CHARLES UNIVERSITY
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
DEPARTMENT OF ANALYTICAL CHEMISTRY

THE AUTOMATION TECHNIQUE LAB-IN-SYRINGE: DEVELOPMENTS AND APPLICATIONS

Habilitation thesis

(COMPLETE AND COMMENTED PUBLISHED SCIENTIFIC WORK)

2023   M.Sc. Burkhard Horstkotte, Ph.D.

UNIVERZITA KARLOVA
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
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AUTOMATIZAČNÍ TECHNIKA LAB-IN-SYRINGE: VÝVOJ A APLIKACE

Habilitační práce

(Soubor publikovaných vědeckých prací doplněný komentářem)

2023   M.Sc. Burkhard Horstkotte, Ph.D.

I declare that this thesis is my original work and that I am the sole author. All literature and other sources from which I have used during preparation are given in the list of references and were properly indicated and cited. This habilitation thesis was not used to obtain another or the same degree at this or another institution.

„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato habilitační práce nebyla využita k získání jiného nebo stejného titulu.“

Hradec Králové, 13th March 2023   M.Sc. Burkhard Horstkotte, Ph.D.

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MOJÍ IVANĚ

Play is the highest form of research

Albert Einstein

Theoretical physicist (1879 - 1955)

Index of abbreviations

ACN	acetonitrile
BI	bead injection
CE	capillary electrophoresis
CPE	cloud point extraction
DES	deep eutectic solvents
DI-SDME	direct immersion single drop microextraction
DLLME	dispersive liquid-liquid microextraction
ELISA	enzyme-linked immunosorbent assays
ETAAS	electrothermal atomic absorption spectrometry
FAAS	flame atomic absorption spectrometry.
FEP	fluorinated ethylene propylene
FIA	flow injection analysis
FIE	flow injection extraction
FT	flow technique
GC	gas chromatography
HC	holding coil
HF	hollow (membrane) fiber
HLLE	homogeneous liquid-liquid extraction
HPLC	high performance liquid chromatography
HS-E	head-space extraction
HS-SDME	head-space single drop microextraction
i.d.	inner diameter
ICP-AES	inductively coupled plasma atomic emission spectrometry
LAV	lab-at-valve
LC	liquid chromatography
LED	light emitting diode
LIS	lab-in-syringe
LLE	liquid-liquid extraction
LOD	limit of detection
LOV	lab-on-valve
LWCC	liquid waveguide capillary cell
MeOH	methanol
MS	mass spectrometry
MSFIA	multisyringe flow injection analysis
NdFeB	neodymium magnets
PEEK	polyether ether ketone
PMMA	poly(methyl methacrylate)
PTFE	polytetrafluoroethylene
RSD	relative standard deviation
SFA	segmented flow analysis

SIA	sequential injection analysis
SLME	supported liquid membrane extraction
SV	(multiposition) selection valve
SWIA	step-wise injection analysis

Content

1. Introduction

Each instrumental technique used in chemical analysis has a sweet-spot field of application, i.e., it ideally fits analytical problems while for other tasks, another technique might be preferred, if at hand, due to higher selectivity sensitivity, cost-efficiency, or robustness. Some techniques are ideal for one analyte only such as cold vapor atomic absorption spectroscopy which is a methodology of choice for the determination of traces of mercury in waters or air. As other techniques, high performance liquid chromatography (HPLC) with mass spectrometric detection (MS) has an extremely wide range of applicability but will be unacceptably costly for simple mixtures of organic analytes present in a ppm concentration range and it is surely not the technique of first choice for the determination of nutrients in seawater.

One smaller branch of instrumental techniques are *flow techniques* (FTs) or *flow approaches*, sometimes addressed with the addition of "non-separative" to distinguish them from capillary electrophoresis (CE) or HPLC, which can be considered separative FTs. The common principle of FTs is the processing of a defined volume of sample in a tubing network and *in-flow.* The most often accomplished tasks are chromogenic assays, separation of the analyte from the sample matrix, or analyte preconcentration where analyte selectivity is achieved either by the chosen reagent, separation approach, or detector.

However, it was stated by two leading professors of the early developments of FTs, Prof. G.D. Christian and Prof. A. Townshend, that: "*Flow injection analysis[1] is more than an analytical technique. It is a technology that provides a platform for the use of most analytical methods*" [1]. The take-home message from this statement is that FTs are tools for the automation of laboratory procedures, and they can be combined advantageously with many other instrumental techniques. Therefore, the application field of FTs has "blurry borders", which becomes apparent considering that one of the main focuses of developing and using FTs today is the automation of sample preparation procedures that are often coupled to what shall be defined as *advanced instrumental techniques*: HPLC, CE, GC, or types of atomic spectrometry.

Automation of laboratory operation comprises in all cases, above all, solution metering, mixing, and transport. So-denoted batch-automation is characterized by performing each sample processing in an individual mixing chamber, generally a vial, and solution handling is accomplished by a computer-controlled syringe acting as an automatic pipette and by the robotic movement of either the sample vial, rack, or pipette needle. Solution mixing, e.g., with water for dilution, chemical agents for derivatization, or an extraction solvent, is always complete, i.e., homogeneous in case of miscible phases. Determinations must be carried out in a reaction *steady state*, given chaotic mixing and implied variability of reaction start. Long equilibration times are over-compensated by possibly processing multiple samples simultaneously.

In contrast, FT automation relies on the sequential processing of samples that are injected or aspirated into a tubing network – *manifold* – and transported in flow by a carrier solution that simultaneously cleans the manifold. The way to introduce the sample, mixing patterns, modalities of carrier flow, and

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[1] Flow injection analysis will be explained later in section 3.2.3.2. The statement can be extended to other FTs

pump characteristics allows to distinguish various types of FTs that have been developed over the last 60 years. However, further principles in contrast to batch automation is the use of a generally laminar carrier flow in which mixing is achieved by solution dispersion. Solution zones merge gradually but follow a highly reproducible pattern so that reliable quantification of a detection product is feasible long before reaction equilibrium. These features offer several important advantages over batch-automation, e.g., following very fast chemiluminescence reactions, determinations based on the catalytic activity of analytes, kinetic differentiation of analytes related to a formed pH gradient, or increasing the lifetime of an enzymatic reactor or electrode that are only briefly exposed to the sample matrix before being regenerated by the carrier solution. Integration of elements in the flow network enhances the operational versatility of FTs compared to vial-based approaches: cartridges for solid phase extraction (SPE), membrane-based phase separators, or radiated tubing coils for analyte mineralization, to name a few.

Gained benefits of sample preparation, in general, are higher method selectivity, detection sensitivity, higher robustness against changes of the sample matrix, and an increased lifetime and reliability of any directly coupled detection technique. Additional advantages achieved by automation, valid for both flow and batch approaches, are generally rendering a higher procedural reproducibility by eliminating arbitrary handling errors, avoidance of user contact with harmful reagents or, vice versa, sample contamination by the user, fast and continuous processing, and the possibility for procedural miniaturization.

Significant advantages of FTs that compensate by far for the drawback of sequential operation include the possibility of connecting the preparative procedure online with the subsequent detection instrumentation to enable higher analyte transfer and in consequence a higher sensitivity, hardly any consumables needed, and far lower costs of the required instrumentation. On the other hand, batch-automation emulates the way of human labor, i.e., homogeneous mixing is a more intuitive concept with predictable operation than a proceeding gradient formation during the transportation in flow. Moreover, using autosampler systems, an increase in sample volume to yield higher preconcentration in automated extractions, and performing procedures that involve multiple steps are far simpler to do than in most FTs.

The flow-batch concept presents a bridging approach between batch and FT-automation. It is based on using a homogeneously mixed chamber as a central element in a flow analyzer, which enables stepwise and homogeneous mixing of solutions and thus, facilitates procedure design and comprehension. While simple, downsides of this approach include the need for lasting chamber cleaning and limitations regarding the automatability of liquid-liquid extractions as the chamber must be atmospherically open. This has led to the idea of using the void of an automatic syringe pump as a mixing, reaction, and extraction chamber for flow-batch procedures as a new FT denoted *Lab-In-Syringe* (LIS). This new automation concept has mainly been used for the automation of a large variety of sample preparation methodologies.

In this habilitation thesis, the development of this latest offspring of the FT family is described and set in comparison to other automation approaches, especially to those based on sample processing *in-flow*. LIS presents a current trend in FT advancement to which end, operational possibilities, advantages, and limitations, are explained in the example of reported applications. Furthermore, a theoretical background to laboratory automation and *in-syringe* automated sample preparation approaches is provided.

2. Objectives of the work

The main aim of this habilitation thesis is to describe the developments and current state of knowledge related to the automation and flow batch technique Lab-In-Syringe. This comprises the main aspects and features of this technique as well as recent advances in terms of instrumentation, methodology, and application. These are predominantly the automation of sample preparation methodologies including the development of automation concepts and the study of new preparative approaches and related elements. These methodologies deviate mostly from the various types of liquid phase microextraction but include also other approaches to enrich the analyte(s) of interest to enhance detection sensitivity and simultaneously to remove matrix components interfering in the analysis, i.e., sample clean-up.

To this end, the thesis aims to describe the main purposes of sample preparation and to discuss the needs of automation and miniaturization as well as to explain the main preparative methodologies in particular from a practical point of view rather than giving a comprehensive overview of published work.

A second objective is to provide a brief overview and timeline of the main non-separative FTs and concepts for automation in-flow including a description of principles, fields of application, and their advantages as instrumental techniques in analytical chemistry, as well as to compare these approaches in terms of operation, characteristics, and specific advantages and limitations. This includes an illustration of the Lab-In-Syringe technique and its key advances and a contrasting juxtaposition to other automation approaches with focus on automation *in-flow*. A demarcation towards an equally termed approach   (LIS) in analytical chemistry used for manual performance of sample preparation shall be given, too.

The third aim is to critically discuss the advances, capabilities, and applications achieved by the Lab-In-Syringe technique on the examples of published works, and to discuss the potential, limitations, room for improvement, and future perspectives of this technique.

Initiated by my colleague Dr. F. Maya, I consider myself lucky for having been at the "birth" of the Lab-In-Syringe technique and for being able to contribute to its progress from the second experimental work. I hope that this thesis will also contribute to the promotion of this technique and the awakening of interest in exploring it as an instrumental tool in chemical analysis and automation of sample preparation methods.

