal-to-fetal transport was done by Sklenářová and co-workers using SIA with fluorometric detection [53]. Few more articles reported the use of FIA-chemiluminescent system in pharmacokinetic study of puerarin [54], streptomycin [55], paclitaxel [56], and matrine [57] in rat plasma. But due to the fact that the blood sample had to be treated first to obtain plasma, sample collection, preparation, and analysis were done individually and system was not fully automated as in case of the previously described work [53].

3.4. Sample preparation as a part of analytical process

It is well known that sample preparation, influencing all later steps of the analytical assay, is usually unavoidable as well as the most time-consuming step of the whole analysis [58]. The need of processing the increasing number of samples including various matrices resulted in the development of new configurations and approaches of well-established conventional sample preparation methods. Complex samples require analytical procedures typically involving sampling, sample preparation, separation, quantification, and data evaluation step while each step is critical to obtain correct results. Those steps have to be subsequently proceeded and thus any error in any step can influence the whole process [59].

Objective of the sample preparation step is both isolation and preconcentration of target analytes from complex sample matrices [58, 59]. Development in instrumentation enabled miniaturization, hyphenation of particular steps, automation, and coupling of analytical methods with minimal human intervention. This can eliminate possible errors and reduce the time and cost per analysis [58, 59].

Sample preparation techniques can be divided into conventional techniques and modern approaches, as described previously by Nováková for bioanalytical samples [58, 60] and shown in Figure 14. Conventional techniques comprehend direct extraction, protein precipitation, SPE, and LLE [58, 60]. Modern approaches comprehend microextractions based on modifications of conventional SPE and LLE techniques, highly selective techniques using molecularly imprinted polymers (MIP) or immunoaffinity SPE, and on-line techniques. Membrane-based extraction techniques [59, 61], missing in this overview, are other conventional sample preparation techniques enabling modern approaches involving on-line techniques as well as membrane-based microextractions [62, 63].

Direct extraction, SPE, and membrane-based extractions are sample preparation methods that were used in experimental works included in this thesis. Brief introduction of those methods together with their modern approaches and automation possibilities are described in following chapters.

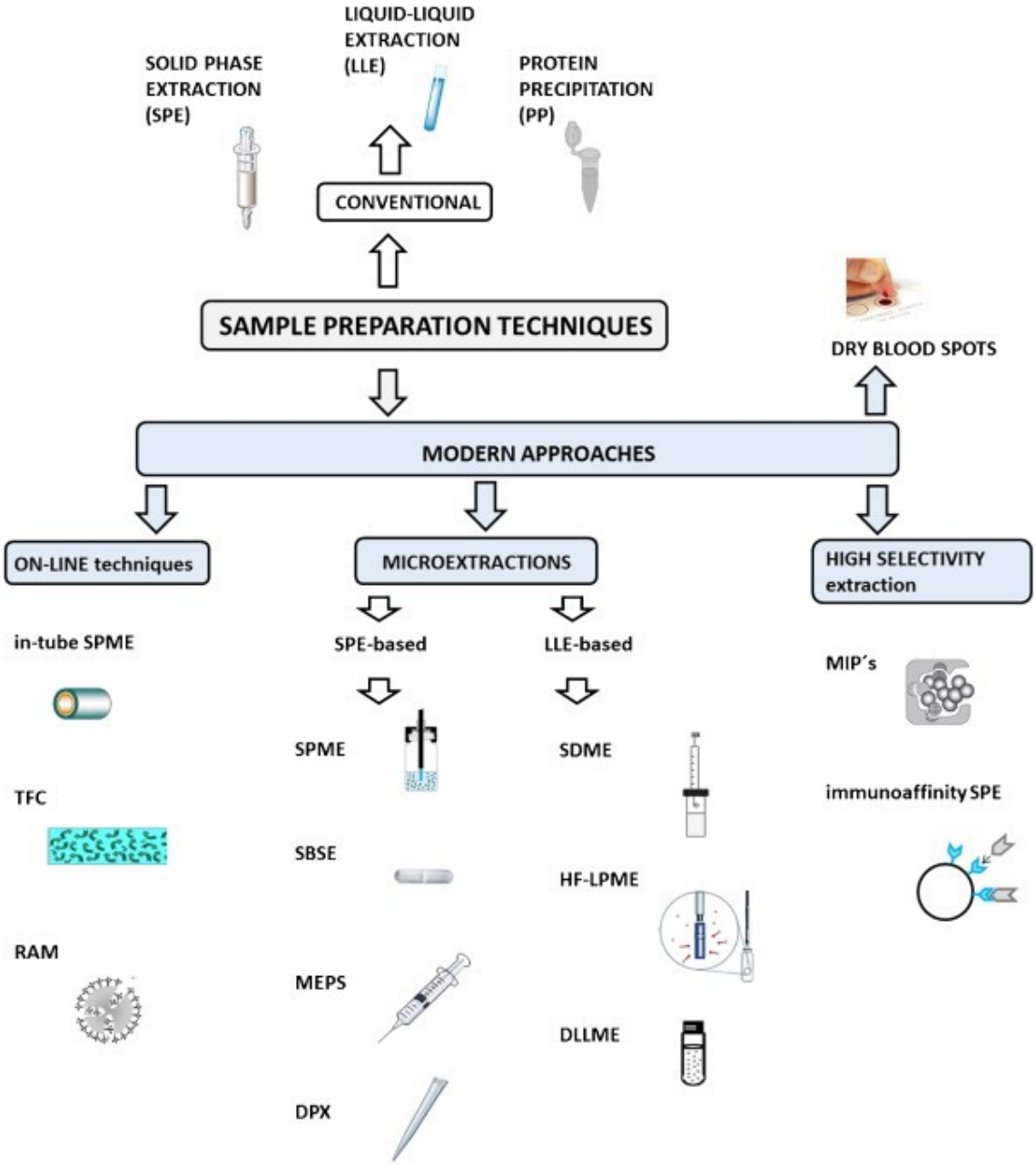

Figure 14: Sample preparation techniques overview. SPME: solid phase microextraction, TFC: turbulent flow chromatography, RAM: restricted access material, SBSE: stir bar sorptive extraction, MEPS: microextraction by packed sorbent, DPX: disposable pipette tips extraction, SDME: single drop microextraction, HF-LPME: hollow-fiber liquid phase microextraction, DLLME: dispersive liquid-liquid extraction, MIP´s: molecularly imprinted polymers. Reprinted with permission from [60].

3.4.1. Direct extraction into solvent

Direct extraction is usually applied with solid samples, for example tablets, food, soil, or plant material. The solid material is grounded into small particles, homogenized, and analytes are extracted into an organic solvent (e.g. methanol, acetonitrile) or its mixture with water or buffer, according to analyte properties. The smaller the particles the more efficient extraction can be achieved. Prior to further sample treatment, the liquid phase can be filtered or centrifuged to remove the solid material [64].

Lovastatin extraction into acetonitrile was used prior automated SPE of the analyte from dietary supplements. After homogenization of tablet/capsule content, powder was weighted, dispensed in acetonitrile, and ultrasound-assisted extraction was applied for 30 min, followed by 24 h of shaking on a platform agitator under laboratory temperature. The acetonitrile extracts were then filtered through PTFE filters and subsequently analyzed by automated SPE procedure [65], as described in chapter 4.4**Chyba! Nenalezen zdroj odkazů.**.

3.4.2. Solid phase extraction

Due to a high extraction efficiency, an analyte preconcentration, a low organic solvent consumption, and a possibility of automation, the SPE is a dominant technique in the sample preparation mostly used prior chromatographic separations. SPE uses analyte adsorption from the liquid sample onto a solid sorbent, usually in a form of a cartridge or a disc. Different retention mechanism take part according to analyte and sorbent type properties. SPE is a multi-step procedure and thus can be found time-consuming in a comparison with protein precipitation or LLE. 96-well plates format benefits from the low sample and solvent consumption, parallel sample processing, and robotic workstation for automation [58, 64].

Typically, these SPE steps follow one another, as shown in Figure 15: a) an SPE sorbent activation with an organic solvent, b) the SPE sorbent conditioning with an aqueous solvent corresponding to the sample matrix composition (water, buffer), c) sample loading and analyte capture onto the SPE sorbent, d) matrix washing out of the SPE sorbent, e) drying of the SPE sorbent with an air stream, f) elution of captured analyte with the organic solvent, g) eluate evaporation under a nitrogen stream, and h) its reconstitution prior analysis [58, 64]. Steps e, g, and h could be avoided, depending on a procedure.

SPE in conventional format was used as preparation of samples of placental tissue lysate in a work described in chapter 4.2.

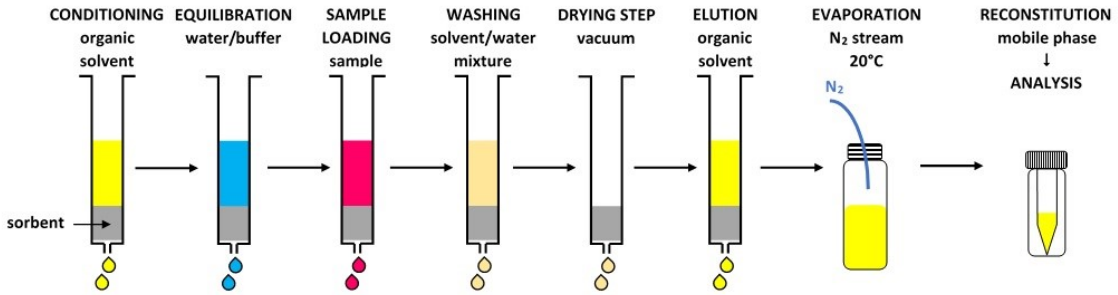

Figure 15: Solid phase extraction scheme.

Microextractions based on SPE mainly comprises miniaturized versions of the conventional SPE such as microextraction by packed sorbent (MEPS), micro-SPE in pipette tips, monolith spin extraction, stir bar sorptive extraction, dispersive SPE or solid phase microextraction. Turbulent flow chromatography, in-tube solid phase microextraction or application of restricted access materials represents on-line SPE approaches.

SPE is widely used in the flow techniques due to the analyte separation from matrix, minimization of interferences, and analyte preconcentration which all helps to improve determination sensitivity. In the flow systems, there are three strategies for SPE accomplishment: a) SPE sorbent packed in a column, b) miniaturized SPE in LOV-BI format, and c) implementation of sensors based on SPE [66].

SPE in the column format uses reusable SPE column commercially produced in a form of disc or 5/10 mm analytical guard column [67] or more often prepared in a laboratory by packing a cartridge with selected sorbent [27]. SPE protocol follows general SPE procedure with sorbent activation, conditioning, sample loading, matrix washing, and analyte elution followed by its direct detection. Column washing has to be efficient enough to prevent cross-contamination between processed samples [68].

When only SPE is automated, SPE column is usually placed prior detection cell, as shown in Figure 16. This assembly enables continuous monitoring of cartridge outlet even during sample loading or matrix washing steps.

SPE using home-made MIP, packed in a column placed within a SIC system for selective preconcentration and on-line determination of drug lovastatin in commercially available dietary supplements [65], was used in a work further described in chapter 4.4.

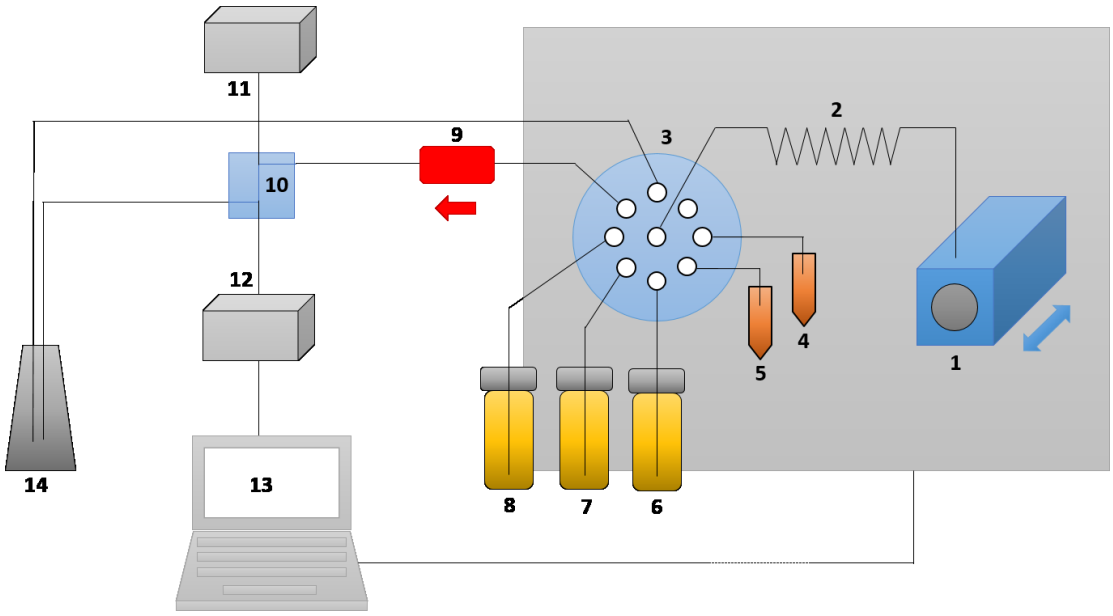

Figure 16: Sequential injection chromatography system used for automated extraction of lovastatin [65]. 1: syringe pump, 2: holding coil, 3: 8-port selection valve, 4: standard/sample, 5: blank, 6: conditioning wash, 7: matrix wash, 8: acetonitrile, 9: extraction column, 10: detection flow cell, 11: UV lamp, 12: UV detector, 13: computer, 14: waste.

When SPE is connected with separation, SPE column is usually placed at a side line and separation column is placed prior the detector as previously mentioned in chapter 3.2.2. Similarly to column-switching LC systems, mobile phase compatibility used for extraction and separation of analytes has to be verified.

At the work of Batista and co-workers [20], SIC system with additional selection valve, shown in Figure 17, was used for sulfonamide antibiotics determination by on-line coupling SPE and SIC separation. First, the SPE column was conditioned, then the sample was loaded. Elution of first six sulfonamides from SPE column and their subsequent separation on the chromatographic column was completed by the first mobile phase. Then, the second mobile phase was used for elution and separation of remaining two sulfonamides. Similar flow system, incorporating commercially available C18 MEPS cartridge for SPE and a monolithic C18 column for separation, was used by Šrámková and co-workers for determination of betaxolol in human urine [19].

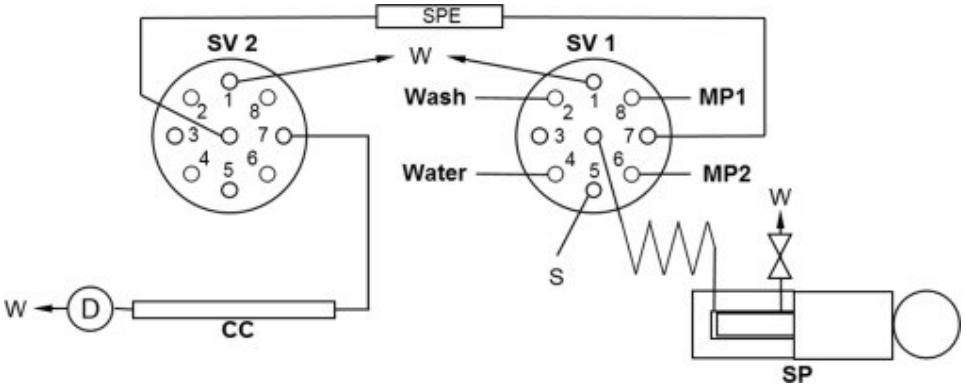

Figure 17: Scheme of SIC setup with on-line SPE for determination of sulfonamides. SV1 and SV2: selection valves; SP: syringe pump; CC: chromatography column; SPE: extraction column; S: sample, Wash: 0.1 mol L^{-1} $NaHCO_3$; Water: water; MP1: first mobile phase 1; MP2: second mobile phase 2; W: waste; D: spectrophotometric detector. Reprinted with permission from [20].

3.4.3. Application of nanoparticles in solid phase extraction

Nanoparticles (NP) as sorbent materials were also studied not only in flow systems. NP with different origins, such as carbon, silica, or metallic NP were used for extraction of various analytes [69]. NP characteristics such as large specific surface area, high adsorptive capacity, and chemical resistance are attractive. The use of NP in flow systems improves sensitivity due to higher analyte preconcentration compared to the common sorbents. Also, NP sorbent amount used for SPE is lower than that of the conventional sorbents with satisfactory extraction capacity. However, limitations of NP use are associated with possible aggregation of NP sorbent, a decrease of surface area and thus extraction efficiency, and pressure generation in the flow system. In continuous-flow configurations, this can be avoided by NP placement within a loop, for example instead of sampling loop, while pumping elution solvent in the opposite direction than the sample. Another possibility is to immobilize NP to avoid overpressure problems and loss of sorbent material as shown in Table 3.