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3. Theoretical part

3.1. Laboratory automation and alternative concepts to flow techniques

Analytical work can be automated by several means and tools, FTs being one of them. An introduction to FTs was given already and the topic will be discussed thoroughly in the following sections. Here, the aims of laboratory automation[2] and existing tools and concepts are discussed as well as where automation can be used in the analytical process. This process can be divided into the following steps:

- *Sampling (and stabilization)*: A representative sample must be taken considering the location, size, time of the day, sampling tools, and storage compartment and conditions. This is often the task that is most difficult to perform correctly and automatable only in a few cases, e.g., in-situ analysis. This is because many factors must be considered, which implies a profound knowledge and understanding of the problematic at hand. For example, soil sampling from a large acre for the determination of organic nitrogen will require sample pooling and disabling of bacterial degradation, the day-night-cycle of plankton must be considered for the sampling of ocean water, and wastewater samples for the analysis of remains of volatile organics and heavy metal must be stored in different containers.

- *Transport and storage*: A sample cannot always be processed without delay or at the location of sampling. Therefore, care must be taken to avoid alteration of the collected material during transport and storage. However, automation can hardly contribute to this step positively by other means than for instance sample transport by an autonomously driving car.

- *Pretreatment and preparation*: These terms are often used as equivalents and a clear distinction does not exist. Sample pretreatment is rather used for the initial steps of sample homogenization, crashing and sieving, drying, representative division, or weighting, in short, tasks that require manual   work.
On the other hand, sample preparation comprises the steps that yield the final solution used for the analysis or procedures carried out in an automated system before analyte quantification. It is these later tasks where most attention concerning laboratory automation goes.

- *Analysis and data-readout*: The determination of the analyte by instrumental techniques.

- *Data evaluation and interpretation* including error analysis and check for result plausibility.

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[2] The word "automation" is related to the ancient Greek word αὐτόματον and means "acting of one's own will. It is used for technologies that reduce human intervention in different kinds of processes. Very often, the denotation "automatization" is used as synonym while a recommendation from IUPAC suggests [2] that "automation" should be used only in case of an existing feed-back or self-regulation in the system and "automatization" is for all self-operating robotic mechanization without human intervention, typically saving personal labor, waste, and costs and improving quality, precision, and accuracy. Nonetheless, I decided to use the denotation "automation" since "laboratory automation" yields over 100-times more results on Web-of-Science than "laboratory automatization" although it can be assumed that in most analyzers used, no such self-regulation was implemented.

It is commonly known that errors committed at the beginning of the analytical process will propagate through the sequence and are, by far, more severe, and less easy to trace and compensate for than errors committed in a later step [3]. It is also more likely that such errors are systematic rather than arbitrary. In terms of the analysis, automatable tasks are the preparation of standards, sample spiking, sample transfer or provision to the detector, and derivatization reactions to convert the analyze into a detectable form. However, it is the automation of tasks related to sample preparation that receives increasing attention from the analyst. In fact, the development of better sample preparation methodologies has been a hot topic in chemical research in the last decade for being a source of significant errors in chemical analysis, which can be at least diminished by their automation.

These include mixing processes (dilution, standard addition, derivatization) as well as sample digestion and approaches for matrix removal and analyte preconcentration. Tasks that are difficult to automate generally require identifying, moving, and carefully positioning a solid element, e.g., replacement of filters, the weighting of sample, vial placement into centrifuges, and other tasks of sample pretreatment.

Important objectives are achieving sample compatibility with the intended detection technique and prolongation of maintenance cycles and lifetime of the respective instrument and improving method selectivity, sensitivity, and determination reliability by avoiding human errors and sample contamination during processing. Furthermore, procedural automation is attractive for being often accompanied by a gain in reproducibility and sample throughput, at least by enabling 24 h, 7 days-a-week operation. It also facilitates procedural downscaling to cut costs for consumables and waste disposal and make analysis feasible when little sample material is available, making it highly attractive for bio- and clinical analysis. Finally, automation contributes to work safety by reducing user exposure to harmful substances.

In terms of automation approaches, the two main concepts of automation *in-batch* and *in-flow*, were already explained in the introduction of the thesis. Considering that the following sections will focus on automation in mesofluidic flow analyzers, i.e., FTs, here only a brief insight into main automation concepts is provided and the concepts, capabilities, and problematics are identified.

Autosamplers / cartesian robots

Autosamplers (Figure 1A) are the workhorses of commercial laboratories and rely on batch processing, i.e., solution mixing in vials aided by airflow, magnetics stirring, or vortexing. Injection syringes of different sizes, possibly eligible by the instrument itself, are used for solution transfer or injection to a coupled detection instrument. Using a gripper, vials can be placed into a heating, vortexing, or pressure chamber, e.g., to promote head-space extraction or degassing by vacuum. Performable extraction techniques include SPE procedures, based on the replacement of single-use cartridges, liquid-liquid-, headspace-, and both types of single drop microextractions. As an important advantage, multiple samples can be processed in parallel, and extract injection can be done directly by the autosampler if positioned on the top of a gas chromatograph or in combination with an injection valve to liquid chromatography. Some instruments even provide the option of automated centrifugation. Drawbacks are surely their costs, exceeding those of a compact car, and the low flexibility in extending the operational possibilities or use for monitoring tasks, as well as the required user training, and costs for maintenance and consumables.

Rotational and anthropomorphic robots

These refer to two further types of robotic automation with higher operational versatility, range, and flexibility of movement. In particular, robotic arms (Figure 1B) with five or six rotational degrees of freedom can truly emulate human work operations: move samples through the free room from one location to another, grip a pipette, weight a sample, measure pH, add solutions, open and close flasks, place a solution on a vortex, position a vial into a centrifuge, and perform diverse types of solvent extractions [4]. For this, all required devices (balance, centrifuge, stirrer, evaporator, etc.), tools (pipettes), consumables (pipette tips, vials), samples, waste deposits, and the injector port of the analytical instrument to be used must be exactly positioned on the working bench and the movement of the robotic arm need precise calibration. More recently, companies are developing mobile manipulators, robotic arms on autonomous guided vehicles, that can move between the analytical instruments and work benches [5,6]. The approach of "robotic-arm" automation can be of interest where the number of samples or the risk of working with them (acids, toxic materials, radioactive substances, etc.) justifies such large investment into instrumentation purchase, setup and calibration, and training of laboratory staff. These likely include clinical facilities, mining, the petrol industry, and company-based medical and pharmaceutical research. The spectrum of capabilities of robotic automation is similarly large as listed operations of interest for sample pretreatment and preparation: cell lysis, protein expression, protein and DNA purification, polymerase chain reaction (PCR), or enzyme-linked immunosorbent assays [7].

Microtiter plate processing

This approach overlaps with former described robotic automation, and it can be said, that pipetting autosamplers and robotics are the tools and microtiter plates are the platform for automation that is directed mainly to bioanalysis with the above-listed automated procedures [8]. Microtiter or well-plates are plastic boards, which feature typically 96 or 384 container positions of sub-milliliter volumes, which makes them ideal for doing dilution series, screening experiments, and achieving high sample throughputs. Their transparency enables determinations by spectrophotometry, fluorimetric, turbidimetric, or chemiluminescence measurements in special readers (Figure 1C). Using purpose robotic systems, sample preparation that can be readily automated include LLE, supported membrane extraction [9]], electromembrane extraction [10],or solid phase microextractions **Error! Reference source not found.** apart from liquid mixing or transfer to advanced analytical instrumentation. However, they are mostly used as platforms for bioassays, e.g., enzyme-linked immunosorbent assays (ELISA). The benefits for pharmaceutical research, proteomics, and clinical analysis were evaluated as immensely positive in a recent article in a laboratory magazine discussing the capabilities of these systems and listing commercial providers [13]. However, their practicability for trace analysis in environmental analysis is surely limited by a low sample volume and achievable preconcentration factors. It should be noted that microtiter plates are also used without automated systems but in manual procedures using multi-pipettes.

Microfluidics

Microfluidics chips are devices with channels in the micrometer range that are produced by micro-milling, etching, or hot embossing. They can be further divided into various categories depending on the way the liquid is manipulated. The variants that are most often used in practical applications and that

show the widest overlap with FT automation, Lab-on-a-chip, Lab-on-a-disk, and paper-devices, are here briefly explained. Other types include electrokinetic platforms, which operate by electroosmotic flow and present platforms for the miniaturization of electromigration techniques, as well as microfluidic droplet formation and electrowetting drop manipulation, which both can be seen as research tools, e.g., to study colloids or single cells confined in aqueous drops [14], are here disregarded. It was stated that "Microfluidics should be merely considered as a toolbox, which is needed to develop innovative new products in the life sciences" [15] as some approaches are rather of academic interest with few practical applications so far.
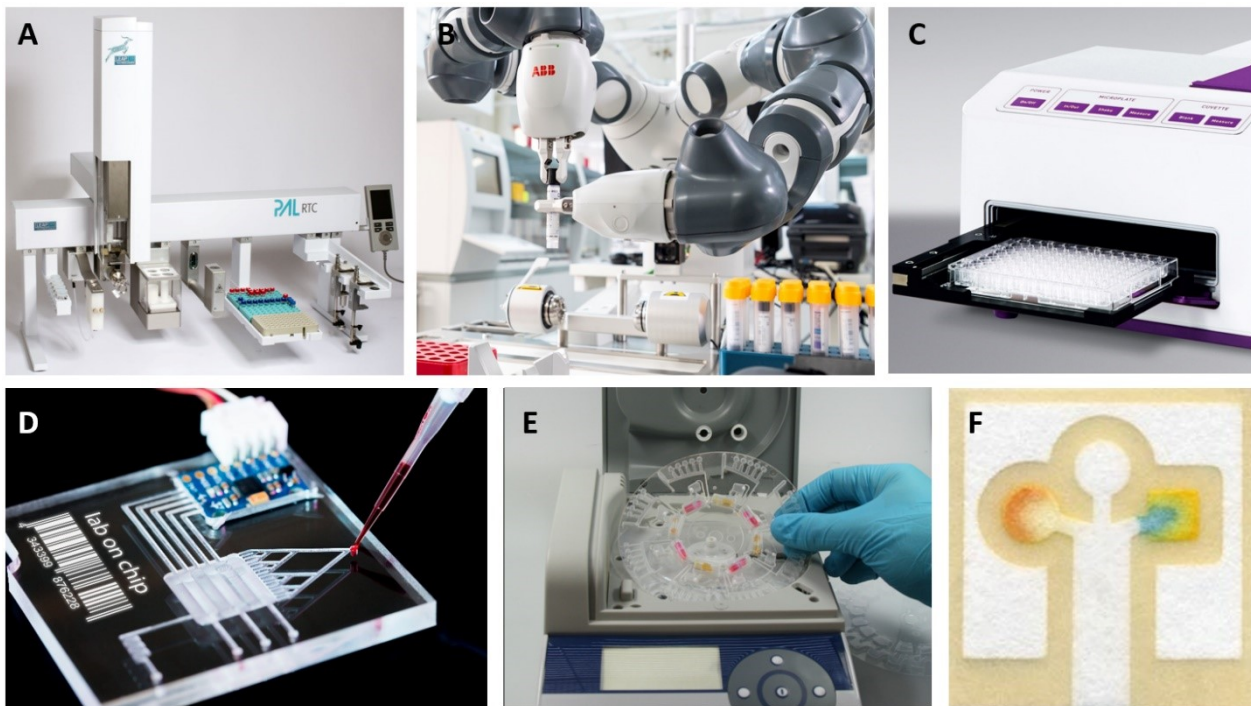
**Figure 1: Examples of online available photos (10.03.2023) to common automation approaches other than FTs. A: advanced autosampler systems, B*: (anthropomorphic) robots, C: microtiter places, D: Lab-On-A-Chip, E: Lab-On-A Disc, F: paper devices.*

A: PAL3 autosampler system form company CTC Analytics AG (Zwingen, Switzerland), found on https://www.leaptec.com/products/pal3, B: Collaborative Robot YuMi® - IRB 14000, company ABB (Zürich, Switzerland), found on https://new.abb.com/news/detail/70465/abbs-collaborative-robot-at-karolinska-university-laboratory C: SPECTROstar Nano Plate reader from company BMG Labtech (Ortenberg, Germany), found on https://noor-scientific.com/products/spectrostar-nano, D: Polytec GmbH (Waldbronn, Germany), found on https://www.polytec.com/eu/surface-metrology/areas-of-application/biology-and-medicine/lab-on-a-chip, E: Centrifugal analyzer from QIAGEN Lake Constance GmbH (Stockach, Germany), found on https://www.gesundheitsindustrie-bw.de/en/article/news/qiagen-lake-constance-a-disk-player-for-rapid-diagnoses, F: from [16], found on https://www.technologyreview.com/2008/05/14/220532/lab-on-a-chip-made-of-paper.

Microfluidics rely in many cases on disposable material but are based on the processing in flow so that they cannot put into the boxes batch automation and flow automation. Following the later explained idea of flow-batch, actually a batch process in flow, they should be considered as flow processes in batch.

Lab-On-A-Chip

This expression refers to automation based on pressure-driven flow with manipulated volumes ranging from a few microliters down to nanoliters while standard-size instrumentation is generally required to control the microfluidic operation from outside, e.g., pumps or pneumatic actuators as the microfluidic chips themselves generally lack integrated pumping. Over the last decades, there has been a significant effort in developing tools for fluid transport, valving, or mixing, as well as for the fabrication of these devices. In terms of use in analytical chemistry, a few examples of automated liquid phase extractions have been reported [17] as well as the integration of enzymatic reactors or monolithic columns for miniaturization of separation or aiming for multi-dimensional liquid chromatography (LC). The author contributed to the development of a chip-like injection valve rotor with an integrated monolithic column that served for analyte stacking in 2D-LC [18,19]. However, most research was focused on the development of chip-based bioassays, point-of-care diagnostics, and the cultivation of cells. Given the high effort needed for the development of these devices and for the required instruments to control them, their future of microfluidics cannot be the downscaling of sample preparation methodologies that are simpler and easier accomplished by FT, sometimes also denoted "mesofluidics" [20,21]. Instead, their potential and benefits are surely utilization in medical and pharmaceutical research for the cultivation of cells to simulate human organs – known as Organ-On-Chip – and provide screening tools for therapeutics [22,23]. Here, the integrable microstructures are of use for the accommodation of cell agglomerates and study them in a controlled medium under fluidic conditions that simulate intestinal or blood flow.

Centrifugal analyzers

Centrifugal analyzers are recently re-gaining popularity as *Lab-on-a-Disc.* This is reflected by the increasing number of publications in the last decade after peaking in the late seventies to early eighties and following abatement. They present a special format of microfluidic devices and are produced by the same technologies but differ in the use of centrifugal forces for liquid driving. Several fluidic micro-conduits of equal architecture are integrated into one transparent disc. For use, it is placed in a special centrifuge that also integrates an optical detector or pickup electrodes for electrochemical detection based on electrodes and slip rings integrated into the disc. Each micro-conduit is loaded with the respective solutions that are positioned close to the center. By increasing the rotation speed over a critical "burst frequency", the liquids are forced e.g., over an elevated barrier to flow from one compartment to the next, possibly aided by capillary forces. Applicable flow rates range over six orders of magnitude. Performable tasks include reagent confluence, immunoassays based on immobilized antibodies, or cell lysis. The detection chambers are located in the outer part of the disk with readout possible during rotation. Practical features are that multiple samples can be processed simultaneously in one run and within a short time, that no connection to an external pump is required, and that centrifugation is an intrinsic feature. This makes *Lab-on-a-Disc* ideal for clinical analysis as cells can be readily separated from serum for lyses or the in-disc purified serum can be used for a chromogenic assay [24]. The same analyzer can be used for different tasks just by changing the discs. Disadvantages are related to method development based on a cycle of micro-conduit designing and simulation, prototyping by micromachining, testing and re-optimization and practically no possibility for method adaption by the final user. They offer versatile pretreatment and automated analysis but do not work

as flexible tools for versatile laboratory automation. Their main field of application is clinical analysis but with an increasing focus also on parameters of environmental interest [25].

Paper devices:

In these devices, the flow is driven by the capillary forces of the medium. The fluidic paths are produced by impregnating the paper with hydrophobic ink (wax printing) that confines the liquid to the hydrophilic wettable areas. The paper itself can act as a filter medium as well as a support for a sorbent or deposited reagents. Paper devices have become popular among FT practitioners since they require less effort in optimization and instrumentation while offering a platform to carry out the same chromogenic reactions as automated by FT analyzers. Moreover, similar unit operations are feasible including chromogenic assays including analyte reaction or gas diffusion [26,27] while they are mostly used for immunoassays [28]. Appealing features include the practical use for rapid and self-applicable diagnostics, portability, simplicity in use and production, low costs, and diverse possibilities for sample preparation and quantification, e.g., by electrochemical detection or colorimetry [29,30]. On the other hand, they remain devices for analyte screening rather than accurate and precise quantification and show a limited ability of analyte preconcentration. In this regard, a combination with FT should be highlighted, where the authors investigated the possibility to replace the tubing manifold with a strip of paper which allowed pumpless miniaturization of the chromogenic determination of peroxide [31].

To choose from the available automation approach the right one is a matter of financial resources, gained benefit of investment, available time for method development, and required sample throughput, among others. It is my opinion that FTs perform very well considering its price-to-benefit ratio, versatility, and fast method development when it comes to tasks that can be broken down into a few unit operations.

3.2. Non-separative analytical flow techniques

An overview will be given on automatization *in-flow* and the related non-separative analytical flow techniques and concepts. For reasons of simplicity, the shorter name *flow techniques* (FTs) will be used knowing that there is in fact a methodical overlap with approaches used for synthesis *in-flow* - flow chemistry [32] – and with the separative liquid chromatographic (LC) and electromigration techniques.

3.2.1. Introduction, common principles, and features

The common feature of FT is solutions transport in a tube assembly, hereafter *manifold*, and treatment of a sample *in-flow*, e.g., by either dilution, chemical reaction, or a type of sample preparation. This distinguishes them from the formerly explained batch automation approaches [33]. Most often, a carrier solution is used that is driven by peristaltic, syringe, or solenoid membrane pumps while other propulsions devices have been investigated including electroosmotic or piezoelectric pumps [34,35]. FT became rapidly established as analytical tools in a time when HPLC was still not a widely utilized nor accessible technique. This is due to their ability to carry out a spectrum of analytical procedures rapidly and in an automated fashion with a minimum of affordable instrumentation. For early approaches, "fluidic mechanization" might be a more appropriate designation since not even computer control was required not available.

Since the automated procedure is carried out in a closed manifold of chemically resistant polymer or glass tubes, sample alteration by oxidation, evaporation, or ambient contamination is prevented and the exposition of laboratory personal and working areas to used hazardous substances is minimal.

FT are non-separative, the best that can be achieved is a selective separation of the analyte from the sample matrix. Most often, FT are used to automate chromogenic assays based on selective reagents, in a majority for inorganic analytes or organic analytes with distinct reactivity or in combination with a preparative procedure or kinetic distinction between analyte and interferent. On the other hand, this makes FT ideal tools for the automation of determination of total indices [36], so procedures aiming to quantify the sum of similarly natured substances. Moreover, FT have often been used for the automation of enzymatic reactions, which are, by nature, analyte selective. Simultaneously, FT provide a platform for reproducible execution and miniaturization of enzymatic assays, thus saving money for needed reagents.

FT have also proven to be ideal tools for the monitoring of fast reactions given their advantage of being able to perform solution mixing fast, highly reproducible, and user-independent for which chemiluminescence assays are good examples [37].

Furthermore, they have been used to automate preparative procedures including SPE and liquid-liquid phase extraction (LLE) and in the last years increasingly liquid phase microextraction (LPME) approaches. Detection in FT analyzers is accomplished by integrated or directly coupled optical[3], electrochemical (potentiometry, amperometry, conductimetry), detectors or online coupled to advanced instrumental techniques atomic absorption-, atomic emission-, and atomic fluorescence spectrometry as well as

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[3] Mainly (spectro)photometry, fluorescence spectrometry, turbidimetry, and chemiluminescence detection

inductively coupled plasma atomic emission spectrometry (ICP-AES) and ICP-MS. All these techniques are compatible with a continuous sample flow and capable of transient detection. Resulting signals are generally peak-shaped and their height, in some cases width, is generally correlated with the analyte concentration. A peculiarity of using spectrophotometry in flow analyzers is that the sample matrix passes the detection flow cell at the same time as the analyte. The resulting baseline disturbance by a difference in refraction index between carrier and sample solvent, the so-denoted *Schlieren effect* [38,39]*,* must be at least partially compensated by measuring light extinction on a reference wavelength, which is optional in HPLC and CE. Today, FT are increasingly used to automate sample preparation for separation techniques HPLC, CE, and gas chromatography (GC). Typically, online coupling to HPLC is normally done via an injection valve that is loaded by the flow system or an appropriate interface for CE or a flow-accessible vial [40].

Initially, FT were used in clinical analysis to deal with high numbers of alike samples but became tools for choice in oceanography, environmental and agricultural analysis (waters, soil extracts), or for process monitoring. Here, the denotation "process" can refer to environmental compartments (ocean water, air, river, ground water, precipitation, …), a technical process (biotechnological cultivation, wastewater treatment plant, …), or a laboratory setup (dissolution of pharmaceutical formulations, soil leaching, permeation of surrogate skin, …). Being able to provide analytical results often in less than a minute and quasi-continuously, they are valuable tools of vigilance, control, and investigative studies of such processes. Today, FT are increasingly used for the automation of sample preparation procedures starting with the dilution of the sample with a loading buffer for HPLC up to matrix removal and analyte preconcentration by precipitation, SPE or liquid phase microextraction approaches (LPME) [41]. Overviews to procedural operations performable by FT are given in sections 3.2.4, 3.3.1, 3.3.2, and, for LIS, in section 3.4.2 while a comprehensive list of scientific publications related to flow automation can be found in an online database at www.flowinjectiontutorial.com with nearly 24.000 entries by the year 2019 [42].