Multi-walled carbon nanotubes (MWCNT) in their raw (r-MWCNT) and oxidized (ox-MWCNT) form, packed in a home-made column, were tested for preconcentration of herbicides metsulfuron methyl and chlorsulfuron from environmental water samples with the use of a FBA system [27]. This work is introduced in this thesis in chapter 4.3.

Table 3: Possible modes of nanoparticles (NP) immobilization considering their nature and possibility of packing preconcentration units. Adapted with permission from [69].

IMMOBILIZATION MODE	CARBON NP	METALLIC NP	SILICA NP
immobilization			✓
multilayer format	✓		
chemical modification		✓	✓
chemical bonding to support	✓	✓	✓
dispersion on inert particles	✓		
retention on filter	✓		
retention by the magnetic field		✓	

✓ - an indication of packing possibility in preconcentration unit

r-MWCNT tent to aggregate and entangle which cause reduction in their effective surface, formation of paths within the SPE column, and overpressure generation in flow system. All those difficulties influence the analytical performance, but can be minimized by r-MWCNT functionalization, for example by oxidation or non-covalent modification with surfactant, small aromatic molecules or ionic liquids. Manipulation with ox-MWCNT is easier due to more hydrophilic structure that prevents agglomeration and loses of adsorbent material which enables more reproducible column packing, decrease of overpressure within the system, and prolonged column life-time and reuse. Also, NP bonding to supportive material or dispersion of NP within inert particles could be another option for their use [69, 70].

From the experimental point of view it should be pointed out that: a) selectivity of MWCNT do not corresponds directly to the C18 sorbents, b) the column packing can be a tricky part of the experimental work, but use of short columns with wide diameter can simplify the work, c) ox-MWCNT are a user-friendly option due to their improved properties compared to r-MWCNT, d) water removal prior elution step in the SPE procedure improves the elution efficiency, and e) back-flush elution can improve the analyte desorption and prevent MWCNT material stacking.

A novel strategy was introduced by Ribeiro and co-workers when solid particles, instead of being packed into a column, were kept in a constant floating and circulation inside a mixing chamber of a multi-pumping flow system [71]. Flowing stream in a chamber was generated by pulses of a micropump. Troubles associated with packed

column use, such as back-pressure generation within the system, preferential solution pathway flow, or sorbent swelling, were thus minimized.

3.4.4. Selective SPE approaches

Application of MIP or immunoaffinity SPE represents highly selective approaches available in the SPE format [58, 64].

Immunoaffinity SPE uses SPE sorbent with immobilized specific antibodies for separation of target analytes from the sample matrix. This approach follows conventional SPE steps and is often used for monitoring of drug concentration level [64]. Antibodies stability and compatibility with solvents together with higher costs can be pointed out as drawbacks of this approach.

MIP as selective sorbents are specifically prepared for extraction of one analyte or group of analytes. Due to MIP preparation by a polymerization using monomers and template, schematically shown in Figure 18, tailor-made selective cavities for analytes are formed. Imprinting process is controlled by the preparation of a non-imprinted polymer using the same synthesis procedure as the MIP in the absence of the template molecule. SPE procedure using MIP sorbent (MISPE) follows conventional SPE steps and can be automated as well [64, 72, 73].

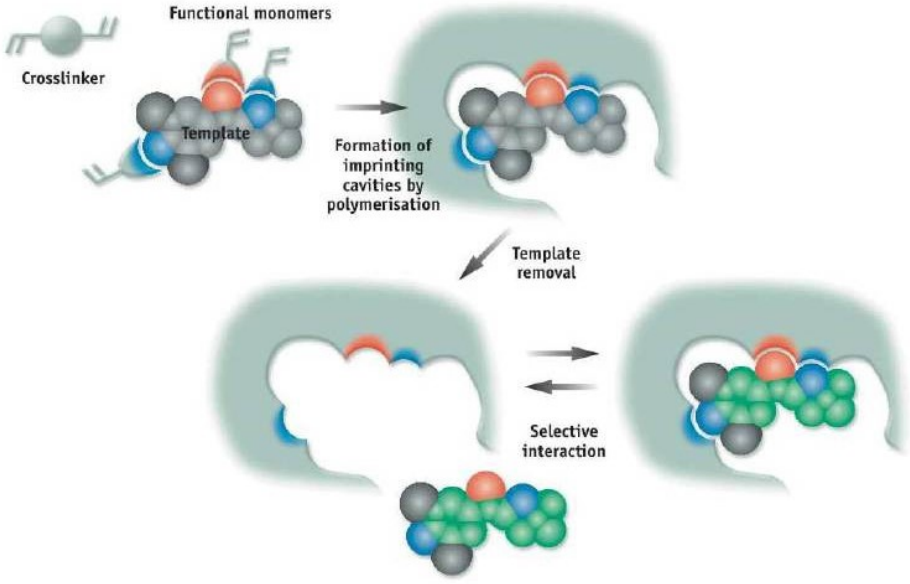

Figure 18: Scheme of molecular imprinting [74].

MIP sorbent selectivity is influenced by several factors such as a) selection of the template molecule (MIP for group of analytes vs. MIP for one analyte only), b) polymer composition (ratios of template, monomer, and cross-linker as well as porogen solvent composition), and c) the MISPE procedure itself.

Various configurations of MISPE were found in literature including columns packed with commercially available or laboratory-prepared sorbents [75, 76]; MIP monoliths prepared in a column [77]; MEPS technique using MIP sorbent in a cartridge [78] or in a needle [79]; immobilized MIP on a stir-bar [80] or MIP placed in a rotating disc [81]; MIP in a format of magnetic beads [82]; MIP-coated silica fibers [73, 80, 82]; and a MIP-coated sensor [83] and an electrode [84]. Also, MISPE was successfully applied for samples with different matrices such as drinking, agricultural, lake, or wastewaters [75, 76, 81]; food [85-87]; soil [88]; plasma or urine [77, 79]; and cosmetic samples [84].

MIP as a highly selective sorbents were applied in flow systems in two formats – solid extractors or flow through sensors [83]. Solid extractors are represented by packed MIP columns placed in a sampling line, sampling port, or in the analytical path towards detector in FIA and SIA systems. MIP columns work as adsorbents retaining the analytes for subsequent elution and detection. Sensors perform selective interactions with the analyte and simultaneous quantification at the same time.

With the proper template choice and synthesis process optimization, MIP could replace other SPE sorbents with the advantage of stability under higher temperature and pressure, higher mechanical resistance, possibility of home-made production, different selectivity than common sorbents, and manufacturing flexibility in the terms of new sorbent types/compositions towards target analytes. Alternative synthesis processes were developed to produce more selective and mechanically stable MIP on the sensor surface. Also, MIP preparation in a form of sensor chip meeting the concept of micro total analysis systems was described [83].

MIP sorbent, prepared at our laboratory by P. Svoboda, was used in automated MISPE determination of drug lovastatin in dietary supplements containing red yeast rice, as described in chapter 4.4. This sorbent was previously evaluated and tested under off-line conditions [89]. When adapting the off-line procedure into the flow system, MIP sorbent was filled in a cartridge prior detection in SIC system. MIP particles, previously cleaned from the dust, did not cause any problem in the off-line arrangement. In the on-line arrangement, sorbent had to be cleaned once more by sedimentation in pure acetonitrile to remove dust particles agglomerated on the surface of the MIP particles. After such treatment, it was possible to use the MIP-filled cartridge in the SIC system without generation of a back-pressure and to continue with procedure optimization.

3.4.5. Membrane-based techniques

Membrane filtration is one of the simplest membrane-based technique. Various types of filters differing in diameter (circular filters for solvent filtration, syringe filters for sample filtration, single step filter vials, or 96-well filtration plates), thickness, filtration area, pore-size (0.2 – 5.0 μm), and material/sorbent type, are nowadays on a market. Some of commercially available ones are summarized in Table 4. Filtration can be accelerated by application of negative pressure or supported by the centrifugation. Filtration can be used as a particular step of sample preparation after simple procedure such as direct extraction into a solvent, or prior sample injection into closed systems or instruments to prevent damage caused by particles.

Table 4: List of commercially available filter materials. Adapted from [90].

MEMBRANE MATERIAL (COMMON ABBREVIATION)	SOLVENT COMPATIBILITY	PROTEIN BINDING
regenerated cellulose (RC)	aqueous, organic	low
cellulose acetate (CA)	aqueous	low
mixed ester cellulose (ME)	aqueous, weak organic	high
cellulose nitrate (CN)	aqueous	-
poly(vinylidene fluoride) (PVDF)	aqueous, organic	low
polypropylene (PP)	aqueous, organic	-
nylon (NYL)	aqueous, weak organic	-
polyether sulfone (PES)	aqueous, weak organic	low
polytetrafluoroethylene (PTFE)	organic	-
polycarbonate (PC)	aqueous, weak organic	low

Some membranes allow passage of specific components based for example on physical state (gas-diffusion membranes), molecular weight (dialysis membranes), or hydrophobicity (e.g. PTFE or PVDF membranes).

In biological samples such as serum, plasma, or blood, proteins can be separated by application of filtration through a semipermeable membrane when only small molecules permeate through the membrane while macromolecules are retained [64]. According to the pore size, microfiltration (pores bigger than 10 nm), ultrafiltration (1 – 10 nm pores), and reverse osmosis (pores smaller than 1 nm) are distinguished.

Membrane extraction with sorbent interface, so-called liquid membranes (LM), are described in following lines. LM combine LLE with membrane-based techniques enabling both extraction and back-extraction of analytes in a single procedure [91]. Two types of units used in LM extraction, one with the supported liquid membrane (SLM) using porous membrane soaked with organic solvent and polymer inclusion membrane (PIM) using extractant incorporated within a polymer structure, are schematically shown in Figure 19.

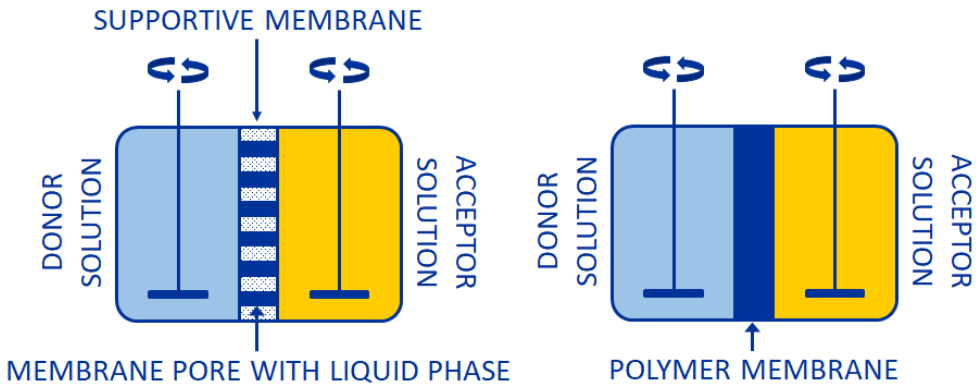

Figure 19: Schemes of extraction cells using supported liquid membrane (left) and polymer inclusion membrane (right).

SLM can be operated in two LLE modes. For hydrophobic species extraction, two-phase extraction with the hydrophobic membrane using the aqueous sample and the same organic solvent as membrane impregnator and acceptor solution can be applied. For extraction of ionic or charged species, three-phase extraction with the hydrophobic membrane using the aqueous sample, membrane impregnated with organic solvent, and aqueous acceptor solution can be used [92]. Due to retention of solvent in membrane pores by capillary forces, acceptor phase destabilization and leaching out of membrane can occur. Thus, long-term stability can be a problem of SLM [91].

PIM differs from SLM significantly. PIM is prepared by a dissolution of a base polymer, an extractant, and if necessary a plasticizer or a modifier, in an organic solvent to form homogeneous mixture. Examples of PIM components are shown in Figure 20 and Table 5. After membrane casting and solvent evaporation, the solid, transparent, and mechanically stable membrane is formed. This provides better membrane stability and easier manipulation over SLM [91]. Extractant leaching out can be observed similarly as in the case of SLM, but this can be influenced by the composition of the aqueous donor/acceptor phase. Also, due to the polymer structure, cellulose triacetate is sensitive to hydrolysis by strong acids/bases, and poly(vinyl)chloride (PVC) and poly(vinylidene

fluoride-co-hexafluoropropylene) (PVDF-HFP) suffer from dechlorination/defluorination under alkaline conditions [93].

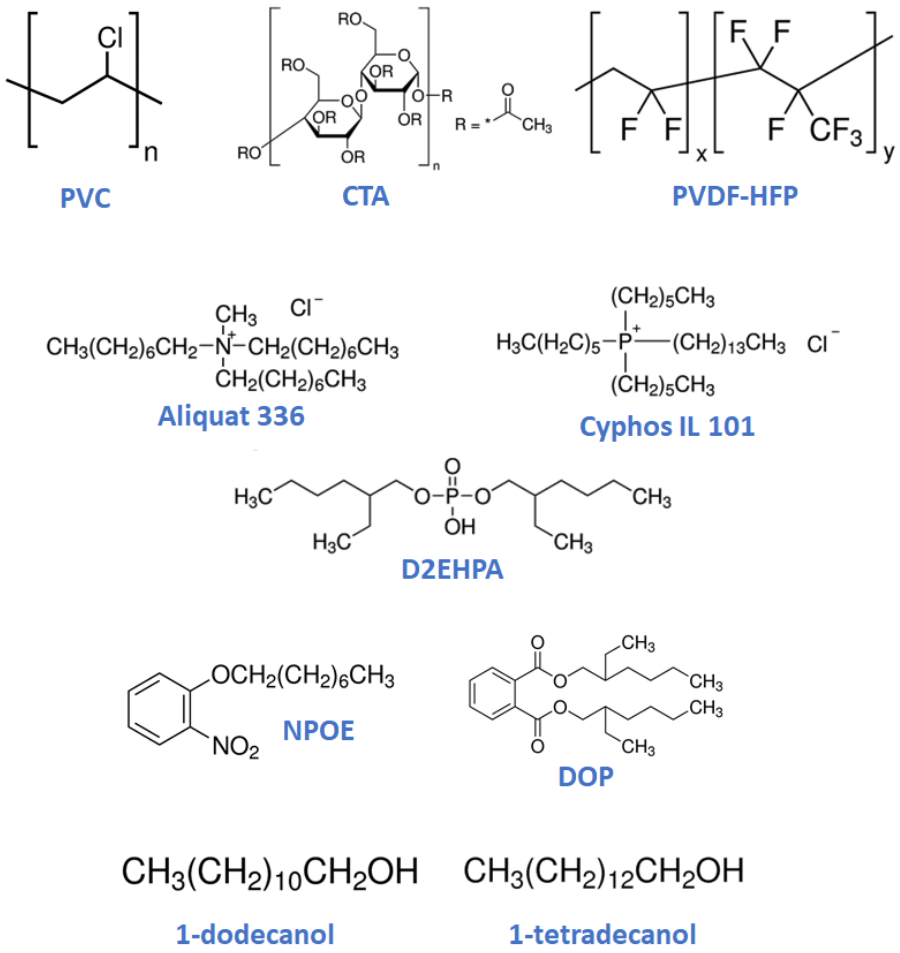

Figure 20: Structures of chosen substances used in the preparation of polymer inclusion membranes. Polymers: PVC – poly(vinyl)chloride, CTA – cellulose triacetate, PVDF-HFP – poly(vinylidene fluoride-co-hexafluoropropylene). Extractants: Aliquat 336, Cyphos IL 101, D2EHPA – di-(2-ethylhexyl) phosphoric acid. Plasticizers: NPOE – 2-nitrophenyloctyl ether, DOP – dioctyl phthalate. Modifiers: 1-dodecanol, 1-tetradecanol.

Table 5: Examples of polymer inclusion membrane (PIM) components [91, 93].