3.2.2. Flow analyzer components and control

All flow analyzers require a pumping element for transporting the sample through the flow network and will often use valves to introduce the sample or reagents to the system or to deviate the flow for solution (re-)cycling or to let it pass through a different part of the flow network. This *manifold* generally consists of flexible tubing of polyfluorinated polymers (today PTFE or more transparent and gas permeable FEP) which connect the different part of the flow system. Figure 2 gives an overview of different pumps and valves used in flow analyzers. Finally, most setups integrate a detector with an appropriate flow cell unless online coupling to a secondary analytical instrument as described is done or only a preparative procedure is automated with possible detection carried out *offline*. Some of these devices are explained below to provide technical background for the reader.

Among the devices used for liquid propelling, peristaltic pumps (Figure 2A) were the first ones available. The liquid is propelled through elastic pumping tubes that are fitted inside a circular pump casing. The tubes are squeezed closed on various points under the compression of rotating cylinders. Liquid displacement is forced by the movement of the compression points. Flow rates are adjustable by choosing pumping tubes of different inner diameters or altering the rotation speed and direction of the

pump. The advantages are that the inner tubing walls are the only part of the pump in contact with the liquid, which allows sterile pumping and usage for medical purposes. Moreover, they can work in continuous mode and mostly feature multiple flow lines in parallel. Their intrinsic drawbacks included peristalsis-related flow pulsation, flow rate drift by progressive wear-out of the pumping tubes, low volumetric precision, pressure robustness, and limitations regarding the pumped media. Today, they are used only for analyzers based on the early FT approaches that are described in the sections 3.2.3.1, 0, and 3.2.3.4.1.

Figure 2: Pump and valve instrumentation used in flow analyzers: A: multichannel peristaltic pump, B: automatic syringe pump (with multiposition head valve), C: multisyringe pump, D: MilliGat® pump, E: solenoid pump, F: computer-controllable low-pressure injection valve with shown rotor (G), H: multiposition selection valve rotor (left) and stator (right), I: solenoid 3-way valve, J: pinch valve.

More frequently used, and exclusively for the FT related to the topic of this thesis, are automatic syringe pumps (Figure 2 B) that became available in the 1990ies. Assuming that the reader is familiar with the concept of a syringe, consisting of a *barrel* and a *piston*, it may be permitted to explain only that in syringe pumps, the piston is moved by a *pusher blocker* or *lever* that is displaced by a *drive screw* connected to a *step motor*. Some syringe pumps must be manually refilled as they are only designed for dispensing liquid and are used for instance to provide a make-up liquid in coupling nano-LC or CE to mass spectrometry (MS) detection. On the other hand, FT analyzers use computer-controlled syringe pumps capable of bidirectional flow with high resolution of piston movement of only a few micrometers. This can correspond, depending on the chosen syringe size, to less than one microliter.

Automatic syringe pumps normally feature a head valve with 2 to 12 positions (most often 2 or 3) that enables filling the syringe with solution aspirated form one port and dispensing the solution through another one. A special variant is a multisyringe pump (Figure 2C) [43] where the same lever displaces the piston of several syringes at once and where each syringe features an individual 2-position head

valve. Such single pump can replace a multichannel peristaltic pump with the option to provide liquid only from some syringes, i.e., into some pumping lines while returning not required solutions from the syringe back into their reservoir. Syringe pumps show generally high volumetric precision, pressure stability up to about 250 psi, and chemical robustness assured by inert materials used including glass, high performance polymers, and ceramics [44]. The neMESYS 1000N syringe pump of the company Cetoni GmbH (Korbußen, Germany) with a syringe made from stainless steel enables operation pressures up to 3000 psi, yet this comes costly. A disadvantage of these pumps is the limited pumping volume and need to aspirate all solutions beforehand preventing continuous flow operation and prolonging method time, as well as an eventual wear out of the piston head by precipitated salts or particles.

An ingenious pumping concept was developed by the company GlobalFIA (WA, USA) by the so-denoted MilliGAT^{TM} pumps (Figure 2D). They enable both continuous, moreover, pulsation-free flow as well as high volumetric precision and bidirectional operation over a wide and computer adaptable flow rate range. Therefore, it has become very popular in the recent years for FT analyzers. It is based on four syringes rotating in a metal casing with the piston being driven by a swashplate so that one syringe is always aspirating, one is providing, and two are in transition. For better understanding of its operation, an online available video animation is recommended [42]. Initial pressure limitations have been overcome with possible values now of up to 1000 psi at low flow rates, making it, "the Missing Link" regarding pump. However, existing downsides are its price and the impossibility of simple visual inspection or maintenance so that pumping aggressive media or solutions of high matrix load might be not recommended.

Finally, solenoid diaphragm micropumps (Figure 2E) are small devices that generate pronounced pulses with the pump membrane lifted and released upon at activation and deactivation of a solenoid. Two integrated check valves guarantee for a unidirectional and intermittently turbulent flow. Small size and power consumption, moderate costs, possibility of quasi-continuous flow, and simplicity of the required control are significant advantages. However, the stroke volume strongly depends on the experienced backpressure [45] and gas content of the solution and bi-directional flow is only possible by using two pumps in counter direction.

In terms of valves, three main types are used: rotary injection valves (Figure 2 F & G), either turned manually or via a computer-controlled step motor, typically featuring 6-10 lateral positions. Two positions are possible where the rotor connects each position with either its clockwise or anticlockwise neighbor. Such valves serve for the insertion of a liquid segment into a separate liquid system, e.g., for sample injection into the mobile phase of an HPLC. 4-position injection valves can be used for intermediate separation of both in- and outlet of membrane-separators or for sample injection with time-controlled volume into a carrier from a continuous sample stream [46].

The second type is a multiposition selection valve (SV), in which the central port is connected to a lateral port. These can be used to aspirate different solutions into the flow system, thus acting as a sampler, or to deviate solutions to different part of the flow system. Additional functions can be added, e.g., by replacing the stator as explained in section 0.

When it comes to process monitoring, attention should be given to the connection of the flow analyzer to the process stream with a minimum dead volume so that the sample is always representative of the

monitored process. This can be achieved via an injection valve or by a flow-through function on one of the lateral position ports, addable by an appropriate adapter [47].

Solenoid valves present the third type of valves that are typically used to "switch" between flow lines (3-way = normally open, normally closed, common) or to close them (open/close = 2-way). They are actuated via an electromagnet with response times below 100 ms. Valving is either by the lifting of a flexible diaphragm that closes in the deactivated state a flow outlet inside the valve or by squeezing a soft flexible silicon tubing, denoted pinch valves (Figure 2J). More valve variants are used in HPLC systems, e.g., for column switching. An injection valve variant working by linear motion will be explained in section 3.2.3.5.1.

The formerly detailed instrumentation has a significant influence on a FT characteristics and robustness, but it is fair to add that FTs have evolved with the available instrumentation, or rather, FT practitioners have experimented with newly available instrumentation, thus exploring made-possible operations. In a similar sense, system control of FT-analyzers has evolved with the technology at hand. Early flow techniques enabled automation of standard laboratory tasks, mainly solution mixing, without computer control; in fact, the FT described in sections 3.2.3.1 and 0 are from *pre-personal computer* times. It was surely part of their success that a high sample throughput was achievable without worrying about software control with data recorded on a paper chart. With the development of automatic syringe pumps and their use in FT systems, aiming for a higher degree of automation, the flow practitioner had to adopt the ability of programming or be able to pay for specialized software. In general, a serial bus connection to a computer is done via USB or RS232 interface to send text-based instructions to the connected instrument, each related to an operational parameter or parameter set, e.g., setting of the flow rate, pumping direction, valve position, volume, etc. Control software generally provides a user-understandable platform. For instance, to empty a Cavro syringe pump completely, a model frequently used by the author, the command "A0R" is sent while the instruction in the used software, selected by mouse click from a menu, reads "SyringePump_Empty" or similar. Highly versatile features of control platforms for setting up an operational method for the flow analyzer include looping of parts of the method to repeat measurements or cleaning cycles, waiting times, user interaction by inquiries, condition statements (if…then…else), e.g., to activate a part of an instruction method, or variables that replace fixed operational parameters. Today, microcontrollers with open-source coding are increasingly used, e.g., to minimize power consumption and system dimensions which facilitates field operations, i.e., using the flow analyzer *at-side* of the process or system to be monitored with wireless data readout via a mobile app. For such applications, solenoid pumps and valves are of interest as they are easily controlled via a relay board.

3.2.3. Main flow techniques and concepts

In the following, the main different approaches to automation *in-flow* are explained[4]. In the content of this thesis, only a short introduction can be given. Extensive descriptions and overviews of the applications and features of each technique can be found in specialist books [48-51] and reviews [33,52-55]. FT approaches differ in the modes of propulsion and sample introduction, operation schemes, and flow characteristics, in particular whether a multichannel or single channel flow network is used, whether flexibility is given for manifold configuration (multicommutation) and method adaptation (programmable sequence of solution mixing), the carrier flow is continuous or sequential, air segmented or unsegmented, mixing is via dispersion of solution "zones" leading to gradient formation or is achieved homogeneously, sample introduction is by injection or by solution stacking, and detection is done in reaction equilibrium or in the kinetic phase of a reaction.

3.2.3.1. Non-injecting approaches: Continuous and Segmented Flow Analysis

Continuous Flow Analysis (CFA) and (Air-) Segmented (Continuous) Flow Analysis (SFA) technique are the oldest approaches to automation or fluidic mechanization of wet chemistry and are based on sample pumping through a tube with confluent mixing of further reagents, i.e., to change the sample, the aspirating tube must be placed into the sample and later into an auxiliary solution, e.g., water, for intermediate cleaning, not only to remove sample remains but also to clearly distinguish between the sample signals.

Publications reporting on CFA fall beyond the reach of modern publication databases and in fact, I was able to access only 5 publications that fit the usual textbook descriptions. The earliest works were proof-of-concepts of using a flowing sample for polarographic analysis and later continuous monitoring of SO_2 in a technical process by this approach [56,57]. Also later work was mostly performed for polarographic determinations requiring as much as 1-2 mL sample to reach a plateau for stable reading (> 10 mL in total) as the system had to be filled completely with the sample [58]. So, any performed chemical reaction required confluent addition of similarly large volumes of reagents.

The concept of air segmentation of the continuous flow, proposed by SKEGGS [59,60], was therefore a significant advantage: The continuous sample flow was divided by air bubbles, introduced by a confluence before any reagent addition, to generate several liquid segments for each sample over a sampling time of less than a minute but taking into account also the dead volume of the aspiration tubing. Due to friction on the tubing wall, the liquid inside each segment is mixed homogeneously by forced inner convection during its transport as shown in Figure 3A. As consequence of air segmentation, carry-over between samples is prevented so that sample and reagent consumptions can be considerably reduced.

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[4] The reader will wonder at some point for what purpose FT and their principles are, even if shortly, explained that might appear to be of little connection to the thesis topic. In flow automation it is mostly about mixing and finding new efficient modes of doing it efficiently. A critical evaluation of a new concept therefore naturally requires knowing the existing concepts before being able to evaluate its apported benefits and shortcomings.

SFA is the first widely utilized FT and is still used, e.g., in clinical, agricultural, and oceanographic analysis. The "AutoAnalyzer" from Technicon Corporation became the first commercially available instrument based on SFA that was continued after 1987 by the company Bran & Luebbe GmbH. On the downside, the introduced bubbles lead to chaotic behavior[5] and irreproducible transport velocity of the segments so that chromogenic assays need quantification in a reaction *steady state.* So, while several readouts[6] can be done for each sample within a minute and sample throughputs of > 100 per hour, the time between sample introduction to the system and readout must be long enough to ensure equilibrium conditions. To improve flow reproducibility and prevent stacking of air bubbles, tubing coils in SFA are made of water-wettable glass, nonionic detergents are added to the reagents, and solenoid valves are used today to increase the reproducibility of bubble size. A further shortcoming is that for some detection techniques, a de-bubbler must be used to remove the air bubbles upstream while for spectrophotometric detection, compensation of baseline disturbance is done by software [49,61].
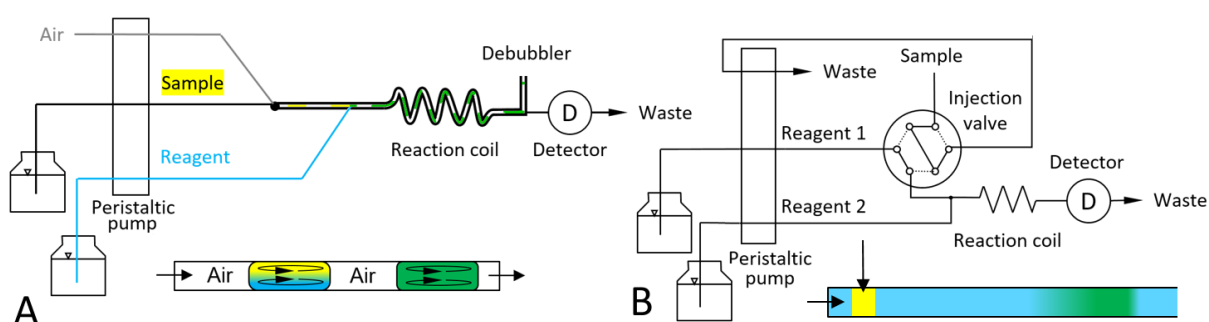
Figure 3: Schemes for typical manifolds in analyzers of the automation FTs (Air-) Segmented Flow Analysis (A) and Flow Injection Analysis (B).

3.2.3.2. Flow Injection Analysis

Flow Injection Analysis (FIA) was firstly proposed by Růžička and Hansen in 1975 [61]. As a new concept, the sample is inserted into a not segmented laminar carrier flow via an injection valve [54]. During the transport in tubing of 0.3 to 0.8 mm i.d. classically by continuous flow, the sample zone disperses in the carrier leading to peak-shaped signals. The friction on the tubing wall leads to a hyperbolic flow profile that causes pronounced peak tailing or, differently interpreted, the formation of a defined concentration gradient of the dispersing solution. This zone dispersion means incomplete mixing (or dilution) with the carrier and related zone broadening. It depends on the injected volume, the solution viscosity, the inner diameter and length of the tubing, and the flow rate. It can be characterized by the so-denoted dispersion factor, which is the dilution factor at peak maximum.

The carrier not only transports the sample and subsequently cleans the manifold from its remains but is generally also a reagent so that under continuing dispersion, the analytes in the sample can form a detectable product. In reverse FIA, it is the reagent, which is injected into the flowing sample which is advantageous if the reagent is costly (enzyme). This can also enable the use of different reagents for the analytes contained in a process stream [62]. As in CFA, more reagents can be added via confluences.

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[5] This behavior can be compared to the movement of individual cars in a stop-and-go traffic situation.

[6] Signals in SFA show in approximation a rectangular shape due to the homogeneity of the solution.

Since no flow segmentation is done and one reagent can be spared by using the carrier solution instead, a simpler manifold setup than in SFA is possible (see Figure 3 B). Injecting a control standard into a sample originating from a monitored process with confluence of needed reagents, continuous quantification of the deviation in concentration of the monitored process from a target value can be done.

The volume of the sample is strictly defined by the injection loop and, in the absence of segmentation bubbles, the liquid content of the manifold is incompressible. Considering this, the liquid flow is constant and therefore the time in which the sample is transported within from the injection valve to the detection flow cell, is highly reproducible, unmatched by other FT. Also mixing pattern depend, at controlled temperature, solely on the fixed tubing dimensions and the sample size. As consequence, FIA enables the quantification of a reaction product at any moment before reaching equilibrium conditions but at the cost of lower sensitivity as less reaction product has formed [63]. Moreover, the time of signal registration can be after the peak maximum has passed the detector. Given that the dispersion and peak profile remain constant for a set system and sample volume, this enables so-denoted "electronical dilution". Furthermore, intermediate reaction products or the presence of a catalyst, possibly the analyte itself, can be quantified. These and other operation modalities, explained in sections 3.2.4, 3.3.1, and 3.3.2, apply to the majority of the hereafter explained FTs and belong to the most outstanding advantages of FTs compared to batch-automation.

Important advantages of FIA are its simplicity, reduction in solution consumption, and the self-cleaning action of the carrier. For instance, many applications of FIA and related FTs use elements in the analyzer system that are prone to aging, passivation, or fouling, e.g., an enzyme cartridge, electrode, or optrode, respectively. In FIA, these are exposed only briefly to a small sample volume that is already partially diluted by undergoing dispersion. Moreover, they are cleaned immediately by the carrier, which prolongs their lifetime and increases method robustness towards the sample matrix. At the same time, a reliable and reproducible determination is carried out. In FIA, only one sample is injected at a time to avoid compromising the method reproducibility. The sample throughout is therefore determined by the residence time of the sample in the manifold but up to 200 injections per hour are possible. Disadvantages are usability for only one analytical parameter at a time, the significant consumption of all continuously added regents, and the need for optimization by manual change of tubing. Therefore, improvements of this successful technique have been aimed for by later explained multicommutation and programmable flow (see sections 3.2.3.4 and 3.2.3.5.3). Downscaling of FIA in form chips has been reported [64] but was rather the beginning for more successful integration of flow manifolds in monolithic conduits, as explained in section 3.2.3.3.2. of has inspired modern Lab-On-A-Chip devices (see section 3.1).

3.2.3.3. Sequential injection techniques

3.2.3.3.1. *Sequential Injection Analysis*

Sequential Injection Analysis (SIA) was proposed for the first time by RŮŽIČKA AND MARSHALL (1990) [65] and is based on a new concept and instrumentation that overcomes the limitations of FIA listed before. An SI-Analyzer (Figure 4A) consists of a bi-directional pump[7] connected via a so-called holding coil (HC, typically 0.8 to 1.5 mm i.d.) to the central port a multiposition SV. Lateral positions were for the required reagents, and sample, for waste discharge, and for sample transport to a detector through a reaction coil or online coupled advanced instrumental technique either directly (atomic spectrometric techniques), via an additional injection valve (HPLC), or an appropriate interface (e.g., CE) [40,67]. The head valve of the syringe pump allowed the refilling of the syringe with water or another appropriate carrier solution.

Former FTs were based on a continuous unidirectional flow while SIA is based on an intermittent flow scheme: aliquots of sample and reagents are aspirated from the SV into the HC undergoing zone dispersions. Zone mixing of the stacked solutions is enhanced by flow direction reversal and the reaction mixture is propelled by the carrier towards the detection flow cell to register the transient peak signal.
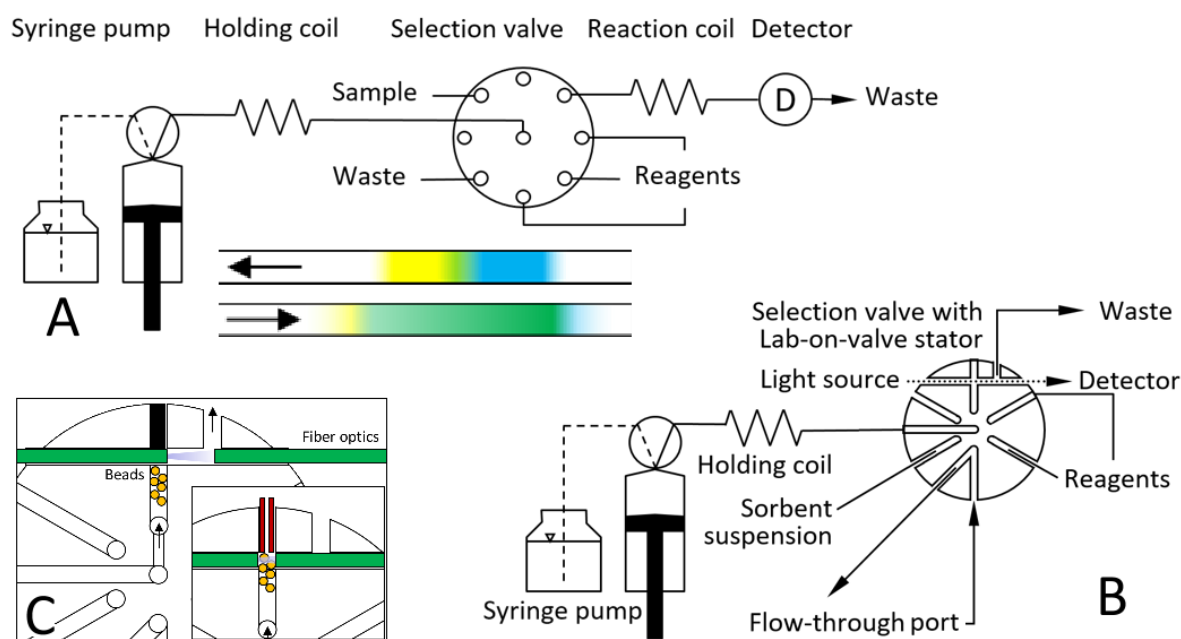
Figure 4: Schemes of manifolds using in Sequential Injection Analysis (A) and Lab-On-Valve (B) as well as of the principle of bead trapping in a flow channel or the multipurpose detection cell of the LOV manifold (C).