COMPONENT:	FUNCTION:	EXAMPLE:
ORGANIC SOLVENT	dissolution of membrane components	chloroform (cellulose-based), dichloromethane (cellulose-based), tetrahydrofuran (PVC[1]-, PVDF[2]-based)
BASE POLYMER	mechanical strength and support of PIM	PVC[1], cellulose derivatives – CTA[2], PVDF[3], PVDF-HFP[4]
BASIC EXTRACTANT	anion-exchanger responsible for anionic analyte extraction into PIM	quaternary ammonium compounds (Aliquat 336[5]), amines
ACIDIC EXTRACTANT	cation-exchanger responsible for cationic analyte extraction into PIM	organophosphorus acid esters (D2EHPA[6]), sulfonic acids, carboxylic acids
NEUTRAL EXTRACTANT	complexing agents with the strong solvating capacity	phosphoric/phosphonic acid esters, ionic liquids (Cyphos IL 101[7])
MACROMOLECULAR EXTRACTANT	selective complexing agents for metal ions	crown ethers, calixarenes, cyclodextrins
PLASTICIZER	increase of PIM flexibility and softness by reduction of intermolecular forces between polymer chains	2-nitrophenyloctyl ether (NPOE), bis(2-ethylhexyl) adipate, dioctyl phthalate, dibutyl sebacate
MODIFIER	solubilization of extracted species within PIM, overcoming of third-phase formation	1-dodecanol, 1-tetradecanol

[1] poly(vinyl)chloride

[2] cellulose triacetate

[3] poly(vinylidene fluoride)

[4] poly(vinylidene fluoride-co-hexafluoropropylene)

[5] trioctylmethylammonium chloride, mixture of C_8 and C_{10} chains with C_8 predominating

[6] di-(2-ethylhexyl)phosphoric acid

[7] tetradecyl(trihexyl)phosphonium chloride

The first applications of PIM are dated back to 70´s when used as sensing components of ion-selective electrodes and optodes. In sensing, fast ion-exchange and low ion transport are required, thus the extractant (so-called ionophore) amount is around 1 - 2 wt%. In extraction, fast ion-exchange together with high ion transport is required and thus extractant (so-called carrier) amount is usually higher than 20 wt% [94].

In sample preparation, extraction and/or preconcentration of an analyte using PIM can be achieved. Traditionally, PIM experiments were done off-line by placing the membrane into donor/acceptor solution with manual sampling. To improve PIM extraction, extraction cells comprising PIM in a sandwich format between donor (sample) and acceptor solution were used. Another possibility to increase extraction efficiency is to apply an electric field. This approach, so-called electromembrane extraction, uses electromigration of target species as a driving force [92]. This approach was used for determination of herbicide glyphosate, commercially available as RoundUp formulation, in water samples by See and co-workers [95].

PIM found its application also as samplers using passive diffusion of analytes through the PIM, so-called passive sampling, mainly in environmental chemistry field [94]. Passive sampling enables analyte collection and preconcentration in one step at the sampling site, e.g. drains, lakes, rivers, or seasides. The sampler is composed of a solution reservoir filled up with acceptor solution and tightly sealed with a membrane. The sampler is immersed into the sample to ensure contact with membrane [94].

3.4.6. Membrane-based techniques in flow systems

On-line sample filtration can be incorporated in the flow systems easily by placing a disc filter into the sampling line to filter the sample, possibly containing small particles, during its aspiration into the system [40, 96]. This was applied in analysis of environmental water samples in work described in chapter 4.3. To prevent carry-over between samples, back-flush of the filter can be used, and to prevent back-pressure generation in the system, periodic replacement of the filter should be done [97].

Membrane separators were already mentioned in previous chapter. For separation of gases, gas-diffusion unit comprising gas-permeable membrane is usually used. For gas-liquid separations, a porous Teflon membrane forming an air-gap barrier between solution and air is one possibility. In automated LLE in flow systems, Teflon membranes are used to promote phase separation as hydrophobic pores allow passage of the organic solvent. In dialysis, hydrophilic membranes with controlled pore size

for exclusion of analytes are used. Membranes, e.g. SLM or PIM, can be also applied as sampling devices for selective preconcentration of target species [13].

Generally, all those membrane separators have similar construction. As shown in Figure 21, the membrane is placed between two blocks with the flow path. The blocks are tightened together by screws or clamps. Donor stream (sample/standard/reference material) circulate on one side of the membrane while analytes permeate through the membrane into the acceptor solution on the other side.

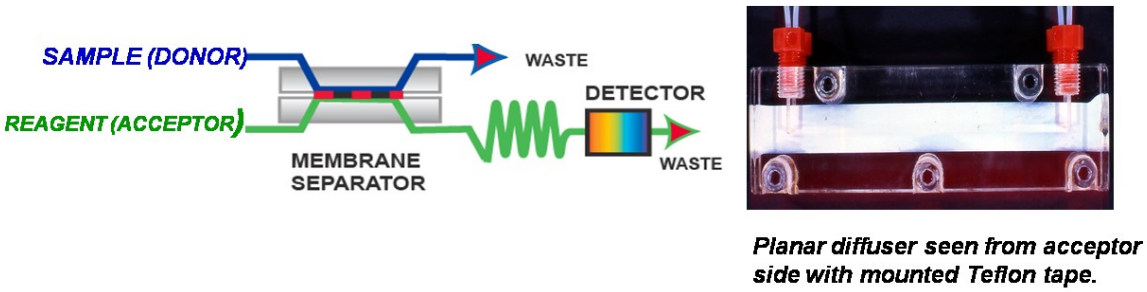

Figure 21: Membrane separator [13].

Automation of extraction with porous supports and impregnated membranes, creating a large and reproducible surface for contact of donor and acceptor phases by filling membrane pores with an organic solvent, were thoroughly discussed in a review from Alexovič and co-workers [92].

SLM was implemented in flow systems in FIA and SIA, and few works also used an on-line coupling with GC or LC [92]. For example, total phenols determination in oil by FIA [98], caffeine determination in solid and slurry coffee and tea by FIA [99], anionic surfactants determination in detergents by FIA [100], local anesthetics determination in plasma by capillary GC [101], or cationic surfactants determination in river and wastewater by SIA-LC [102].

Extraction selectivity can be enhanced by the addition of extractant into a solvent for membrane impregnation, as was used for lanthanides determination [103], or by combination with immunologic recognition, so-called immune-SLM-extraction. Using this approach, 2,4,6-trichlorophenol was determined in wastewaters using immuno-SLM extraction followed by fluorescence flow immunoassay [104], 4-nitrophenol in wastewaters using a fluorescence flow immunoassay in SIA [105], or progesterone in saliva using magnetic immune-SLM assay [106].

PIM extractions were implemented within flow systems as well, using mainly FIA systems and flow-through extraction cell. Extraction cell formed by a membrane placed between two blocks with channels or by a membrane placed between two spacers with channel placed between two blocks can be used. All parts must be tightened by screws or clamps to prevent liquids leakage.

Such system was used for determination of zinc in pharmaceuticals and samples from galvanizing industry by Zhang and co-workers [107] or by Nagul and co-workers for trace phosphate determination in natural waters [108]. This system configuration, shown in Figure 22, could be operated in two modes – FIA and continuous flow – allowing either preset sample volume injection or continuous sample introduction in predefined time interval [108].

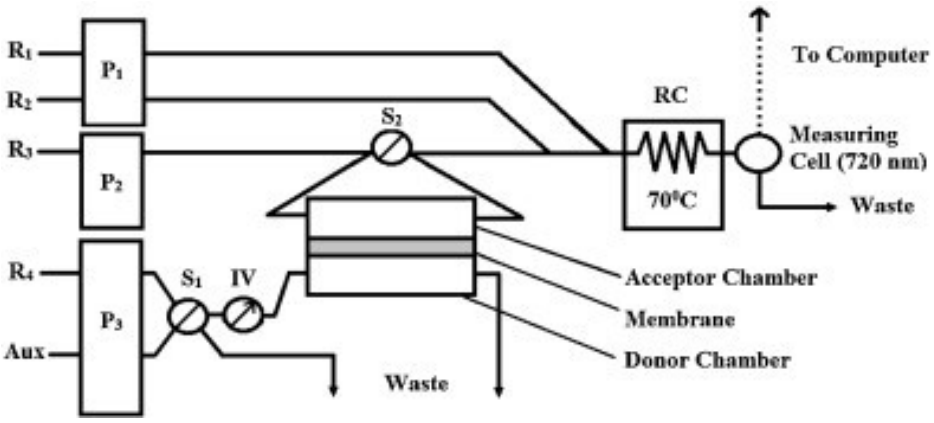

Figure 22: Schematic of the flow analysis manifold for phosphate determination. P_1, P_2, P_3: reagent, acceptor, and donor line pumps; S_1, S_2: selection valves; IV: sample injection valve; RC: reaction coil; R_1: reductant stream; R_2: molybdate stream; R_3: acceptor stream; R_4: deionized water; Aux: auxiliary stream (used only in the continuous flow method). Reprinted with permission from [108].

Kahina and co-workers used a micro-channel cell (see Figure 23) with the volume of only 164 µL with flat sheet membrane for cadmium determination [109].

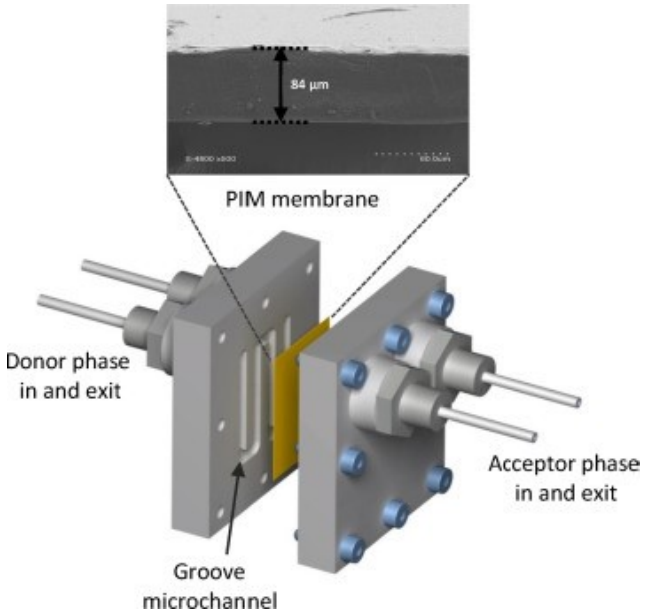

Figure 23: Membrane extraction micro-device based on the PIM sandwiched between two serpentine micro-channels built in Teflon plates. SEM micrograph of the PIM cross-section. Reprinted with permission from [109].

4. WORKS INCLUDED IN DISSERTATION THESIS

Dissertation thesis is presented as a commented collection of four original research papers and one unpublished project dealing with automation of analytical procedures with the use of various modifications of flow systems, mainly sequential injection analysis, except the work describing efavirenz determination by HPLC-UV method in the pharmacological study. Four articles were published in peer-review international journals with impact factor and one project is currently being finished and prepared for publication, but the experimental results are presented as a part of the dissertation and enclosed as supplementary material. The candidate is author of three papers and co-author of one paper. Author contributions to the works follow.

1. Fully automatic flow-based device for monitoring of drug permeation across a cell monolayer (Supplement 1)
L. Zelená, S. S. Marques, M. A. Segundo, M. Miró, P. Pávek, H. Sklenářová, P. Solich
Analytical and Bioanalytical Chemistry 408 (2016) 971-981
IF_{2015}: 3.125
Times cited: 2, JCR category: analytical chemistry, Quartile in category: Q1,
Rank in category: 16 of 81 (from the Web Of Science Core Collection, 30.03.2019)
Candidate´s contribution: supervising of master student S. Marques, carry out of the experiments, data processing, writing and revisions of the manuscript.

2. Universal efavirenz determination in transport study, rat placenta perfusion and placenta lysate by HPLC-UV (Supplement 2)
L. Zelená, J. Řezníček, M. Čečková, H. Sklenářová
Journal of Pharmaceutical and Biomedical Analysis 137 (2017) 70–77
IF_{2016}: 3.255
Times cited: 1, JCR category: analytical chemistry, Quartile in category: Q2,
Rank in category: 23 of 81 (from the Web Of Science Core Collection, 30.03.2019)
Candidate´s contribution: carry out of the experiments (HPLC method optimization and validation, efavirenz determination in real samples from the pharmacological study), data processing, writing and revisions of the manuscript.

3. An integrated on-line method for the preconcentration and simultaneous determination of metsulfuron methyl and chlorsulfuron using oxidized carbon nanotubes and second order fluorescent data (Supplement 3)
C.C. Acebal, M. Grünhut, N.E. Llamas, M. Insausti, L. Zelená, H. Sklenářová, P. Solich, B.S. Fernández Band
Microchemical Journal 129 (2016) 90-97
IF_{2015}: 2.893
Times cited: 4, JCR category: analytical chemistry, Quartile in category: Q2,
Rank in category: 24 of 81 (from the Web Of Science Core Collection, 30.03.2019)
Candidate´s contribution: carry out of the experiments (metsulfuron methyl preconcentration) during 6-weeks study stay, manuscript comments and revisions.

4. Fully automated method based on on-line molecularly imprinted polymer solid-phase extraction for determination of lovastatin in dietary supplements containing red yeast rice (Supplement 4)
L. Novosvětská, P. Chocholouš, F. Švec, H. Sklenářová
Analytical and Bioanalytical Chemistry 411 (2019) 1219-1228
IF_{2017}: 3.307
Times cited: 0, JCR category: analytical chemistry, Quartile in category: Q1,
Rank in category: 16 of 81 (from Web Of Science Core Collection, 30.03.2019)
Candidate´s contribution: carry out of the experiments, data processing, writing and revisions of the manuscript.

5. Sequential injection determination of 2,4-dichlorophenoxyacetic acid herbicide using preconcentration with a polymer inclusion membrane (Supplement 5)
L. Novosvětská, M. I. G. S. Almeida, R. W. Cattrall, H. Sklenářová, P. Solich, S. D. Kolev
Unpublished project
Candidate´s contribution: carry out of the experiments (4-months research stay at the University of Melbourne, Australia; work continuation at the Faculty of Pharmacy, Hradec Králové), data processing, writing and preparation of the reports as bases for manuscript, work introduction to S. Zatrochová (PhD student proceeding with the project).

4.1. Fully automatic flow-based device for monitoring of drug permeation across a cell monolayer

Automation and on-line monitoring of cell permeation studies were realized as a joined project with colleagues from Department of Chemistry, Faculty of Pharmacy, University of Porto, colleague from Department of Chemistry, University of Balearic Islands, and colleagues from Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University. This project was also supported by the Grant Agency of the Charles University as a GAUK project 159415.

This work follows the conventional batch-wise procedure of permeation studies widely used in pharmacology and toxicology for evaluation of: a) drug absorbance/transport, b) drug-transporter interaction, or c) drug-drug interactions.

In this work, transport of a fluorescent dye Rhodamine 123 (Rho123) was used as a marker for evaluation of a function of a membrane transporter P-glycoprotein. Transport of Rho123 alone or in combination with verapamil, an inhibitor of P-glycoprotein, was studied. Structures of both analytes are shown in Figure 24.

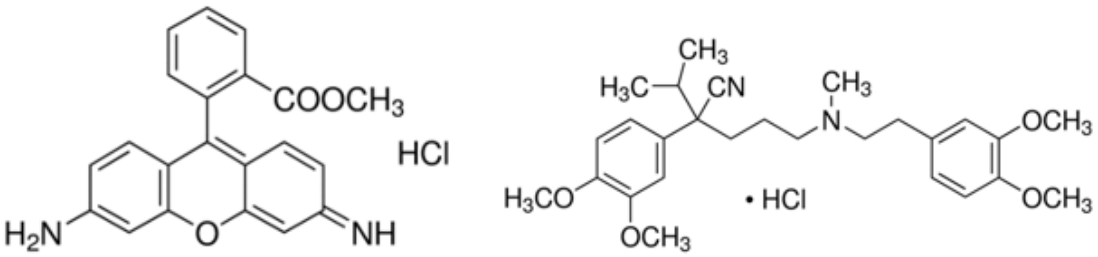

Figure 24: Structure of Rhodamine 123 (left) and verapamil hydrochloride (right).

Flow-based apparatus consisted of sequential injection instrument MicroSIA with a fluorometric detector connected via a circulation loop to FDC. FDC was filled with OptiMEM® cultivation medium that served as an acceptor solution. Transwell® insert with cultured cell monolayer and filled with marker solution was placed in FDC. Circulation of OptiMEM® medium was ensured by a stirring of the solution in FDC and by medium circulation within the circulation loop via auxiliary peristaltic pump. Detailed scheme of the circulation loop and FDC is shown in Figure 25.

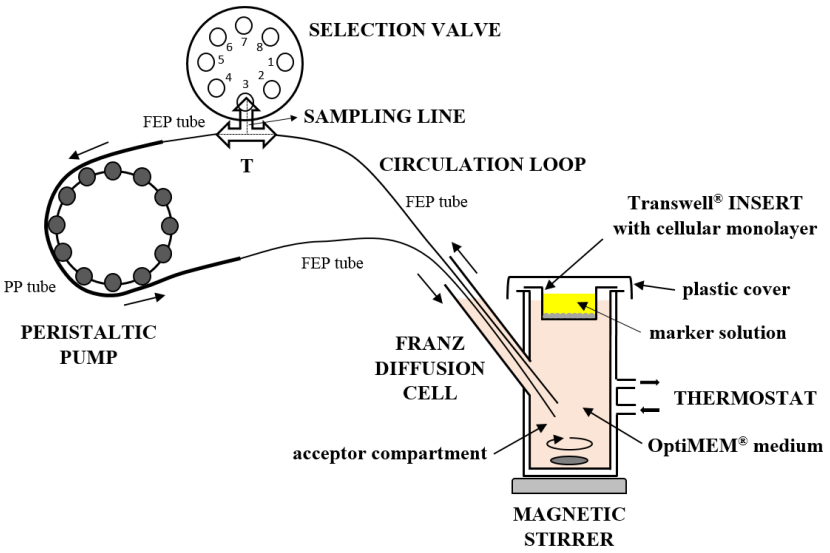

Figure 25: Detailed scheme of the circulation loop integrated in the automatic flow system and Franz diffusion cell [14]. PP tube: peristaltic pump tube, FEP: fluorinated ethylene propylene tube, T: T-connector, 1-8: ports of MicroSIA selection valve.

Samples were analyzed in 10 min intervals and after 4 hours the kinetic profile of Rho123 transport was obtained. Pharmacokinetic assay was carried out with cellular monolayer of MDCKII-MDR1 cells (Madin-Darby canine kidney type II cells transfected with human P-glycoprotein). Inserts with the cellular monolayers were incubated in a laboratory at the Department of Pharmacology and Toxicology and transported to our laboratory at the day of experiment. Rho123 passive diffusion through cell-free insert was fast and thanks to Rho123 yellow color also visible which enabled to identify monolayer disruption if occurred.

Three types of protocols, shown in Figure 26, were used. Protocol A: Rho123 alone where no significant fluorescence signal increase was expected. Protocol B: Rho123 with addition of verapamil where signal increase was expected due to inhibition of P-glycoprotein by verapamil and easier transport of Rho123 into FDC. Protocol C: Rho123 with verapamil addition after 30 min from the start of the experiment where signal increase was expected with time delay.

As can be seen from Figure 27, a difference in signal increase was observed between protocols B and C where verapamil was added at different times. In protocol B, Rho123 and verapamil were added at the same time and signal increase occurred from the start of experiment. In protocol C, signal increase occurred approximately after 60 min. This is most likely explained by the previous binding of Rho123 to the transporter prior delayed verapamil addition in the contrast to the protocol B where direct competition of both substances occurs from the start of the experiment.

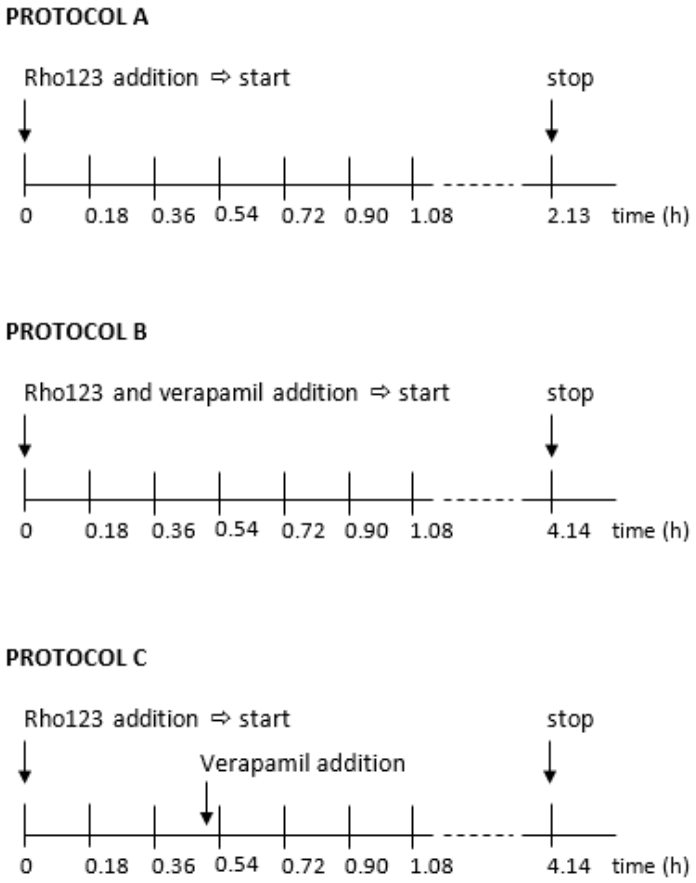

Figure 26: Schemes of protocols used in pharmacokinetic assays with Rhodamine 123.

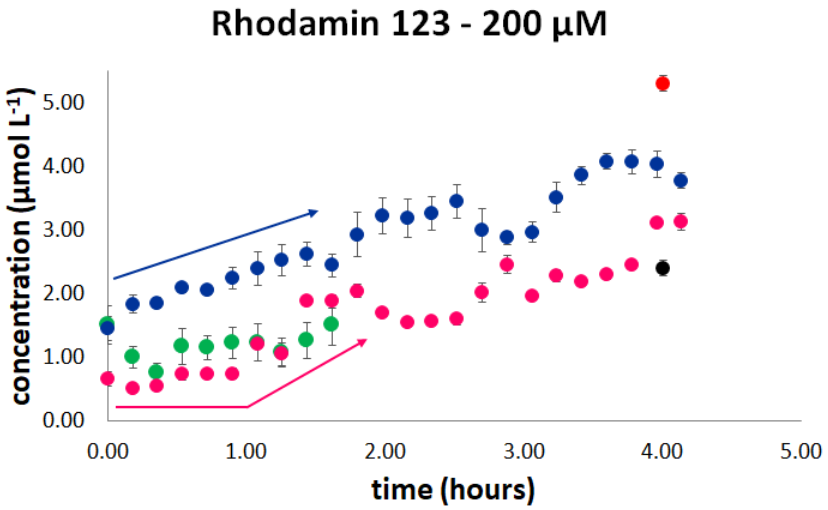

Figure 27: Kinetic profiles of Rhodamine 123 transport using protocol A (●), B (●), and C (●). Comparison with batch-wise results corresponding with protocol A (●) and B (●).

Presented work enabled real time Rho123 concentration measurement based on on-line sampling from FDC followed by detection within MicroSIA system with temperature control necessary for cellular viability during experiments. Experimental results proved that the method is comparable with batch-wise experiments (see Figure 27) with the benefit of more detailed kinetic profile monitoring in a real time. Also, the first 10 points of measurement could be used for inhibition evaluation in case of application of the marker and inhibitor at the same time.

On one side, the described flow system showed possibility of time-based procedure automation including automated sampling from/re-filling of liberation unit together with marker real time detection. On the other hand, the same system enabled only a single permeation experiment at the time which meant time-consuming experiments to obtain enough experimental data. Therefore, further research done in this field is described as well even though the work has not been summarized and published in a scientific manuscript yet.

Further steps in this research work were: a) to set up a multiple system for real-time monitoring of permeation in parallel using three FDC units, b) to use automated system for evaluation of Rho123 transport using different P-glycoprotein inhibitors (verapamil and quinidine), and c) to use the automated system for evaluation of Rho123 transport testing different cell lines.

MicroSIA system had to be slightly modified to connect three FDC units. This time, three single-wall glass FDC units were placed in a plastic holder into a water bath placed on the multi-position magnetic stirrer, as shown in Figure 28. Thermostat was placed in the water bath to maintain the temperature of 37°C necessary for experiments with living cells. Circulation loops were constructed exactly in the same way as previously placing the T-connectors to the port 3, 5, and 7 of the selection valve. Other ports were used as connections to the detector, the waste, the OptiMEM® medium reservoir, and the reservoir of Rho123 standard solutions for calibration curve determination. The whole system is shown in Figure 29.

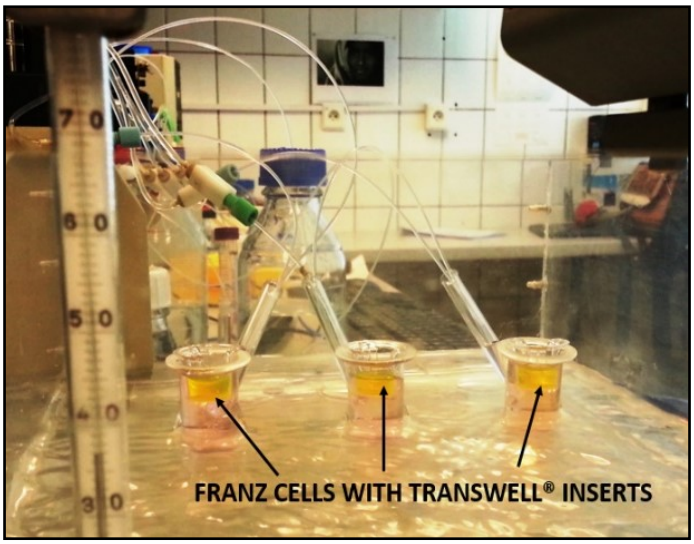

Figure 28: Detailed photo of three Franz diffusion cells placed in a water bath.

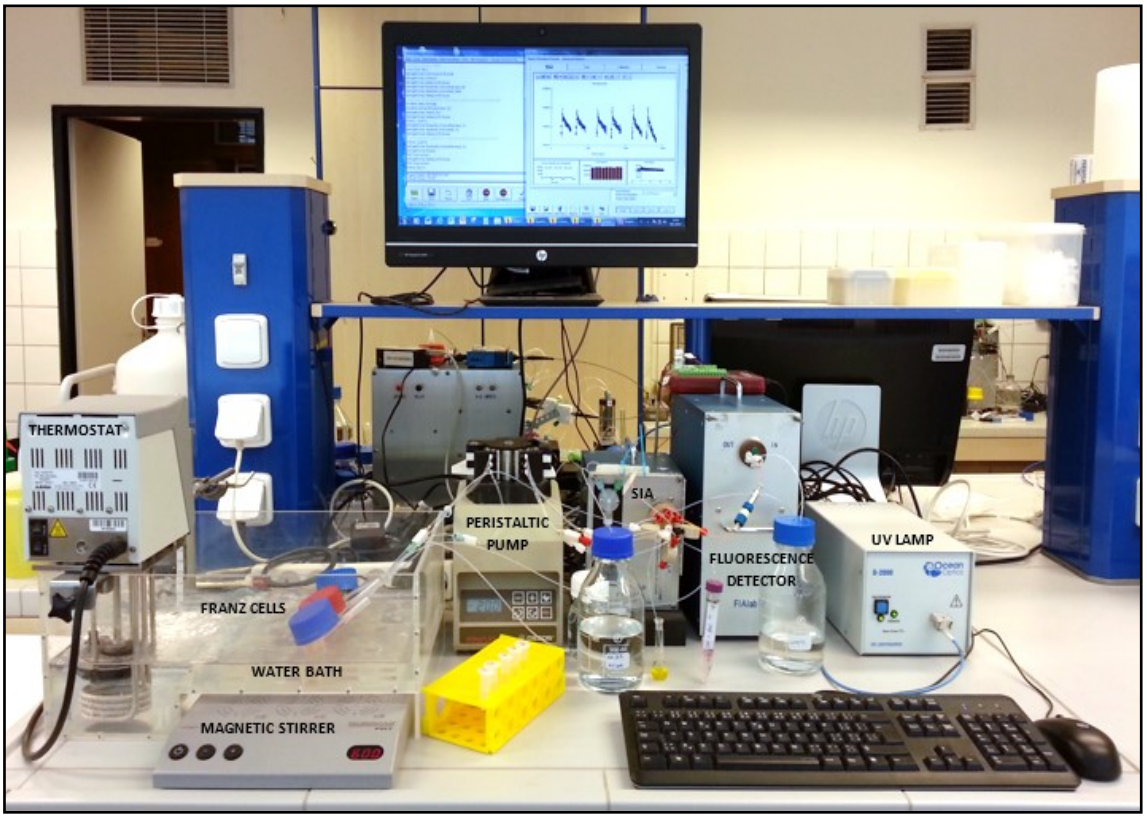

Figure 29: Photo of MicroSIA system with three Franz diffusion cell units.

When using three FDC units, two options of sampling are available. First one when all inserts are placed into FDC units at the beginning of the experiment and each sampling from FDC 1, FDC 2, and FDC 3 corresponds to the different time interval. The second option, used at our work and shown in Figure 30, is to consequently place the inserts and measure from time zero at each FDC.

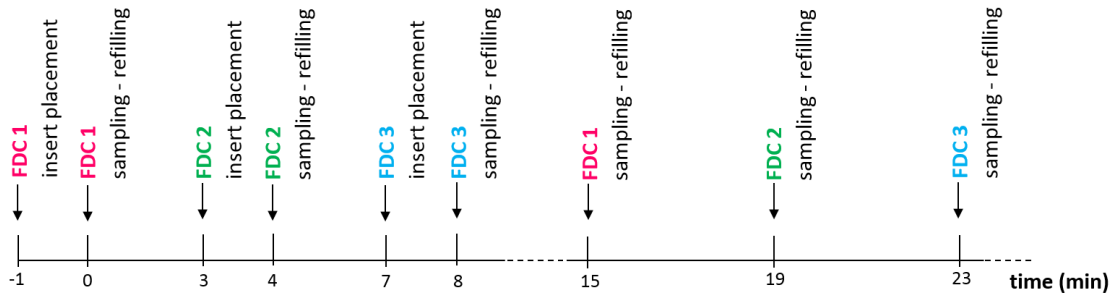

Figure 30: Scheme of sampling from the system using three Franz diffusion cells.

Separation of the analytes and matrix was a further step in this work to ensure that the evaluated fluorescence signal: a) corresponds to the marker itself or to the substances used in the experiments and b) is not influenced by the matrix interference from OptiMEM® medium or inhibitor transported through cells. SIC system SIChrom™ was used for this application placing the separation column in between the selection valve and the detection cell.

Some requirements for such separation had to be followed: a) carrying out separation in a medium-pressure system using short analytical column, b) separation of marker and inhibitors with max. analysis time of 5 min, c) sampling from FDC, separation and detection of analytes in triplicate within max. 15 min interval, d) exclusion of medium matrix and DMSO interference, e) maximal mobile phase volume of 3.5 mL according to the syringe pump volume limit, and f) maximum of 50% (v/v) of organic modifier in the mobile phase as prevention of medium components precipitation. Partial results from separation experiments can be found in Supplement 1B.

Flow system arrangement comprising liberation unit, enabling automation of the transport experiments, and separation column, enabling more precise evaluation of tested substances content, shown versatility and variability of flow techniques that was used in dealing with this experimental task.

4.2. Universal efavirenz determination in transport study, rat placenta perfusion and placenta lysate by HPLC-UV

This work, dealing with the determination of antiretroviral drug efavirenz in a pharmacological study, was a joined project with colleagues from Group of Experimental Pharmacology and Drug Interactions, Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University.

Work was divided into two parts. At first, fast and universal chromatographic method with UV detection (HPLC-UV) was developed and validated for efavirenz (see Figure 31) determination in three different complex matrices. Second, the validated method was used for the analysis of real samples obtained during the transport experiments on cellular monolayers, rat placenta perfusion experiments, and from rat placenta tissue lysates. Experiments with cell cultures and rats were carried out at Department of Pharmacology and Toxicology and collected samples were then analyzed at Department of Analytical Chemistry. Pharmacological characteristics were calculated and evaluated from the chromatographic data. During experimental work, more than 800 real samples were processed.

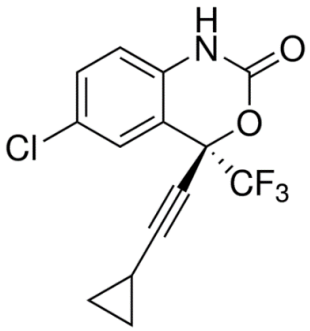

Figure 31: Structure of antiretroviral drug efavirenz.

During HPLC-UV method development there were a few requirements that had to be fulfilled: a) fast analysis in terms of series of samples that had to be processed after experiments with cells or rats (ranging from 25 to 80 per day), b) simple preparation of matrix samples with low volume (cultivation medium for cells – 50 µL, Krebs buffer for perfusion – 100 µL, placenta lysate – 1 mL), and c) universal method for three types of sample matrix.

Optimization of chromatographic conditions included mobile phase composition, flow rate, sample volume, and internal standard (IS) selection. Using Kinetex® C18 (50 × 2.1 mm, 2.6 µm) analytical column, analysis of 2 µL of the sample took 2 min using

65% acetonitrile as a mobile phase under 0.3 ml/min flow rate, laboratory temperature, and detection at 245 nm. Using Discovery® HS C18 (150 × 4.6 mm, 5 µm) analytical column, analysis of 10 µL of sample took 5 min using 65% acetonitrile as a mobile phase under 1.6 ml/min flow rate, laboratory temperature, and detection at 245 nm. When analytical column damage and lifetime was considered calculating approximate matrix samples amount, Discovery® HS C18 column with higher pore size was chosen despite the longer time of analysis and higher sample/solvent consumption.