The new requirement in FT for computer control of syringe pump and SV came with the benefit that most influencing parameters were defined by software instructions, together being the operation method. These include solution volumes, flow rates, pump direction, and optionally pausing to prolong

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[7] For the first publications, linear syringe pumps, used predominately in SI-Analyzers, were unavailable so that a syringe pump was constructed where the piston was driven by a crankshaft and computer-controlled flywheel thus featuring sinusoidal flow. The success of SIA started when linear syringe pumps (see chapter 3.2.2) became widely available [66] Furthermore, the detection flow cell was integrated into the HC.

the reaction time. Therefore, the influence of the manifold and HC geometry became secondary so that analyzer adaptation by simply changing the instructing protocol was possible. So, sample dilution can be simply performed, e.g., by the aspiration of water between sample and reagent solutions segments, zone penetration can be increased by repeated flow reversal, and volumes and ratios of sample to reagents are easily adapted[8]. What is more, it was easy to switch between methods and perform multi-parametric analysis on the same analyzer without the need for manual re-configuration. Moreover, new operation modes (see section 3.2.4), were facilitated, e.g., taking advantage of the controlled formation of concentration gradients to enable kinetic distinction between analytes, mono-segmentation (see section 3.2.3.5.1), of determinations in stop-flow mode. Finally, either multiple detectors or different paths to the detector are eligible via the SV. This enables circumventing, e.g., an SPE cartridge integrated in one path, and in consequence analyte determination either with or without preconcentration.

The invention of SIA presents a significant advance in automation *in-flow* in terms of operational flexibility whereas a lower consumption of sample and reagent and pulsation-free pumping with higher pressure robustness are other advantages [69,70]. Specialized treatises review the potential of SIA for process monitoring [10,70,71] as well as gradient techniques, stop-flow, and mono-segmentation [72,73] that are explained in detail in section 3.2.4. The disadvantages of SIA compared to FIA include the impossibility of confluence mixing (later elegantly solved by the concept of Programmable Flow - section 3.2.3.5.3), the need to constant syringe refilling, and, by mixing based on stacking multiple solution zones, lower reproducibility and increased Schlieren effect.

3.2.3.3.2. Lab-On-Valve and Bead Injection

The Lab-On-Valve (LOV) technique was proposed by Růžička (2000) [74,75]. It is based on the replacement of the multiposition valve stator by an integrated micro-conduit or monolithic manifold that is equally denoted Lab-On-Valve. The LOV device is fabricated from transparent polymers PMMA or Ultem® for higher chemical stability and features straight, and smooth flow channels equal to the outer diameter of the typical flexible tubing used in FT analyzers (1.5 mm). It features a multipurpose detector flow cell, used generally for spectrophotometric or fluorimetric detection based on inserted fiber-optics as well as a flow-through port that can be used to recirculate a solution or suspension or to connect the analyzer to a process stream with minimal dead volume (Figure 4B). Further functionalities have been integrated into the LOV design by drilling additional holes for electrodes or via stereolithographic 3D [76,77]. A LOV-like platform for multisyringe pumps was proposed by the author as Chip-On-Valve [78], integrating HC and reaction coil as well as confluences and produced by lamination of pre-carved PMMA slides. LOV can be seen as an SIA variant that enables procedural downscaling, and an increase in injection throughput by minimization of solution consumption that also aids automation of enzymatic assays [77]. The most noteworthy advantage is the simplification of handling sorbents as suspensions for *in-system* packing of microcolumns compared to earlier approaches [80,81]. The short channels and transparency of the LOV device are advantageous in method optimization.

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[8] The concept of zone penetration or "merging" was reported for FIA applying double injection in 1978 [68]

Being so closely related to LOV, the main principle of this technique, termed Bead Injection (BI) [82,83], is described here with further explanation given in section 3.3.2. In BI, a small volume of a 0.5 to 5%(w/v) suspension of particles of typically 30 to 200 μm, hereafter *beads,* is aspirated and, by flow reversal, is passed through a path of the LOV manifold with integrated frit. When DI is done in the channel leading to the integrated detection cell, the inserted optical fibers can be used to restrict the passage of the beads (Figure 4C). In consequence, they are trapped and form a microcolumn of a few milligrams of material. Depending on the nature of the beads – a sorbent, particles with surface-immobilized enzymes, proteins, antibodies etc., or glassy carbon beads – an active surface is created to be used for SPE [82], a bioassay [83-85], or as an electrode [86], respectively. After a single or repeated use, the beads are re-aspirated and discharged. BI has been used most-often to automate renewable SPE in microformat for analyte pre-concentration and sample clean-up. Trapping transparent beads inside the detection cell, on-sorbent detection is possible so that the analytes do not even have to be eluted. Review articles that have discussed the applications, characteristics, and potentials of LOV and BI techniques are highlighted [20,21,75,82,87]. Shortcomings of LOV that differ from the ones listed for SIA include the difficulty to include further manifold elements or to perform chemical reactions of an analyte that was preconcentrated by BI-SPE. Different concepts have been proposed can overcome this issue, among these Programmable Flow (see section 3.2.3.5.3), the combination with MSFIA [88], or a methodical development by the author, in which a second pump is used to add loading reagents in the HC and the outlet of the SPE microcolumn is connected to the flow-through port of the LOV that enables eluate re-aspiration for a color reaction [89].

3.2.3.4. Multicommutated approaches

Multicommutation means the ability of repeated and rapid activation or deactivation of a pumping line or manifold path as a function of time for which solenoid valves [90] or individual pumps [91] are used. The operation method can be described by binary numbers [90-93] that indicate all de-/activation states of the individual actuators. This concept is applicable in many FT [52] but it is assigned mostly to the approaches explained in the following. Typical manifold structures are given in Figure 5 A-D.

3.2.3.4.1. Multicommutated Flow Injection Analysis

Multicommutated Flow Injection Analysis (MCFIA) was proposed in 1994 by REIS ET AL. [90] with characteristics, potentials, and applications comprehensively reviewed [92,93]. In this technique, 3-way solenoid valves are used to increase the operational flexibility of FIA systems, e.g., to enable re-cycling of not required solutions to their reservoir, thus deactivating the respective pump line, or to select a specific manifold path, e.g., that circumvents an enzymatic reactor or a preconcentration column. A characteristic manifold structure is given in Figure 5A. Possible operations in MCFIA, common also for the subsequent multicommutation FT, are confluent mixing and so-denoted binary sampling schemes, i.e., alternating loading of sample and reagent aliquots into a flow path to improve mixing efficiency. Another possibility is zone splitting by precise time control of valve activation that yields very small sample volumes and enables high dilution factors. Carrier recycling allows stopping the sample in the manifold or detection cell without the need for pump control to prolong reaction times or to monitor the chemical reaction, respectively. MCFIA allows the construction of software-adaptable "flow networks" by simply changing the commutation sequence. A limitation is the use of peristaltic pumps

and stroke pulse of the valves in specific applications as well as heat generation of the solenoid, which can lead to valve failure.
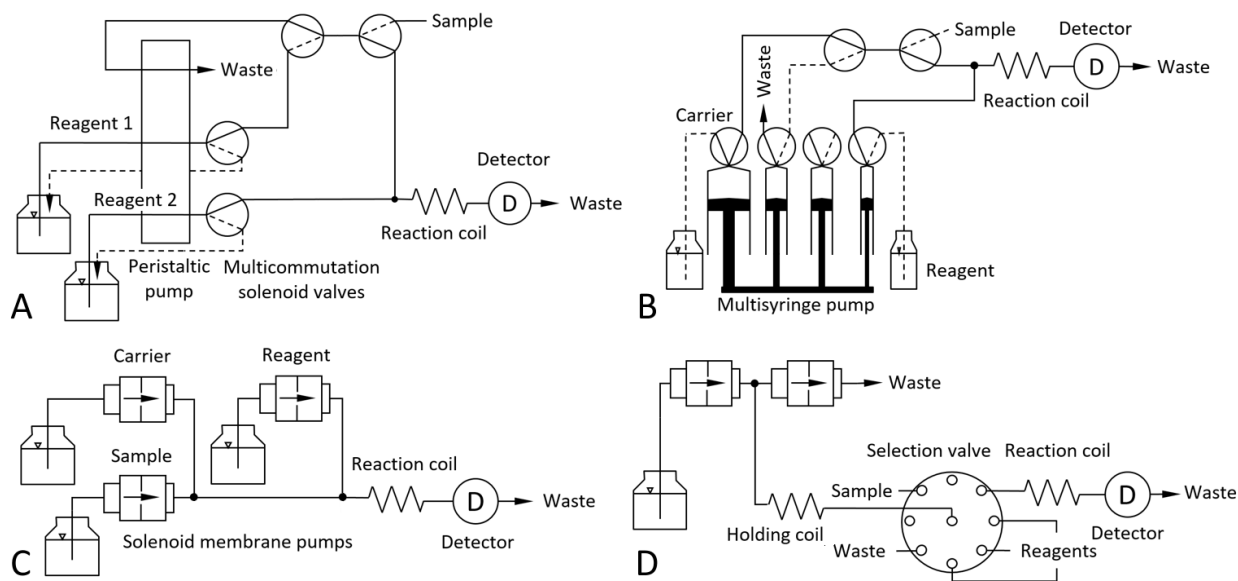
Figure 5: Schemes of typical manifolds of the automation concepts and techniques Multicommutated Flow Injection Analysis (A), Multisyringe Injection Analysis (B), and Multipumping Flow Analysis following a FIA-like operation scheme (C) or SIA-like operation scheme (D).

3.2.3.4.2. Multisyringe injection analysis

The first application of Multisyringe flow injection analysis (MSFIA) was by ALBERTÚS ET AL. (1999) [94,95] It is based on the use of multiple syringes operated by the same actuator, thus operating in parallel. Solenoid head valves on each syringe enable to connect each one to the flow network or recycle the respective solution as required. One syringe is typically used for loading an injection loop with the sample. For such aim purposes, additional solenoid valves can be controlled by the pump module. The other syringes are used for the carrier and confluent addition of reagents following FIA and MCFIA operation schemes.

MSFIA combines features from MCFIA such as feasible confluent solution, binary sampling schemes, and flow-network adaptation and from SIA and syringe pumps such as software-controlled change of flow rates and direction, flow rate stability, pressure robustness, and chemical resistance. Volume ratios between flow lines can be adapted by a change of the syringe sizes. The pressure robustness has motivated the combination of MSFIA with column-based separation as described for SIA in section 0. Regarding this thesis, it should be highlighted that the first experimental works with LIS were carried out with a multisyringe pump with one syringe used to in-syringe liquid phase extraction. A typical structure of a MSFIA manifold is shown in Figure 5 B and review publications to MSFIA are highlighted [95-97].

3.2.3.4.3. Multipumping flow systems

Multipumping flow systems (MPFS) were proposed by LAPA ET AL. in 2002 [91] with reviewa on the characteristics, potentials, and applications being highlighted [98]. Instead of multicommutated flow lines, individual solenoid micropumps with stroke volumes between a few to hundred microliters are

used for solution provision or aspiration. The pulsation strokes were found to cause an intermediate turbulent flow regime that improved solution mixing and consequent signal increase compared to the laminar flow regime as typical in prior FTs. An intrinsic advantage of the technique is the easy adaptation of the flow rate of the individual pump. The drawbacks are that the costs for the analyzer increase significantly with the number of used pumps (about 3 times higher prices than for solenoid valves). The need to use sample volumes that are a multiple of one stroke volume to achieve reproducible results, and the micropumps' susceptibility towards particle load and air bubbles, as well as a slow pressure stability are further drawbacks. Improving their pressure robustness and reliability has been studied by the author [99].

3.2.3.5. Merging concepts

FT are tools of inventive science [41]. Flow practitioners have steadily invented new approaches, either merging the existing automation concepts or inventing new concepts. In this chapter, four examples of such merging are described, three aiming at the benefits of homogeneous mixing in FT. This is of relevance for this thesis describing developments, advantages, and applications of a FT also focused on this aim. Schematics of used elements and representative manifolds of the techniques are shown in Figure 6.

3.2.3.5.1. Monosegmentation

Monosegmentation is an automation concept merging SFA with other, typically unsegmented FTs, which has been by PASQUINI AND OLIVEIRA (1985) [100]. The aim was to combine the advantages of homogeneous solution mixing and zero dispersion obtained by air segmentation with the high reproducibility and low sample consumption of FIA. For this, two air bubbles are introduced simultaneously with the sample or sample and reaction mixture. To tis aim, a purpose-designed linear injection valve was designed shown in Figure 6A. Sliding the middle part of the valve separates one liquid segment by two air bubbles so that during transport toward the detector the solutions encapsulated by the segmentation bubbles mix homogeneously. Improved variants of this valve were developed to inject simultaneously also needed reagents or an immiscible solvent into the sample segment [101]. The approach becomes more flexible when automated on SIA, where simply air must be aspirated in the zone stacking sequence [73]. No overviewing article is known to the author, but the concept is still applied today for being simple and efficient. High reproducibility is achieved despite air compressibility by using only two bubbles and controlling their size.

3.2.3.5.2. Flow-Batch Analysis

Flow-Batch automation [102] is based on the use of a mixing chamber as a central element in the flow analyzer to mix sample and reagents stepwise and in volumes as needed. The use of flow-through chambers dates back to times before the invention of FIA and was used to promote mixing in CFA [103]. However, a batch-wise filled and emptied reaction chamber was, to the best of my knowledge, firstly proposed by SWEILEH & DASGUPTA in 1988 [104,105], who used an air-pressure driven MCFIA system for solution handling and a continuously stirred mixing chamber with integrated spectrophotometric detection for solution mixing. In 1999, HONORATO ET AL. proposed the entitlement of Flow-Batch as a hyphenation of MCFIA with batch automation [106]. Later, the concept was transferred to other FTs including SIA (Figure 4 B1) and MPFA (Figure 4 B2) with always the same premise of flow operations for filling and emptying the chamber and homogeneous mixing within, possibly aided by stirring.
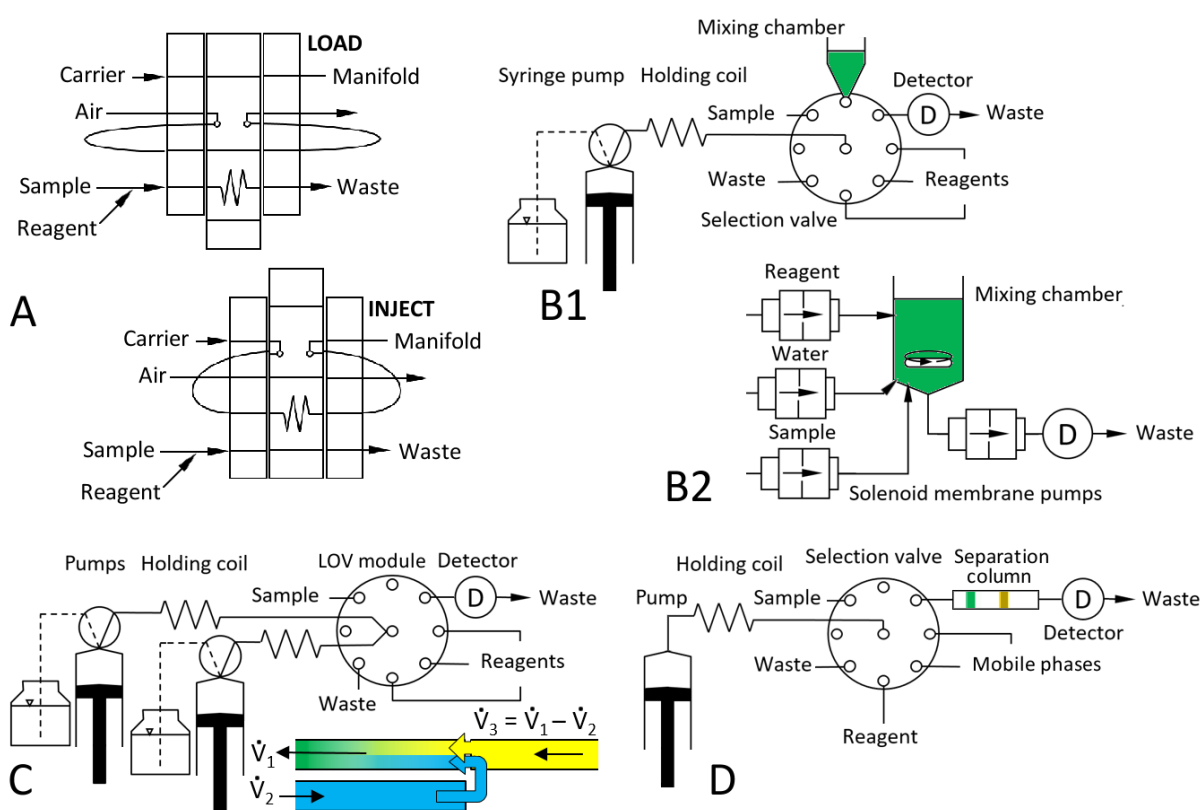
Figure 6: Schemes of a purpose-designed injection valve for monosegmention (A) and of typical manifolds of the automation concepts and techniques of Flow-Batch based on using mixing chambers in a SIA system (B1) or in a Multipumping Flow Analyzer (B2), of Programmable Flow (C), and Sequential Injection Chromatography (D).

A partial overlap with the LIS technique was the development of a piston-propelled flow-batch chamber by ALMEIDA ET AL. (2007), into which solutions were aspirated by the displacement of a plunger integrated into the chambers top [107]. Solution flow was controlled by solenoid valves but no extra pump was required. The similarity between this approach and an automatic syringe used upside-down was in addressed in the first article such approach in LIS [108]. Furthermore, miniaturization of Flow-Batch Analysis was done with a chamber of ca. 200 µL and a rotating nylon fiber for solution mixing [109].

Flow-Batch Analysis has been proven a versatile approach especially for automated titrations, sample dilutions or standard preparation, and chromogenic assays. The automation of LLE procedures using

mixing chambers in various combinations with SI-Analyzers has also been reported [110-112]. Outstanding advantages of Flow-Batch automation are the conceptional simplicity and higher predictability of operation since mixing is not achieved by zone dispersion. Homogenous mixing minimizes any effect of sample viscosity, which can affect signal shape and reproducibility in dispersion-based FTs considerably. Moreover, solutions can be added at-will as long as the chamber integrates a mixing element for homogeneous solution mixing. Disadvantages are the need for a purpose-designed and machined mixing chamber (although commercial solutions are available), and the need for individual valves or pumps for each solution if solution handling is not by a SIA system.

   Carrying out detection inside a SIA-based mixing chamber or in its outflow, the respective approach is sometimes referred to as Lab-At-Valve (LAV), proposed by BURAKHAM ET AL. (2005) for the automation of LLE [113]. Another modality is the use of a peristaltic pump for liquid propulsion placed between the SV and the mixing chamber, which was proposed as Stepwise Injection Analysis (SWIA) concept by MOZZHUKHIN ET AL. (2007) [114,115]. Other advantages are that a sensing element, e.g., an electrode, can easily be integrated into the system by just placing it into the chamber [116]. On the other hand, as no carrier provides a self-cleaning effect, there is a higher risk for carry-over and, considering an atmospherically open mixing chamber, of contamination. Finally, the chamber must be cleaned after each analysis and possibly manually if the sample matrix adsorbs to the chamber walls [116]. Two comprehensive reviews by ZAGATTO ET AL. (2009) on the use of mixing chambers in FTs [117] and by DIAZ DINIZ ET AL. (2012) on Flow Bach Analysis [102] are highly recommended.

3.2.3.5.3.   Programmable Flow Injection

   Programmable Flow injection (by its inventor abbreviated pFI) is a recently proposed operation concept by RŮŽIČKA in 2018 [118,119]. It is based on using a LOV analyzer with two MilliGAT pumps (yet also individual syringe pumps would be possible) connected to the central port of the SV, each one with its own HC [89,120-122]. Possible fluid operations are solution mixing by zone stacking as in SIA, confluence as in FIA, or binary sampling schemes as in multicommutation FT as well as zone splitting. The beauty of the concept is in the differential flow that enables multiple confluence mixing. This works by aspirating with pump 1 a solution, e.g., a reagent, at a flow rate $\dot{V}_1$ from one of the SV ports while providing another, previously aspirated solution, e.g., the sample, at a lower flow rate $\dot{V}_2$ so that the flow rate and aspirated volume of the reagent are in effect reduced, the flow rate being $\dot{V}_3=\dot{V}_1-\dot{V}_2$. Both solutions mix homogeneously by confluence in the HC of pump 1. Differently explained, the opposite of zone splitting [93,43] is carried out. This procedure can be performed repeatedly to add, by confluence, further reagents. In combination with BI, this technique is a powerful approach to achieving high preconcentration factors by renewable SPE. Limitations are the laminar flow regime so that fully homogeneous mixing might require the transfer of the entire solution into the other HC once more by applying equal flow rates for both pumps but in opposite directions. The time required for mixing increased with each solution significantly and the operation is surely less intuitive than homogeneous mixing in flow-batch. Moreover, usability for the automation of liquid phase (micro)extractions appears to be limited.