Type A samples were obtained from *in vitro* transport assays on cellular monolayers and used for interaction evaluation of efavirenz with ABC transporters, a group of human transporters involved in the cellular transport. Sample clean-up was limited by the sample volume of 50 µL and matrix composition of OptiMEM® medium including nutrients, trace elements, and grow factors for cells. Filtration on 96-well microplate format was considered as an option. Minimal volume for such treatment is 150 to 200 µL to have enough sample after filtration, which in this case requires 3- to 4-fold sample dilution and 96-well plate autosampler to avoid transfer of filtered sample back into vials.

Thus, sample filtration using 50 µL of the matrix in small syringe filters accelerated by centrifugation (see Figure 32) was tested. High loss of analyte and minimal matrix removal using four membrane materials (PVDF, PTFE, nylon, cellulose) was found insufficient for such sample preparation.

Finally, 50 µL of standard/sample was mixed with 50 µL of IS β-estradiol 17-acetate solution in pure acetonitrile and injected directly into 5 µm pore analytical column. Real sample analyses did not indicate any contribution of tested human transporters to efavirenz transport.

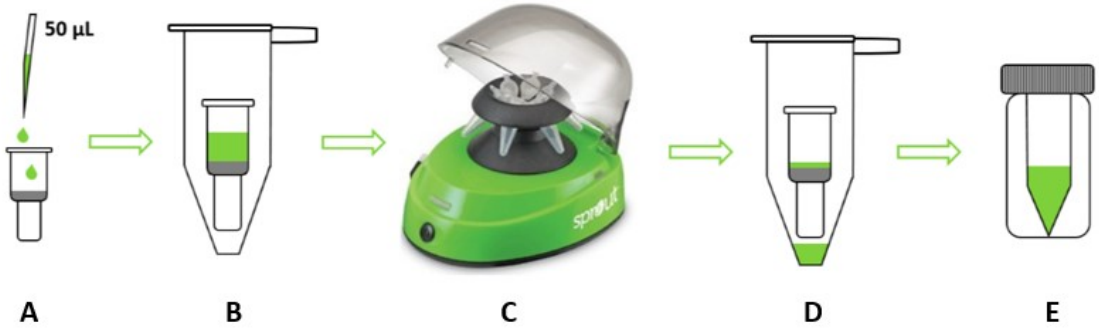

Figure 32: Scheme of filtration in syringe filters accelerated by centrifugation. A) sample transfer into syringe filter, B) syringe filter placement into the Eppendorf tube, C) 5 min centrifugation at Sprout Mini centrifuge, Heathrow Scientific, USA, D) syringe filter removal, E) filtered sample transfer into the vial with insert prior HPLC-UV analysis.

Type B samples were obtained from *in vivo* placenta perfusion experiments and were used for confirmation of *in vitro* results at the organ level. Krebs solution, a buffer containing salts and maintaining pH during perfusion experiment, was the matrix. After dilution of Krebs solution with the IS solution in pure acetonitrile, precipitation occurred. β-estradiol 17-acetate revealed limited solubility in both water and 50% acetonitrile, so it was not possible to follow sample A preparation. No compound was found suitable as IS and thus the validation was carried out without IS. A 10 min gradient elution was incorporated in the analysis sequence when samples in Krebs matrix were analyzed. Surprisingly, a large decrease of efavirenz was observed in the fetal side of the placenta suggesting fetal-to-maternal efavirenz transport which was in a contrary with *in vitro* results obtained on ABC transporters. Perfusion experiments incorporating various inhibitors of ABC transporters were carried out, but efavirenz decrease was observed again. Thus, experiments with lysates of placenta tissue followed.

SPE procedure was developed to determine efavirenz in type C samples, rat placenta lysates prepared from the perfused placental tissue. In this case, the matrix contained disrupted tissue, lysis agent, and efavirenz. Sample volume was higher (approx. 1 mL) and number of samples to be processed was lower in comparison to samples A and B. Developed SPE procedure consisted of usual protocol using C18 cartridges, pure acetonitrile, 30% acetonitrile, and water as solvents. Eluate evaporation under nitrogen stream and reconstitution in mobile phase after SPE followed.

Five lysis approaches using various agents and efavirenz stability in lysates were tested. Application of 0.02% sodium dodecyl sulfate solution for placenta lysis was chosen due to minimal changes in efavirenz concentration during both lysis and SPE procedure. No detectable efavirenz amount was found in type C samples, which supported the results from *in vitro* experiments.

Organ-free perfusion experiments, maintaining the experimental design but excluding the rat placenta, shown efavirenz concentration profile very similar to the one from *in vivo* experiments. Efavirenz binding onto the surface of PVC cannula tubes, used during perfusion experiments, was confirmed when high efavirenz amount was detected after washing the tubes with pure methanol.

From our analytical point of view, easy, fast and universal HPLC-UV method for efavirenz determination in three matrix types was developed, validated and used in the pharmacological study. However, due to the proven efavirenz adhesion to PVC cannulas, it was not possible to completely evaluate the *in vivo* results from the pharmacological point of view. Anyway, this technical issue is important for further experiments with perfused organs.

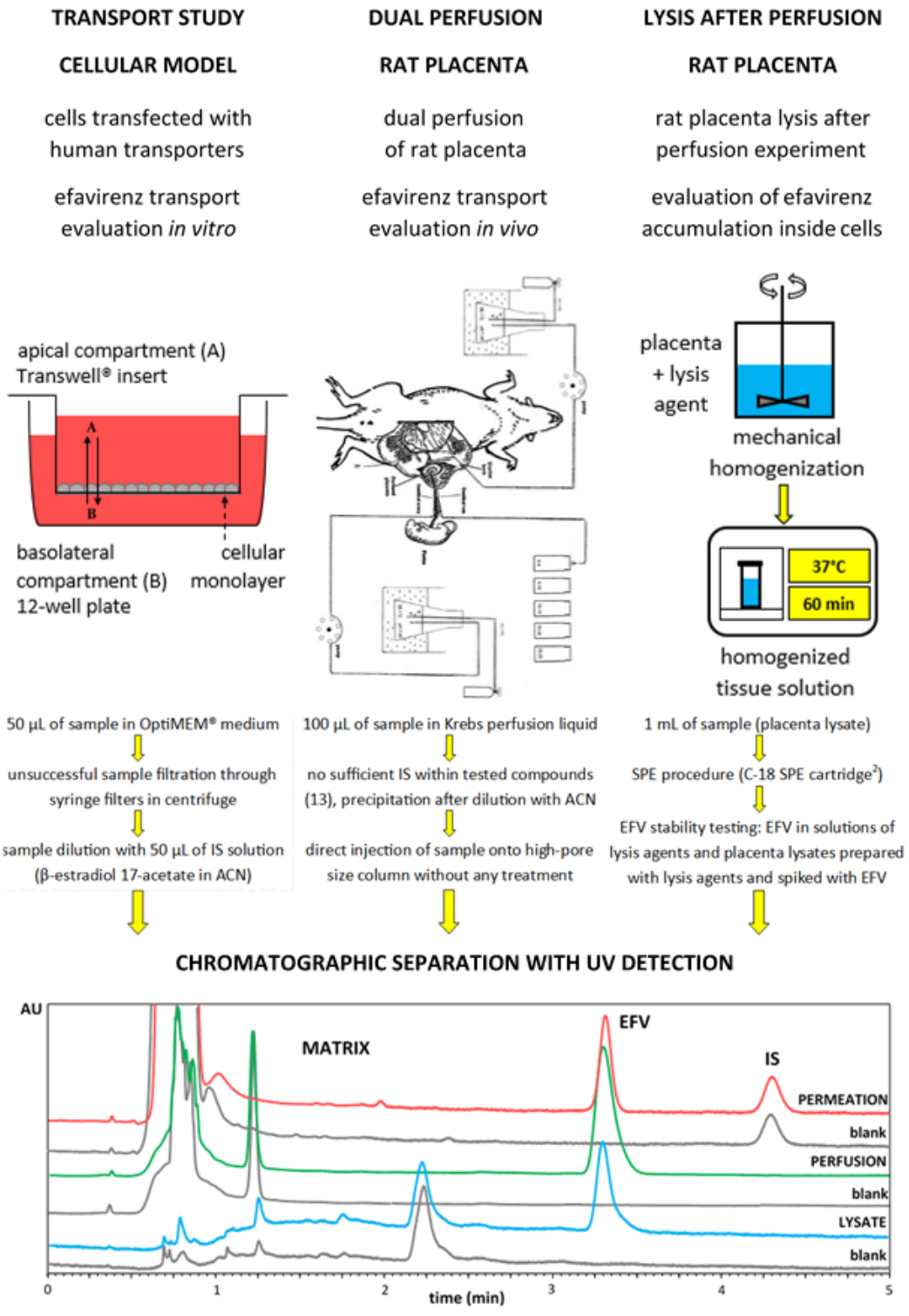

Figure 33: Comparison of sample processing for three different matrices at pharmacological study of efavirenz (EFV). ACN: acetonitrile, IS: internal standard, SPE: solid phase extraction.

4.3. An integrated on-line method for the preconcentration and simultaneous determination of metsulfuron methyl and chlorsulfuron using oxidized carbon nanotubes and second order fluorescent data

This work was part of the cooperation with the Institute of Chemistry, National University of South, Bahía Blanca, Argentina in a range of the project Mobility 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. During 6 weeks study stay, I was involved in the experimental part of the work dealing with the automation of SPE using raw and oxidized MWCNT as a sorbent within automated FBA system.

This work presents an automated method for preconcentration and determination of two herbicides, metsulfuron methyl (MSM) and chlorsulfuron (CSF), using chemometric second order fluorescent data treatment for simultaneous quantitation of the analytes in water samples from the south part of Buenos Aires province. Presented work follows the previous work [110] of working groups cooperation in which FBA-based flow system connected with the mixing chamber was used for MSM preconcentration on C18 extraction column followed by its photodegradation and photoproducts detection in the mixing chamber.

MSM and CSF (see Figure 34) do not exhibit native fluorescence, but after the photodegradation using UV irradiation at proper pH, the photoproducts can be determined. In this case, acetonitrile-NaOH medium with the pH of 12.5 had to be used. Thanks to different kinetics of MSM and CSF photodegradation, the fluorescent emission spectra could be used as a function of photo-irradiation time for analytes quantitation after application of chemometric modeling.

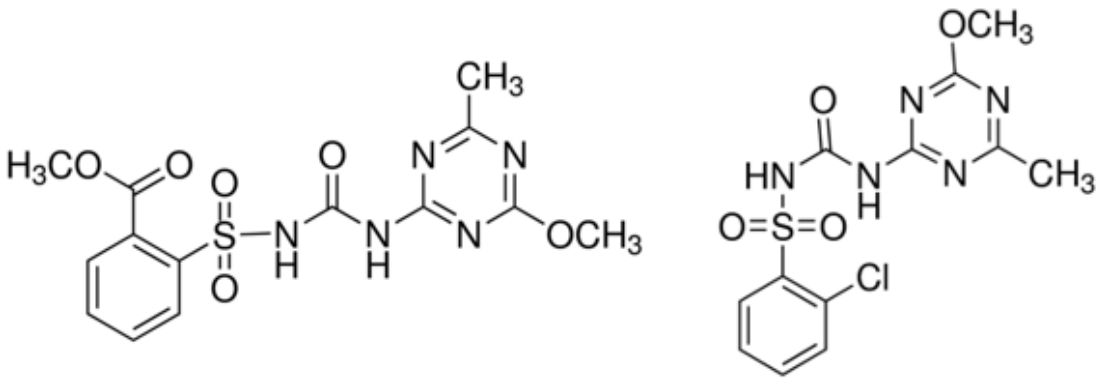

Figure 34: Structures of metsulfuron methyl (left) and chlorsulfuron (right).

FBA-based flow system was composed of three main parts, as shown in Figure 35: 1) preconcentration part, 2) photodegradation part, and 3) detection part.

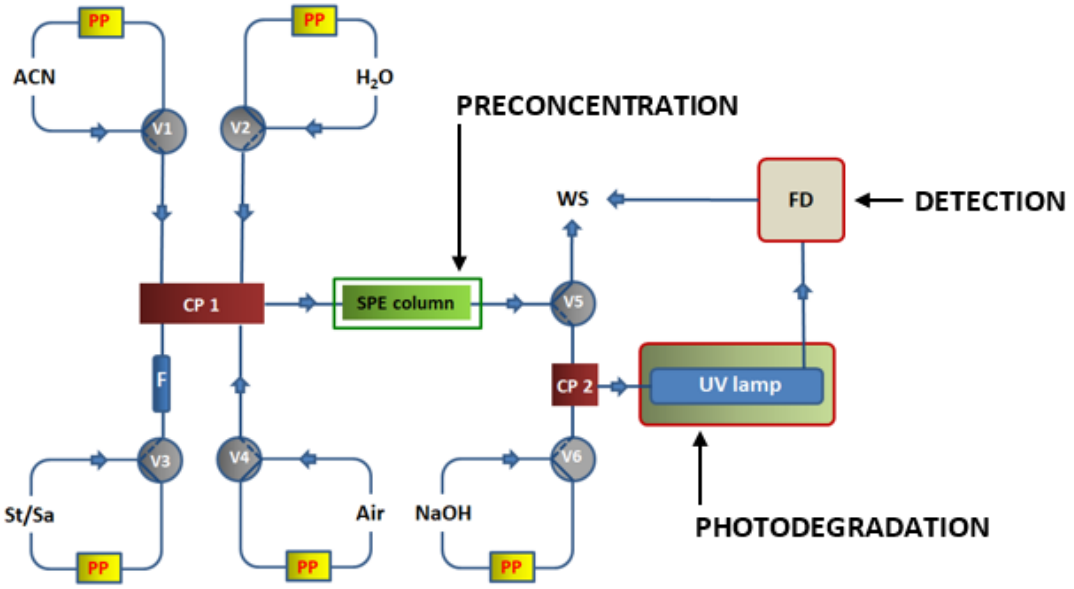

Figure 35: Flow-batch analysis system used for determination of metsulfuron methyl and chlorsulfuron in water samples [27]. PP: peristaltic pump, V: 3-way solenoid valve, CP: confluence point, SPE column: extraction column filled with carbon nanotubes sorbent, FD: fluorescence detector, ACN: acetonitrile, H_2O: distilled water, St/Sa: standard/sample solution, NaOH: sodium hydroxide solution pH 12.5, WS: waste.

The SPE procedure included those steps: a) filling the channels with all solutions, b) column conditioning, c) standard/sample loading, d) column washing, e) column drying with air, f) elution of analytes and pH conditioning by mixing the eluate with NaOH, and g) photodegradation followed by fluorescent detection. Single analysis was completed in 21 minutes. In-line filter was added into the sample line to filter the samples prior the analysis.

In a preconcentration part, elution conditions, sample volume, and sample loading flow rates were investigated as well as the sorbent type. Home-made SPE column was prepared from both r-MWCNT and ox-MWCNT. Ox-MWCNT shown better results according to fluorescence signal (lower limit of MSM determination, higher correlation coefficient), but also handling with the column in the system was easier due to lower back pressure generation within the manifold causing the risk of SPE column clogging. Thus, ox-MWCNTS column was used for further experiments.

Chemometric data analyses were carried out using chemometric modeling and fluorescent spectra of MSM, CSF, photodegradation media, and synthetic mixtures of MSM, CSF, NaOH, acetonitrile, and water. Calibration set was performed due to 2 × 2 full factorial design evaluating MSM and CSF concentration at 0.77 and 7.70 μg L^{-1}. Set of 20 spiked samples were prepared using non-spiked matrix and matrix spiked according to 2 × 2 full factorial design using two concentrations for both MSM (2.50 and 5.96 μg L^{-1}) and CSF (12.5 and 29.8 μg L^{-1}).

Prepared solution sets were analyzed by the proposed method in random order, employing two algorithms for the second order data treatment. Analytical performance of MSM and CSF determination was similar for both chemometric analyses. Recovery of spiked samples, LODs, LOQs, and relative errors of predictions achieved with both models showed acceptable values when compared with previously published methods.

This work showed the capability of flow techniques in sample preparation automation and miniaturization pointing out low sorbent amount used for SPE, savings of reagents and solvents by recirculation in FBA system, and minimal operator demands by connection of all three parts of the system – preconcentration, photodegradation, and detection.

4.4. Fully automated method based on on-line molecularly imprinted polymer solid-phase extraction for determination of lovastatin in dietary supplements containing red yeast rice

Presented work was part of the student grant GAUK 274216 and followed work of Svoboda et al. [89]. Previously, a) the synthesis of lovastatin lactone-selective MIP sorbent was optimized, b) off-line extraction protocol using 1 mL syringe with 20 mg of MIP sorbent was optimized, c) matrix effects were evaluated in different food matrices naturally containing lovastatin such as red yeast rice, Oyster mushroom, and Pu-erh tea, and d) off-line extraction followed by UHPLC separation with MS detection (MISPE UHPLC-MS/MS) was applied for lovastatin determination in food samples.

Presented work included further step – automation and miniaturization of the extraction – towards connection of SIA (extraction step) with LC-MS (separation and detection). In presented work, a) highly selective automated extraction of lovastatin lactone (see Figure 36) before spectrophotometric determination was developed, b) dietary supplements were analyzed by proposed method and results were verified by UHPLC-MS/MS analysis, and c) on-line extraction within the flow system (MISPE-SIA-UV) and off-line MISPE UHPLC-MS/MS were compared in terms of analytical performance and method capability using three matrices – red yeast rice, oyster mushroom, and Pu-erh tea.

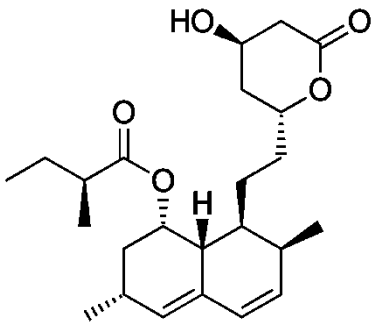

Figure 36: Structure of lovastatin lactone, a blood cholesterol decreasing drug.

The extraction was operated on-line using miniaturized extraction column, filled with 4.5 mg of home-made MIP lovastatin lactone-selective sorbent. The column was placed between selection valve and detection flow cell of SIChrom^{TM} instrument, which represents SIC system, utilizing medium-pressure syringe pump and robust selection valve for trouble less work under generated back-pressure in the system generated during extractions or separations [19, 20].

MIP sorbent was prepared by P. Svoboda using thermally initiated free radical polymerization described previously [89]. Simvastatin was used as a template molecule, methacrylic acid as a monomer, ethylene dimethacrylate as a cross-linker, azobisisobutyronitrile as an initiator, and 0.1% aqueous formic acid-acetonitrile 20-80 (v/v) as a porogen.

When such prepared MIP was used for SPE column preparation, problems with high pressure generation were observed. This can be explained by finest particles sticking to the surface of the MIP particles. To prevent this, an elimination of fine particles was done by dispersing and sedimentation of MIP in pure acetonitrile three times. After such treatment it was possible to use prepared SPE column in SIChrom^{TM} system with reasonable back pressure generation, which prevented crossing of the system pressure limit.

Prior automated extraction, it was necessary to prepare acetonitrile extracts from dried matrices (see Figure 37). Red yeast rice, oyster mushroom, and Pu-erh tea matrices were used for the comparison of on-line and off-line methods. Red yeast rice was used as a matrix which is common to selected dietary supplements. Dietary supplements were in form of tablets or gelatin capsules with powdered content. Extracts were prepared according to previously published procedure [94].

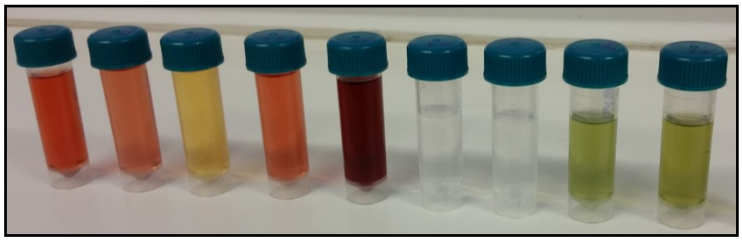

Figure 37: Acetonitrile extracts of different matrices naturally containing lovastatin. From left: 4 dietary supplements, red yeast rice, 2 oyster mushrooms, 2 Pu-erh teas.

The MISPE-SIA-UV procedure itself consisted of a typical five-step protocol including sorbent activation, sorbent conditioning, sample loading, matrix washing, and analyte elution. Thanks to the setup in the flow system, it was possible to carry out the MISPE in three cycles: a) sorbent activation and conditioning, b) sample loading followed by matrix washing, and c) lovastatin elution followed by its direct UV detection.

Flow system precisely controlled volumes and flow rates in all steps of the procedure by the program. Due to direct connection of SPE column with detection cell, the absorbance signal of eluates was monitored continually during whole procedure.

This was used with benefit to verify lovastatin retention during sample loading step, lovastatin hydroxyl acid/matrix/interferences elution from the column during matrix washing optimization, and finally lovastatin elution during optimization of procedure. Using the flow-based system, eluate evaporation and reconstitution necessary prior UHPLC-MS/MS analysis was not necessary due to on-line detection, and avoiding this step the analysis time was shortened.

The extraction procedure was based on previous work, but the composition of solvents was studied together with parameters such as aspiration volumes and flow rates, flow through the extraction column, and elution volume, to fit in the flow-through arrangement. Optimized analytical run, comprising sample preparation followed by on-line detection, used only 250 µL of sample, 1.4 mL of acetonitrile, and took 7.5 min.

Less sensitive and selective UV detection compared to MS is the main drawback of our MISPE-SIA-UV method. But, all tested dietary supplements were based on the red yeast rice and thus presence of other statins was not expected. Thanks to that, separation of matrix and lovastatin using MIP sorbent is sufficient for dietary supplements analysis including sufficient sensitivity of UV detection. After MISPE optimization, eluates were collected, and it was verified by the UHPLC-MS/MS measurement that lovastatin hydroxy acid and matrix interferences were washed out during matrix washing step while lovastatin lactone was extracted and eluted by acetonitrile in following step.

Four dietary supplements bought in Czech pharmacies were analyzed by the MISPE-SIA-UV. For comparative measurements the eluates were collected, diluted 100-times, and subsequently analyzed by the UHPLC-MS/MS. Correlation of both MISPE-SIA-UV and UHPLC-MS/MS results was proven by F-test and t-test ($\alpha = 0.05$), while statistical data comparison did not show a significant difference. Considering determined lovastatin content and daily dose recommended by the manufacturer, only one dietary supplement (supplement B, 16 mg of lovastatin per day) exceeds 10 mg recommended lovastatin daily dose [111]. In the case of combined treatment with supplement B and statin containing medicinal drug, the risk of overdose and side effect appearance is increased and should be taken into consideration.

As it was already stated, the MISPE-SIA-UV vs. MISPE UHPLC-MS/MS method enabled miniaturization and automation of the extraction using selective MIP sorbent in terms of low sorbent amount (4.5 mg vs. 20 mg), sample volume (250 µL vs. 1 mL), and solvent consumption (1.4 mL vs. 4.0 mL) together with analysis time shortening (7.5 min vs. approx. 40 min) and computer control of each particular procedure step. On the other hand, the MISPE-SIA-UV shown higher LOD (0.15 µg/mL vs. 0.30 ng/mL) and LOQ (0.5 µg/mL vs. 1.0 ng/mL) together with narrowed concentration range (0.5-5.0 µg/mL vs. 1-500 ng/mL) in comparison with MISPE UHPLC-MS/MS. MISPE-SIA-UV arrangement

provides cheaper and automated alternative to LC-MS methods for statins determination in dietary supplements formulations. Our automated method could be considered as a base for further on-line connection of automated MISPE with LC method. Such connection will benefit from automated sample preparation with sensitive detection for analysis of matrices with low lovastatin content.

This work again demonstrated capability of flow techniques in miniaturization and automation of sample preparation not only by savings of sorbent/sample/solvents, but mainly by time efficiency in the automated procedure.

4.5. Sequential injection determination of 2,4-dichlorophenoxyacetic acid herbicide using preconcentration with a polymer inclusion membrane

Presented work, dealing with automated determination of a model herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), is a project partly done at Kolev Research Group at the School of Chemistry, Faculty of Science, University of Melbourne, Australia, during my 4-month research stay. Project continuation was enabled in Laboratory of Flow Methods at my home faculty. This project is under supervision of prof. Kolev and was partly supported by the STARSS project and the Mobility Fund of the Charles University. Work has not been finished and published in a form of a manuscript yet but will be continued in the range of ongoing cooperation between both departments.

Flow-based system was comprised of a SIA analyzer with a UV detector, an extraction cell with polymer membrane, and an external peristaltic pump (see Figure 38). A home-made extraction cell, shown in detail in Figure 39, was composed of two holders, two spacers, and studied polymer inclusion membrane strip. Extraction cell was connected to the port 3 of the selection valve and to the external peristaltic pump. The use of extraction cell where membrane strip was placed in sandwich format enabled simultaneous extraction and back-extraction of 2,4-D followed by its detection in automated way. LabJack module U12 controlled the peristaltic pump actuation during measuring procedure. FIAlab software for Windows was used for instrument control and data collection and processing.

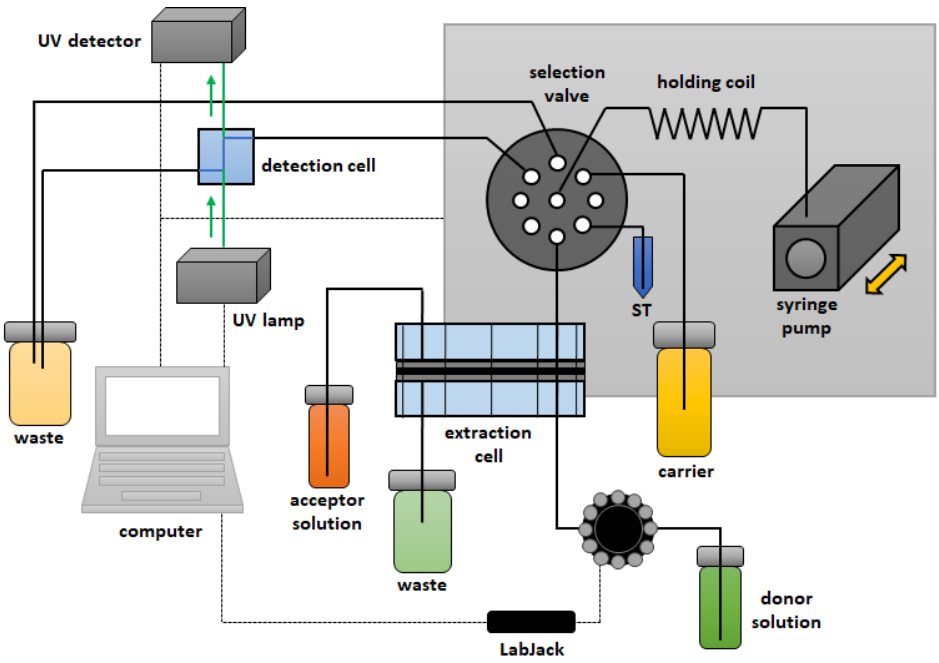

Figure 38: Scheme of the SIC system, used in 2,4-dichlorophenylacetic acid determination, connected to the extraction cell, peristaltic pump, and LabJack module.

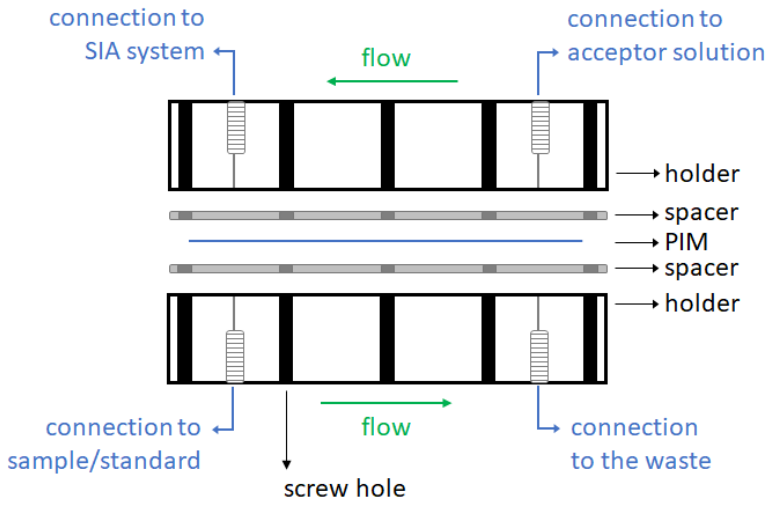

Figure 39: Scheme of the home-made extraction cell composition. PIM – polymer inclusion membrane strip 1.5 × 12 cm, SIA system – sequential injection analysis system.

Flow system operated under the stop-flow conditions. First, sample was introduced in the extraction cell via peristaltic pump while acceptor solution was stagnant. After sample loading, stop interval was applied followed by analysis of zone of acceptor solution with extracted analyte.

Optimization of system parameters as well as testing of polymer membranes of different characteristics is briefly described in Supplement 5.

The limit of 2,4-D in fresh waters according to Australian law is 0.28 ppm [112]. Proposed method should be evaluated with respect to calibration range, repeatability and accuracy, together with study of interferences emerging during water samples analyses. Validated method should be used for real-sample determination. All those steps are aims of further work.

Described system pointed out the versatility of flow techniques for connection with additional parts such as extraction cell, peristaltic pump or LabJack module.

5. CONCLUSION

This experimental dissertation thesis was focused on new applications in the field of pharmaceutical and environmental analysis based mainly on the sequential injection and flow-batch approach from the field of the flow techniques. Dissertation also included one application of fast chromatographic method for processing of series of samples obtained in the complex pharmacological study including various sample matrices.

It was proven that flow methods are universal and efficient tools in the automation and/or miniaturization of sample preparation methods based on solid phase extraction, using home-made molecularly imprinted polymer or carbon nanotube sorbents, as well as membrane-based extraction using home-made polymer membrane. Also, automation of permeation study with real-time monitoring of the marker of transport through cellular monolayer was carried out using flow system with liberation unit and living cells which opened a new insight into this topic.

This thesis presented new contributions to the analytical field of flow-based techniques pointing out versatility of such techniques in various automated applications including different analytes, sample matrices, and sample preparation techniques.

This dissertation thesis comprised a theoretical background to five experimental works carried out during my doctoral studies. Partial experimental results were presented at national and international conferences in forms of oral lectures or posters (chapter 6) and four works were published in the scientific journals with impact factor in the range of 2.893 – 3.307.

The works were joined projects with our colleagues from the Department of Chemistry, Faculty of Pharmacy, University of Porto, Porto, Portugal (chapter 4.1), the Department of Chemistry, University of Balearic Islands, Palma de Mallorca, Spain (chapter 4.1), the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University (chapter 4.1 and 4.2), the Department of Chemistry, University of South, Bahía Blanca, Argentina (chapter 4.3), and the School of Chemistry, Faculty of Science, University of Melbourne, Melbourne, Australia (chapter 4.5).

6. LIST OF OTHER OUTPUTS OF CANDIDATE

6.1. Oral presentations at domestic and international conferences

9th meeting of Division of Analytical Chemistry Analítica 2018, Porto, Portugal, 26.3.-27.3.2018: *Automated approach for the sequential injection determination of the herbicide 2,4-D based on on-line preconcentration using a polymer inclusion membrane*, L. Zelená, M.I.G.S. Almeida, R.W. Cattrall, H. Sklenářová, P. Solich, S.D. Kolev.

8th postgraduate and 6th postdoctoral conference, Faculty of Pharmacy in Hradec Králové, Charles University, Czech Republic, 24.1.-25.1.2018: *2,4-dichlorophenoxyacetic acid determination using preconcentration with a polymer inclusion membrane*, L. Zelená, M.I.G.S. Almeida, R.W. Cattrall, H. Sklenářová, P. Solich, S.D. Kolev.

Sanofi prize for Pharmacy 2017, Sanofi/Embassy of France in the Czech Republic, 1.6.2017, competition of nominated Ph.D. research works in all fields of pharmaceutical sciences: *Lovastatin determination in dietary supplements by a fully automated MIP-SPE procedure*, L. Zelená, P. Svoboda, L. Nováková, H. Sklenářová.

7th postgraduate and 5th postdoctoral conference, Faculty of Pharmacy in Hradec Králové, Charles University, Czech Republic, 7.2.-8.2.2017: *Lovastatin determination in dietary supplements by a fully automated MIP-SPE procedure*, L. Zelená, P. Svoboda, L. Nováková, H. Sklenářová.

6th postgraduate and 4th postdoctoral conference, Faculty of Pharmacy in Hradec Králové, Charles University, Czech Republic, 9.2.-10.2.2016: *Sequential injection manifold as a tool for automated performance of drug permeation studies*, L. Zelená, L. Hyršová, J. Fibigr, P. Pávek, H. Sklenářová.

5th postgraduate and 3rd postdoctoral conference, Faculty of Pharmacy in Hradec Králové, Charles University, Czech Republic, 3.2.-4.2.2015: *Sample pre-treatment in determination of efavirenz in OptiMEM® Reduced Serum Media using high-performance liquid chromatography with UV detection*, L. Zelená, J. Řezníček, M. Čečková, H. Sklenářová.

6.2. Poster presentations at domestic and international conferences

19th International Symposium on Advances in Extraction Technologies (ExTech 2017), Santiago de Compostela, Spain, 27.6.-30.6.2017: *On-line determination of Rhodamine 123 using separation in low-pressure SIA system*, L. Zelená, J. Fibigr, L. Hyršová, H. Sklenářová. *MISPE procedure for lovastatin determination – comparison*

of on-line and off-line approaches, L. Zelená, P. Svoboda, K. Plachká, L. Nováková, H. Sklenářová.

20th International Conference on Flow Injection Analysis and Related Techniques (ICFIA 2016), Palma de Mallorca, Mallorca, Spain, 2.10.-7.10.2016: *Automated SPE procedure for lovastatin determination using laboratory-made molecularly imprinted polymer as a sorbent*, L. Zelená, P. Svoboda, L. Nováková, H. Sklenářová.

18th International Symposium On Advances In Extraction Technologies & 22nd International Symposium On Separation Sciences (ExTech-ISSS 2016), Toruń, Poland, 3.7.-6.7.2016: *Drug permeation studies performed automatically using SIA manifold and Franz diffusion cell as an analytical tool* , L. Zelená, L. Hyršová, J. Fibigr, P. Pávek, H. Sklenářová. *Determination of efavirenz in rat placenta lysate samples using HPLC-UV,* L. Zelená, J. Řezníček, M. Čečková, H. Sklenářová.

45th Synthesis and Analysis Conference, Faculty of Pharmacy in Hradec Králové, Charles University, Czech Republic, 22.6.-24.6.2016: D*etermination of efavirenz using HPLC-UV method: a pharmacological study*, L. Zelená, J. Řezníček, M. Čečková, H. Sklenářová.

9th International Conference on Instrumental Methods of Analysis - Modern Trends and Applications (IMA 2015), Kalamata, Greece, 20.9.-24.9.2015: *Automation of drug transport monitoring using sequential injection manifold*, L. Zelená, E. Lopes, M. Segundo, M. Miró, L. Hyršová, P. Pávek, H. Sklenářová.

9th International Conference on Instrumental Methods of Analysis - Modern Trends and Applications (IMA 2015), Kalamata, Greece, 20.9.-24.9.2015: *Efavirenz determination in samples obtained in transport studies using HPLC with UV detection*, L. Zelená, J. Řezníček, M. Čečková, H. Sklenářová.

Flow Analysis XIII, Prague, Czech Republic, 5.7.-10.7.2015, active participant and member of organizing committee: *Automation of drug permeation studies using Rhodamine 123 as a marker*, L. Zelená, E. Lopes, M. Segundo, M. Miró, P. Pávek, H. Sklenářová. *Testing of carbon nanotubes as a sorbent for fully automated SPE procedure using metsulfuron methyl as a target analyte* (the best poster award), L. Zelená, C.C. Acebal, N.E. Llamas, M. Grünhut, A.G. Lista, H. Sklenářová, B.S. Fernandéz Band, P. Solich.

XVI International Symposium on Luminescence Spectrometry, Rhodos, Greece, 24.9.-27.9.2014: *Automated monitoring of drug interactions across cell monolayer using fluorometric assay of the Rhodamine* 123, L. Zelená, S. Marques, M. Segundo, M. Miró, P. Pávek, H. Sklenářová.

6.3. Grant projects and scholarships

Grant Agency of the Charles University, project 159415, 2015-2017: *In-line monitoring of permeation studies in sequential injection analysis system*. Leading researcher of the project.

STARSS project, CZ.02.1.01/0.0/0.0/15_003/0000465, 2017-2019: *Establishment of Specialized Team for Advanced Research on Separation Science*. Ph.D. student co-worker of the project, project guarantor prof. RNDr. Petr Solich, CSc.

Grant Agency of the Charles University, project 860216, 2016-2019: *Development of new types of biocompatible hemodialysis membranes for separation of biomolecules*. Student co-worker of the project, leading researcher Mgr. Michaela Kohlová.

Grant Agency of the Charles University, project 274216, 2016-2017: Synthesis of molecularly imprinted polymers for selective SPE extraction of lovastatin from food samples and elimination of matrix effects in LC-MS analysis. Student co-worker of the project, leading researcher Mgr. Pavel Svoboda.

Grant Agency of the Czech Republic, project GA15-10781S, 2015-2017: *On-line hyphenation of automated extraction processes with liquid chromatography for complete sample analysis*. Student co-worker of the project, leading researcher prof. RNDr. Petr Solich, CSc.

TEAB project, CZ.1.07/2.3.00/20.0235, 2013-2016: *Establishment of Research Team for Experimental and Applied Biopharmacy*. Student co-worker of the project, leading researcher prof. RNDr. Petr Solich, CSc.

Bilateral cooperation project MOBILITY, 7AMB14AR029, 2014-2015: *Application of modern flow methods for the determination of biologically active compounds in complex matrices*. Student co-worker of the project, leading researcher assoc. prof. PharmDr. Hana Sklenářová, Ph.D.

6.4. Foreign experience

University of Melbourne, Melbourne, Australia, 08/2017 – 12/2017: Study and research stay (4 months) at the Kolev Research Group with the support of the STARSS project and the Mobility Fund of the Charles University. Work topic: preconcentration of model herbicides (glyphosate, 2,4-dichloroacetic acid) from water samples with the use of polymer inclusion membranes within the flow system manifold. Supervisors: prof. Spas D. Kolev, dr. M. Inés G. S. Almeida.

University of Balearic Islands, Palma de Mallorca, Spain, 03/2015: Study stay (1 week) at the Department of Chemistry in the range of a short stay of the project TEAB at working groups of prof. Manuel Miró and prof. Victor Cérda.

The National University of South, Bahía Blanca, Argentina, 10/2014 – 12/2014: Study and research stay (6 weeks) at the Department of Chemistry in the range of bilateral project Mobility. Work topic: automation of solid-phase extraction within a flow system using carbon nanotubes as a sorbent. Supervisor: dr. Carolina Acebal.

Faculty of Pharmacy, University of Porto, Porto, Portugal, 09/2012 – 02/2013: Study stay (5 months) at the Department of Analytical Chemistry. Diploma thesis project: development of a new pyruvate-selective electrode for wine control. Supervisors: prof. Maria da Conceição Branco da Silva, dr. Célia Maria Pinto Gomes Amorim.

7. REFERENCES

[1] L.T. Skeggs, An automatic method for colorimetric analysis, American journal of clinical pathology 28 (1957) 311-322.
[2] J. Ruzicka, E.H. Hansen, FLOW INJECTION ANALYSES .1. NEW CONCEPT OF FAST CONTINUOUS-FLOW ANALYSIS, Analytica Chimica Acta 78 (1975) 145-157.
[3] J. Ruzicka, G.D. Marshall, SEQUENTIAL INJECTION - A NEW CONCEPT FOR CHEMICAL SENSORS, PROCESS ANALYSIS AND LABORATORY ASSAYS, Analytica Chimica Acta 237 (1990) 329-343.
[4] P. Chocholous, P. Solich, D. Satinsky, An overview of sequential injection chromatography, Analytica Chimica Acta 600 (2007) 129-135.
[5] G.R.D. Prabhu, P.L. Urban, The dawn of unmanned analytical laboratories, TrAC Trends in Analytical Chemistry 88 (2017) 41-52.
[6] B. Horstkotte, M. Miró, P. Solich, Where are modern flow techniques heading to?, Analytical and bioanalytical chemistry 410 (2018) 6361-6370.
[7] L. Krcmova, D. Solichova, P. Solich, Microplates in liquid chromatography–New solution in clinical research?–A review, Talanta 115 (2013) 973-979.
[8] W.E. van der LINDEN, Classification and definition of analytical methods based on flowing media (IUPAC Recommendations 1994), Pure and applied chemistry 66 (1994) 2493-2500.
[9] V. Cerdà, J. Avivar, A. Cerdà, Laboratory automation based on flow techniques, Pure and Applied Chemistry 84 (2012) 1983-1998.
[10] W.R. Melchert, B.F. Reis, F.R. Rocha, Green chemistry and the evolution of flow analysis. A review, Analytica Chimica Acta 714 (2012) 8-19.
[11] P.C. Pinto, M. Lucia, M. Saraiva, J.L. Lima, Sequential injection analysis hyphenated with other flow techniques: A review, Analytical Letters 44 (2011) 374-397.
[12] S. Clavijo, J. Avivar, R. Suárez, V. Cerdà, Analytical strategies for coupling separation and flow-injection techniques, TrAC Trends in Analytical Chemistry 67 (2015) 26-33.
[13] J. Ruzicka, Flow Injection analysis, Tutorial and news on flow based micro analytical techniques. http://www.flowinjectiontutorial.com/index.html, 2018 (Accessed 03 October 2018 2018).
[14] L. Zelená, S.S. Marques, M.A. Segundo, M. Miró, P. Pávek, H. Sklenářová, P. Solich, Fully automatic flow-based device for monitoring of drug permeation across a cell monolayer, Analytical and Bioanalytical Chemistry 408 (2016) 971-981.
[15] J. Ruzicka, Lab-on-valve: universal microflow analyzer based on sequential and bead injection, Analyst 125 (2000) 1053-1060.
[16] J. Wang, E.H. Hansen, Sequential injection lab-on-valve: the third generation of flow injection analysis, TrAC Trends in Analytical Chemistry 22 (2003) 225-231.
[17] D. Šatınský, P. Solich, P. Chocholouš, R. Karlíček, Monolithic columns—a new concept of separation in the sequential injection technique, Analytica Chimica Acta 499 (2003) 205-214.
[18] P. Chocholous, L. Kosarova, D. Satinsky, H. Sklenarova, P. Solich, Enhanced capabilities of separation in Sequential Injection Chromatography - Fused-core particle column and its comparison with narrow-bore monolithic column, Talanta 85 (2011) 1129-1134.
[19] I. Sramkova, P. Chocholous, H. Sklenarova, D. Satinsky, On-line coupling of Micro-Extraction by Packed Sorbent with Sequential Injection Chromatography system for direct extraction and determination of betaxolol in human urine, Talanta 143 (2015) 132-137.

[20] A.D. Batista, P. Chocholouš, D. Šatínský, P. Solich, F.R.P. Rocha, On-line hyphenation of solid-phase extraction to chromatographic separation of sulfonamides with fused-core columns in sequential injection chromatography, Talanta 133 (2015) 142-149.
[21] C. Fernández-Ramos, D. Šatínský, B. Šmídová, P. Solich, Analysis of trace organic compounds in environmental, food and biological matrices using large-volume sample injection in column-switching liquid chromatography, TrAC Trends in Analytical Chemistry 62 (2014) 69-85.
[22] M.A. Obando, J.M. Estela, V. Cerda, Simultaneous determination of hydrochlorothiazide and losartan potassium in tablets by high-performance low-pressure chromatography using a multi-syringe burette coupled to a monolithic column, Analytical and Bioanalytical Chemistry 391 (2008) 2349-2356.
[23] V. Cerdà, J.M. Estela, R. Forteza, A. Cladera, E. Becerra, P. Altimira, P. Sitjar, Flow techniques in water analysis, Talanta 50 (1999) 695-705.
[24] B.F. Reis, M.F. Gine, E.A.G. Zagatto, J. Lima, R.A. Lapa, MULTICOMMUTATION IN FLOW-ANALYSIS .1. BINARY SAMPLING - CONCEPTS, INSTRUMENTATION AND SPECTROPHOTOMETRIC DETERMINATION OF IRON IN PLANT DIGESTS, Analytica Chimica Acta 293 (1994) 129-138.
[25] P. Diniz, L.F. de Almeida, D.P. Harding, M.C.U. de Araujo, Flow-batch analysis, Trac-Trends in Analytical Chemistry 35 (2012) 39-49.
[26] E. Zagatto, J. Carneiro, S. Vicente, P. Fortes, J. Santos, J. Lima, Mixing chambers in flow analysis: a review, Journal of Analytical Chemistry 64 (2009) 524-532.
[27] C.C. Acebal, M. Grünhut, N.E. Llamas, M. Insausti, L. Zelená, H. Sklenářová, P. Solich, B.S.F. Band, An integrated on-line method for the preconcentration and simultaneous determination of metsulfuron methyl and chlorsulfuron using oxidized carbon nanotubes and second order fluorescent data, Microchemical Journal 129 (2016) 90-97.
[28] P. Solich, M. Polasek, J. Klimundova, J. Ruzicka, Sequential injection technique applied to pharmaceutical analysis, Trac-Trends in Analytical Chemistry 23 (2004) 116-126.
[29] A.M. Pimenta, M. Montenegro, A.N. Araujo, J.M. Calatayud, Application of sequential injection analysis to pharmaceutical analysis, Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 16-34.
[30] R.W. Min, J. Nielsen, J. Villadsen, On-line monitoring of glucose and penicillin by sequential injection analysis, Analytica Chimica Acta 320 (1996) 199-205.
[31] X. Liu, E.H. Hansen, Sequential injection determination of D-glucose by chemiluminescence using an open tubular immobilised enzyme reactor, Analytica Chimica Acta 326 (1996) 1-12.
[32] P.J. Baxter, G.D. Christian, J. Ruzicka, MULTIVARIATE APPROACH FOR THE SIMULTANEOUS DETERMINATION OF TOTAL BIOMASS AND GLUCOSE FROM A YEAST FERMENTATION BY SEQUENTIAL INJECTION-ANALYSIS, Chemia Analityczna 40 (1995) 455-471.
[33] R.W. Min, J. Nielsen, J. Villadsen, Simultaneous monitoring of glucose, lactic acid and penicillin by sequential injection analysis, Analytica Chimica Acta 312 (1995) 149-156.
[34] N.W. Barnett, S.W. Lewis, D.J. Tucker, Determination of morphine in process streams by sequential injection analysis with chemiluminescence detection, Fresenius' journal of analytical chemistry 355 (1996) 591-595.
[35] N. Barnett, C. Lenehan, S. Lewis, D. Tucker, K. Essery, Determination of morphine in water immiscible process streams using sequential injection analysis coupled with acidic permanganate chemiluminescence detection, Analyst 123 (1998) 601-605.

[36] A. Kovács, É. Petró, I. Erős, I. Csóka, The role of dissolution testing in quality control, J Periodica Polytechnica Chemical Engineering (2014) 17-20.
[37] O. Anand, X.Y. Lawrence, D.P. Conner, B.M. Davit, Dissolution testing for generic drugs: an FDA perspective, The AAPS journal 13 (2011) 328.
[38] Council of Europe, European Directorate for the Quality of Medicines & HealthCare (EDQM), European Pharmacopoeia, English edition 9.0, (2017).
[39] Ministry of Health of the Czech Republic, Bohemian Pharmacopoeia 2017, GRADA, Praha, 2017.
[40] X.-Z. Liu, Z.-L. Fang, Sequential-injection system for drug-dissolution studies of ibuprofen tablets and sustained-release formulations, Analytica Chimica Acta 358 (1998) 103-110.
[41] H. Pasekova, M. Sales, M. Montenegro, A. Araujo, M. Polášek, Potentiometric determination of acetylsalicylic acid by sequential injection analysis (SIA) using a tubular salicylate-selective electrode, Journal of Pharmaceutical and Biomedical Analysis 24 (2001) 1027-1036.
[42] X.-Z. Liu, S.-S. Liu, J.-F. Wu, Z.-L. Fang, Simultaneous monitoring of aspirin, phenacetin and caffeine in compound aspirin tablets using a sequential injection drug-dissolution testing system with partial least squares calibration, Analytica Chimica Acta 392 (1999) 273-281.
[43] Z. Legnerova, D. Satinsky, P. Solich, Using on-line solid phase extraction for simultaneous determination of ascorbic acid and rutin trihydrate by sequential injection analysis, Analytica Chimica Acta 497 (2003) 165-174.
[44] D. Kremr, D.J. Cocovi-Solberg, P. Bajerova, K. Ventura, M. Miro, On-line monitoring of in-vitro oral bioaccessibility tests as front-end to liquid chromatography for determination of chlorogenic acid isomers in dietary supplements, Talanta 166 (2017) 391-398.
[45] P. Solich, H. Sklenarova, J. Huclova, D. Satinsky, U.F. Schaefer, Fully automated drug liberation apparatus for semisolid preparations based on sequential injection analysis, Analytica Chimica Acta 499 (2003) 9-16.
[46] S. Azarmi, W. Roa, R. Löbenberg, Current perspectives in dissolution testing of conventional and novel dosage forms, International Journal of pharmaceutics 328 (2007) 12-21.
[47] J. Klimundova, H. Sklenarova, U.F. Schaefer, P. Solich, Automated system for release studies of salicylic acid based on a SIA method, Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 893-898.
[48] J. Klimundova, K. Mervartova, H. Sklenarova, P. Solich, M. Polasek, Automated sequential injection fluorimetric set-up for multiple release testing of topical formulation, Analytica Chimica Acta 573 (2006) 366-370.
[49] J. Klimundova, D. Satinsky, H. Sklenarova, P. Solich, Automation of simultaneous release tests of two substances by sequential injection chromatography coupled with Franz cell, Talanta 69 (2006) 730-735.
[50] A.C. Alves, I.I. Ramos, C. Nunes, L.M. Magalhães, H. Sklenářová, M.A. Segundo, J.L. Lima, S.J.T. Reis, On-line automated evaluation of lipid nanoparticles transdermal permeation using Franz diffusion cell and low-pressure chromatography, 146 (2016) 369-374.
[51] L. Zelena, J. Reznicek, M. Ceckova, H. Sklenarova, Universal efavirenz determination in transport study, rat placenta perfusion and placenta lysate by HPLC-UV, Journal of Pharmaceutical and Biomedical Analysis 137 (2017) 70-77.

[52] S.A. Motz, J. Klimundova, U.F. Schaefer, S. Balbach, T. Eichinger, P. Solich, C.M. Lehr, Automated measurement of permeation and dissolution of propranolol HCl tablets using sequential injection analysis, Analytica Chimica Acta 581 (2007) 174-180.
[53] H. Sklenářová, P. Pavek, D. Šatínský, P. Solich, R. Karlíček, F. Štaud, Z.J.T. Fendrich, Determination of rhodamine 123 by sequential injection technique for pharmacokinetic studies in the rat placenta, 58 (2002) 1145-1149.
[54] R. Yang, Q. Wang, H. Zeng, Z. Qin, J. Li, L. Qu, Determination of puerarin in biological samples and its application to a pharmacokinetic study by flow-injection chemiluminescence, Luminescence 26 (2011) 368-373.
[55] B. Du, H. Li, J. Jin, T. Wang, Y. Li, G. Shen, X. Li, Chemiluminescence determination of streptomycin in pharmaceutical preparation and its application to pharmacokinetic study by a flow injection analysis assembly, Spectrochimica Acta Part A: Molecular Biomolecular Spectroscopy 115 (2013) 823-828.
[56] X. Xiong, X. Zhao, Y. Li, Z. Song, A Fast and Efficient Chemiluminescence Method for Determination and Pharmacokinetic Study of Paclitaxel in Rat Plasma, Current Pharmaceutical Analysis 10 (2014) 246-254.
[57] X.-Y. Xiong, H.-J. Yu, Y.-F. Nan, A Highly Sensitive Chemiluminescence Method and Application in Rapid Pharmacokinetic Study of Matrine in Rat Plasma, Current Pharmaceutical Analysis 13 (2017) 452-461.
[58] L. Nováková, H. Vlčková, A review of current trends and advances in modern bio-analytical methods: Chromatography and sample preparation, Analytica Chimica Acta 656 (2009) 8-35.
[59] H.L.L. Janusz Pawliszyn, Handbook of Sample Preparation, John Wiley & sONS, Inc., Hoboken, New Jersey, USA, 2010.
[60] L. Nováková, Challenges in the development of bioanalytical liquid chromatography–mass spectrometry method with emphasis on fast analysis, Journal of Chromatography A 1292 (2013) 25-37.
[61] L.M. Gonçalves, I.M. Valente, J.A. Rodrigues, Recent advances in membrane-aided extraction and separation for analytical purposes, Separation & Purification Reviews 46 (2017) 179-194.
[62] M. Miró, W. Frenzel, Automated membrane-based sampling and sample preparation exploiting flow-injection analysis, TrAC Trends in Analytical Chemistry 23 (2004) 624-636.
[63] E. Carasek, J. Merib, Membrane-based microextraction techniques in analytical chemistry: A review, Analytica chimica acta 880 (2015) 8-25.
[64] M. Douša, L. Nováková, Moderní HPLC separace v teorii a praxi II., 1st ed., Lucie Nováková, Michal Douša, Europrint a.s., Pod Kotlářkou 3, Praha 5, 2013.
[65] L. Novosvětská, P. Chocholouš, F. Švec, H. Sklenářová, Fully automated method based on on-line molecularly imprinted polymer solid-phase extraction for determination of lovastatin in dietary supplements containing red yeast rice, Analytical and Bioanalytical Chemistry 411 (2019) 1219-1228.
[66] R. Rodriguez, J. Avivar, L.O. Leal, V. Cerda, L. Ferrer, Strategies for automating solid-phase extraction and liquid-liquid extraction in radiochemical analysis, Trac-Trends in Analytical Chemistry 76 (2016) 145-152.
[67] J.L. Ammerman, J.H. Aldstadt, Monolithic solid-phase extraction for the rapid on-line monitoring of microcystins in surface waters, Microchimica Acta 164 (2008) 185.
[68] S.S.M.P. Vidigal, I.V. Tóth, A.O.S.S. Rangel, Sequential injection lab-on-valve platform as a miniaturisation tool for solid phase extraction, Analytical Methods 5 (2013) 585-597.

[69] C.C. Acebal, B. Simonet, M. Valcárcel, Nanoparticles and continuous-flow systems combine synergistically for preconcentration, TrAC Trends in Analytical Chemistry 43 (2013) 109-120.
[70] C.C. Acebal, SOLID-PHASE EXTRACTION IN AUTOMATED FLOW SYSTEMS USING CARBON NANOTUBES AS SORBENTS, a lecture, Flow Analysis XIII, 5-10 July 2015, Prague, Czech Republic, 2015.
[71] M.F.T. Ribeiro, A.C.B. Dias, J.L.M. Santos, J.L.F.C. Lima, E.A.G. Zagatto, Fluidized beds in flow analysis: use with ion-exchange separation for spectrophotometric determination of zinc in plant digests, Analytical and Bioanalytical Chemistry 384 (2006) 1019-1024.
[72] V. Pichon, Selective sample treatment using molecularly imprinted polymers, Journal of Chromatography A 1152 (2007) 41-53.
[73] A. Martín-Esteban, Recent molecularly imprinted polymer-based sample preparation techniques in environmental analysis, Trends in Environmental Analytical Chemistry 9 (2016) 8-14.
[74] Biotage, MIPs - Molecularly Imprinted Polymers. http://www.biotage.com/product-page/mips---molecularly-imprinted-polymers, (Accessed 10 October 2018 2018).
[75] M.J. Liu, Y.N. Li, S. Lin, X.C. Dong, Determination of tetracycline residues in lake water by on-line coupling of molecularly imprinted solid-phase extraction with high performance liquid chromatography, Analytical Methods 6 (2014) 9446-9452.
[76] E. Rodriguez, F. Navarro-Villoslada, E. Benito-Pena, M.D. Marazuela, M.C. Moreno-Bondi, Multiresidue Determination of Ultratrace Levels of Fluoroquinolone Antimicrobials in Drinking and Aquaculture Water Samples by Automated Online Molecularly Imprinted Solid Phase Extraction and Liquid Chromatography, Analytical Chemistry 83 (2011) 2046-2055.
[77] M. Javanbakht, M.M. Moein, B. Akbari-adergani, On-line clean-up and determination of tramadol in human plasma and urine samples using molecularly imprinted monolithic column coupling with HPLC, Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 911 (2012) 49-54.
[78] S.M. Daryanavard, A. Jeppsson-Dadoun, L.I. Andersson, M. Hashemi, A. Colmsjo, M. Abdel-Rehim, Molecularly imprinted polymer in microextraction by packed sorbent for the simultaneous determination of local anesthetics: lidocaine, ropivacaine, mepivacaine and bupivacaine in plasma and urine samples, Biomedical Chromatography 27 (2013) 1481-1488.
[79] M.M. Moein, D. Jabbar, A. Colmsjo, M. Abdel-Rehim, A needle extraction utilizing a molecularly imprinted-sol-gel xerogel for on-line microextraction of the lung cancer biomarker bilirubin from plasma and urine samples, Journal of Chromatography A 1366 (2014) 15-23.
[80] E. Turiel, A. Martin-Esteban, Molecularly imprinted polymers for sample preparation: A review, Analytica Chimica Acta 668 (2010) 87-99.
[81] V. Manzo, K. Ulisse, I. Rodríguez, E. Pereira, P. Richter, A molecularly imprinted polymer as the sorptive phase immobilized in a rotating disk extraction device for the determination of diclofenac and mefenamic acid in wastewater, Analytica Chimica Acta 889 (2015) 130-137.
[82] Y.L. Hu, J.L. Pan, K.G. Zhang, H.X. Lian, G.K. Li, Novel applications of molecularly-imprinted polymers in sample preparation, Trac-Trends in Analytical Chemistry 43 (2013) 37-52.

[83] A.C.B. Dias, E.C. Figueiredo, V. Grassi, E.A.G. Zagatto, M.A.Z. Arruda, Molecularly imprinted polymer as a solid phase extractor in flow analysis, Talanta 76 (2008) 988-996.
[84] Y. Wang, J. Tang, X.Y. Luo, X.Y. Hu, C. Yang, Q. Xu, Development of a sensitive and selective kojic acid sensor based on molecularly imprinted polymer modified electrode in the lab-on-valve system, Talanta 85 (2011) 2522-2527.
[85] T. Jing, J.W. Niu, H. Xia, Q. Dai, H.Y. Zheng, Q.L. Hao, S.R. Mei, Y.K. Zhou, Online coupling of molecularly imprinted solid-phase extraction to HPLC for determination of trace tetracycline antibiotic residues in egg samples, Journal of Separation Science 34 (2011) 1469-1476.
[86] M. Zougagh, A. Rios, M. Valcarcel, Automatic selective determination of caffeine in coffee and tea samples by using a supported liquid membrane-modified piezoelectric flow sensor with molecularly imprinted polymer, Analytica Chimica Acta 539 (2005) 117-124.
[87] J.L. Suarez-Rodriguez, M.E. Diaz-Garcia, Flavonol fluorescent flow-through sensing based on a molecular imprinted polymer, Analytica Chimica Acta 405 (2000) 67-76.
[88] L.Q. Yang, X.M. Zhao, J. Zhou, Selective enrichment and determination of nicosulfuron in water and soil by a stir bar based on molecularly imprinted polymer coatings, Analytica Chimica Acta 670 (2010) 72-77.
[89] P. Svoboda, D. Sander, K. Plachka, L. Novakova, Development of matrix effect-free MISPE-UHPLC-MS/MS method for determination of lovastatin in Pu-erh tea, oyster mushroom, and red yeast rice, Journal of Pharmaceutical and Biomedical Analysis 140 (2017) 367-376.
[90] GE Healthcare Bio-Sciences Corp., Filtration for HPLC, Sample preparation, General Electric Company, Marlborough, USA, 2017.
[91] SD Kolev, M.I.G.S. Almeida, RW Cattrall, Polymer Inclusion Membranes, Handbook of Membrane Separations: Chemical, Pharmaceutical, Food, and Biotechnological Applications, Second Edition, CRC Press, Taylor & Francis Group, Boca Raton, Florida, USA, 2013, pp. 723-739.
[92] M. Alexovic, B. Horstkotte, P. Solich, J. Sabo, Automation of static and dynamic non-dispersive liquid phase microextraction. Part 2: Approaches based on impregnated membranes and porous supports, Analytica Chimica Acta 907 (2016) 18-30.
[93] L.D. Nghiem, P. Mornane, I.D. Potter, J.M. Perera, R.W. Cattrall, S.D. Kolev, Extraction and transport of metal ions and small organic compounds using polymer inclusion membranes (PIMs), Journal of Membrane Science 281 (2006) 7-41.
[94] M.I.G. Almeida, R.W. Cattrall, S.D. Kolev, Polymer inclusion membranes (PIMs) in chemical analysis-A review, Analytica Chimica Acta 987 (2017) 1-14.
[95] H.H. See, P.C. Hauser, Electric field-driven extraction of lipophilic anions across a carrier-mediated polymer inclusion membrane, Analytical chemistry 83 (2011) 7507-7513.
[96] Z. Legnerová, H. Sklenářová, P. Solich, Automated sequential injection fluorimetric determination and dissolution studies of ergotamine tartrate in pharmaceuticals, Talanta 58 (2002) 1151-1155.
[97] C.K. Zacharis, A. Verdoukas, P.D. Tzanavaras, D.G. Themelis, Automated sample preparation coupled to sequential injection chromatography: On-line filtration and dilution protocols prior to separation, Journal of pharmaceutical and biomedical analysis 49 (2009) 726-732.

[98] J. Van Staden, H. Britz, In-line flow injection extraction-preconcentration through a passive hydrophilic membrane. Determination of total phenols in oil by flow-injection analysis, Fresenius' journal of analytical chemistry 357 (1997) 1066-1071.
[99] E. Luque-Pérez, A. Ríos, M. Valcárcel, L.-G. Danielsson, F. Ingman, Spectrophotometric flow injection determination of caffeine in solid and slurry coffee and tea samples using supported liquid membranes, Laboratory Automation Information Management 34 (1999) 131-142.
[100] L. Jing-Fu, J. Gui-Bin, Determination of anionic surfactants in detergents by microporous membrane liquid–liquid extraction and flow injection spectrophotometry, Microchemical journal 68 (2001) 29-33.
[101] Y. Shen, J.̊ke Jönsson, L. Mathiasson, On-line microporous membrane liquid− liquid extraction for sample pretreatment combined with capillary gas chromatography applied to local anaesthetics in blood plasma, Analytical Chemistry 70 (1998) 946-953.
[102] J. Norberg, E. Thordarson, L. Mathiasson, J.Å. Jönsson, Microporous membrane liquid–liquid extraction coupled on-line with normal-phase liquid chromatography for the determination of cationic surfactants in river and waste water, Journal of Chromatography A 869 (2000) 523-529.
[103] J. Doležal, C. Moreno, A. Hrdlička, M. Valiente, Selective transport of lanthanides through supported liquid membranes containing non-selective extractant, di-(2-ethylhexyl) phosphoric acid, as a carrier, Journal of Membrane Science 168 (2000) 175-181.
[104] M. Tudorache, J. Emnéus, Selective immuno-supported liquid membrane (ISLM) extraction, enrichment and analysis of 2, 4, 6-trichlorophenol, Journal of membrane science 256 (2005) 143-149.
[105] E. Thordarson, J.Å. Jönsson, J. Emnéus, Immunologic trapping in supported liquid membrane extraction, Analytical chemistry 72 (2000) 5280-5284.
[106] M. Tudorache, I.A. Zdrojewska, J. Emnéus, Evaluation of progesterone content in saliva using magnetic particle-based immuno supported liquid membrane assay (m-ISLMA), Biosensors Bioelectronics 22 (2006) 241-246.
[107] L.L. Zhang, R.W. Cattrall, S.D. Kolev, The use of a polymer inclusion membrane in flow injection analysis for the on-line separation and determination of zinc, Talanta 84 (2011) 1278-1283.
[108] E.A. Nagul, C. Fontàs, I.D. McKelvie, R.W. Cattrall, S.D. Kolev, The use of a polymer inclusion membrane for separation and preconcentration of orthophosphate in flow analysis, Analytica Chimica Acta 803 (2013) 82-90.
[109] K. Annane, A. Sahmoune, P. Montels, S. Tingry, Polymer inclusion membrane extraction of cadmium (II) with Aliquat 336 in micro-channel cell, Chemical engineering research design 94 (2015) 605-610.
[110] C.C. Acebal, M. Grünhut, I. Šrámková, P. Chocholouš, A.G. Lista, H. Sklenářová, P. Solich, B.S.F. 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.
[111] European Food Safety Authority, EFSA Panel on Diabetic Products, Nutrition and Allergies, Scientific opinion on the substantiation of health claims related to monacolin K from red yeast rice and maintenance of normal blood LDL cholesterol concentrations (ID 1648, 1700) pursuant to Article 13 (1) of Regulation (EC) No 1924/2006, EFSA Journal 9 (2011) 2304.

[112] Australian and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and NEw Zealand, Australian and New Zealand Guidelines for Fresh and Marine Water Quality, National Water Quality Management Strategy, No. 4, 2000.

8. SUPPLEMENTARY MATERIAL

Supplement 1A: Fully automatic flow-based device for monitoring of drug permeation across a cell monolayer – manuscript

Supplement 1B: Separation of Rhodamine 123 and verapamil – unpublished results

Supplement 2: Universal efavirenz determination in transport study, rat placenta perfusion and placenta lysate by HPLC-UV – manuscript

Supplement 3: An integrated on-line method for the preconcentration and simultaneous determination of metsulfuron methyl and chlorsulfuron using oxidized carbon nanotubes and second order fluorescent data – manuscript

Supplement 4: Fully automated method based on on-line molecularly imprinted polymer solid-phase extraction for determination of lovastatin in dietary supplements containing red yeast rice – manuscript

Supplement 5: Sequential injection determination of 2,4-dichlorophenoxyacetic acid herbicide using preconcentration with a polymer inclusion membrane – unpublished data