3.2.3.5.4. Sequential Injection Chromatography

Sequential Injection Chromatography has been proposed in 2003 by ŠATÍNSKÝ ET AL. [44] by using a monolithic column in a SIA system, thus hyphenating FT with liquid chromatography (Figure 6D). This way, the intrinsic issue of FT, not being able to separate compounds of similar properties, i.e., not exhibiting specific reactivity, was overcome while the flexibility of SIA in terms of solution handling was preserved. Easier than in HPLC, the sample volume can be altered, or chemical reactions can be performed before injection [123,124] or for post-column derivatization [125]. Consequent variants followed by a combination with MSFIA and FIA [126,127]. Improvements in instrumentation have allowed to increase the applicable pressure and thus the robustness of the techniques significantly, while limitations of usable mobile phase to a few milliliters in one syringe stroke and lower flexibility for gradient separation than in HPLC still remain. On the other hand, the higher operational flexibility towards HPLC for integrating sample preparation steps in the run method, e.g., analyte preconcentration by SPE, has to be highlighted. The benefits of the technique have been proven in multiple experimental works, one review is cited [123].

3.2.4. Main operations and modes in flow analyzers

The present thesis deals with a young flow automation concept applied to the automation of sample preparation approaches. Therefore, a short overview of common operations in flow analyzers should be given. As the listed operations have been used many times, it is impossible to make comprehensive or even representative citations. So, the reader is referred mostly to specialized literature [48-51, 128].

Solution mixing

This is the fundamental operation in all FTs and is required to dilute the sample, enable chemical reactions, or adjust its pH with buffer. These appear as little tasks but actually represent much of the working time in the laboratory. Metallurgic wastewaters can show gram per liter concentrations of inorganic contaminants so that dilution by several orders of magnitude is required and much effort has been spent in developing flow concepts to do so. The applied principles depend on the used FT, the required dilution factor, and the homogeneity of the mixture. At times, there are special demands for uniform mixing, e.g., between the sample and a standard for matrix match calibrations [47] or, using spectrophotometric detection, to decrease the influence of the salinity, i.e., the refraction index, of either the sample or the reagent mixture on the signal (Schlieren effect) [38,39]. A variety of approaches have been developed, each typically but not necessarily associated with one FT: zone dispersion in the carrier and confluence of reagents in FIA, solution stacking and zone dispersion in SIA, binary sampling and splitting in multicommutation FT, and homogeneous mixing in flow-batch approaches and SFA. Instrumental devices to increase mixing include flow-through mixing chambers or static mixers that increase the dilution factor or knotting of tubing that improves zone penetration without significant zone broadening. It should be highlighted that all dispersion-based approaches depend significantly on sample viscosity and all approaches apart from homogeneous mixing are affected by unintentionally introduced air to the flow network or bubble formation, e.g., by heating. In 2007, I proposed a simple approach to dilute a highly concentrated sample in SIA: after aspiration of a small sample volume, a similar or larger volume is discharged. By dispersion, a reproducible part remains in the HC, available to react with a subsequently aspirated reagent [129].

Stop-Flow

Stop-Flow is useful to eliminate the sample background in optical detection of a reaction product [72]. The mixture of sample and chromogenic reagents is rapidly pushed into the detection flow cell. After blank measurement, the increasing signal is registered with the flow stopped. The slope of the signal is due to the progressing reaction and is proportional to the analyte concentration. This way, the genuine coloration of the sample does not contribute to the measurement. In contrast, a transient zone would result in a peak and the requirement to measure the sample with and without reagent to compensate for its blank signal [47]. Stop-flow can also be useful to study reaction catalysis or inhibition. In the same way, determinations can be done by spectrophotometric detection in the mixing chamber of a flow-batch analyzer. An earlier approach is *flow reversal* where the sample mixture was forced to pass the detector multiple times [130].

Titrations

Many FT applications were directed to automation of titrations, among those many being based on flow batch, i.e., homogeneous mixing or mixing sample and titrant in varying ratios in a mono-segmented flow. Another approach that enables titrations to be completed in 30 s is based on the injection and dispersion of, e.g., an acidic sample into an alkaline indicator solution that is used as a carrier in FIA. By zone dispersion, the sample neutralizes a part of the carrier leading to a color change of the indicator. The half-height width of the obtained peak is proportional to the logarithm of the proton concentration [131]. Using a buffered indicator solution, also the peak height can be used [43].

Kinetic differentiation and speciation

Kinetic differentiation between two analytes is possible if their reaction in the same assay proceeds at different speeds or requires different reaction media, e.g., different acidity. An example is the simultaneous determination of PO_4^{3-} and SiO_4^{2-} by the molybdenum blue reaction. Here, PO_4^{3-} reacts faster and at higher acidity than SiO_4^{2-}. Therefore, the stop-flow approach could be used for analyte differentiation by their reaction speed - PO_4^{3-} already reacted [132] - as well as by forming a proton gradient by dispersion of an acidic masking solution that separated two zones of the sample. At the higher acidity in the front sample, only PO_4^{3-} was able to form the reaction product while the silicate reaction was favored in the second sample zone by the lower acidity [133]. In addition, it is possible to determine very low concentrations of an analyte if it acts as a catalyst and so can, even at trace level in traces, have a significant effect on the kinetics of the de facto observed reaction. An overview to this topic is given [134].

Digestions

Digestions by microwave, ultrasound, UV-irradiation, or/and assisted by a strong oxidant or acid can be accomplished by placing a tubing coil, consequently, into a microwave, an ultrasonic bath, or wrapping it around a UV lamp, thus creating a *photoreactor* [135]. This approach is used e.g., to determine the sum of an organically bonded element, e.g., organic phosphorus or mercury, or the speciation of the oxidation status of some half- or transition metals, e.g., As(III)/A(V), Se(IV)/Se(VI), or V(IV)/V(V)[9]. The principle is using a quantification method that reacts only with one species, the inorganic compound, or one oxidation state of the element. It is used once without and once after sample digestion, thus yielding the total concentration of the element. In the case of mineralization of organic compounds, the difference in the found concentrations corresponds to the organically bonded element [136].

Photoconversion

Using optical detection, *photoconversion* aims to differentiate the signals from an analyte and the matrix background by either destructing the photoactive analyte or generating a photoactive species by radiation. Ideally, the light source of the spectrophotometer or fluorimeter itself suffices for this derivatization so that measurement is consequently done in stop-flow. The process can be promoted by adjusting the reaction pH and adding reagents similar to those used for photodegradation [137,138].

Membrane separations

Gas diffusion [138] and *pervaporation* [140,141] are two approaches to gas phase extraction. In both cases, the sample, containing an analyte that is volatile at a given pH, is passed through a shallow flow channel covered with a hydrophobic nano-porous membrane. On the other side of the membrane, a similar channel is used for an appropriate acceptor. The membrane separator and system setup differ for both techniques: in gas diffusion, donor and acceptor solutions are in contact with the membrane. In consequence, analyte transfer is generally fast and efficient. In pervaporation only the acceptor solution is in contact while a headspace separates the donor from the membrane. This way, membrane blockage or fouling by matrix components is avoided. However, analyte transfer through headspace and usually a thicker membrane to support the weight of the acceptor is very slow. Analytes of question include CO_2/carbonate, NH_3/NH_4^+, SO_2/SO_3^-, H_2S/S^{2-}, HCN/CN^-, and smaller organic amines and carboxylic acids. The achieved matrix separation enables interference-free chromogenic reactions or even directly a sufficiently high selectivity to determine the analyte by the unspecific detection of the acceptor conductivity [142]. Similarly, *sample dialysis* and *tangential flow filtration* [143] can be carried out by the utilization of a more porous hydrophilic membrane and an increase of backpressure that forces the liquid to pass through the membrane. Finally, membranes are also used for bubble elimination or the separation of organic and aqueous phases in flow-automated LLE based on fluid segmentation (see section 3.2.3.1). In all membrane separations, pressure control in both compartments is important to avoid bending or even permanently damaging the membrane. This is most critical when aiming for analyte enrichment in which case the acceptor is stopped while the donor

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9 It will be known that some oxidation states of metallic analytes can be readily distinguished by species-selective chromogenic reagents, e.g., Cr(III)/Cr(IV) or Fe(II)/Fe(III) [MERCK].

is continuously passed along the membrane. The use of membranes as separators in flow systems was a topic of several reviews; two are highlighted [144,145]

Vapor and hydride generation

Given their high selectivity, the use of atomic adsorption and fluorescent detection in hydride generation and cold vapor modes has been repeatedly reported for the determination of half-metals and mercury where the FT analyzer was used for analyte preconcentration and possible speciation as well as for the generation of the half-metal hydrides or atomic mercury by mixing the sample with borohydride, acid in a gas-liquid separator.

Thermosetting

Heating of tubing coils by water bathes or by winding the coil around a heating block is used to increase the reaction speed, thus yielding thermostated reactor. As a downside, solution *debubbling* before the detector can be required to avoid baseline disturbance by partial solution degassing and bubble formation.

Solid reactors

Solid reactors are filled with immobilized "reagents" that are integrated into a flow line as a cartridge [87]. These can be enzymes that have been immobilized on glass beads to convert an analyte in the sample into a detectable product or to quantify the consumed oxygen or co-enzyme, e.g., NADH [146]. Another example is immunoreactors that are based on sorbents with immobilized antibodies or antigens to specifically bind to the analyte (the antigen or antibody, respectively), e.g., to perform an ELISA assay. Release of the analyte and simultaneous reactor regeneration is done by a change of medium or replacing the sorbent entirely following the BI approach [81,83-85]. Another frequently used component are reduction columns, e.g., containing copperized cadmium or zinc for the reduction of NO_3^- to NO_2^-, which can be photometrically determined by Griess reaction [78]. A final example is catalytic reactors, e.g., a column filled with precipitated Co_2O_3 that has been used by the author for green determination of ClO^- based on its catalytic decomposition and consequent signal decrease [147].

(Co-)Precipitation

Precipitation yields a surprisingly high count of over 100 publications in the online database on published FT applications [42]. In most cases, co-precipitation of metal cations present in extremely low concentrations is performed which enables its quantitative recovery on an *in-tube* filter. For analytes at higher concentration, direct precipitated and enrichment can be done by the same mean. Filter regenerator and analyte release is done by dissolving the precipitate [148]. Precipitation can also be used for matrix elimination with two examples of LIS automated milk and serum deproteination contemplated in this thesis (see section 3.4.2) [149,150]. It is also an essential step for turbidimetric determinations or for the determination of dissolved oxygen according to Winkler [151,152].

Sample leaching and process monitoring

In general, FT operate with liquid samples. However, a significant number of applications reported on dynamic leaching studies [153]. Analytes and matrices of interest were mostly metal cations in soil, wood, and tissue samples but also plasticizers leached from microplastics were studied [154]. The homogenized material is packed into a cartridge (different formats and respective advantages were described) and integrated into the FT system. Repeated passage of an extraction medium, e.g., seawater for microplastics, diluted acid and gastrointestinal surrogates for soil samples, etc., and analyte determination in the obtained fraction allows estimating their bioaccessibility and bioavailability under various conditions. Advantages of FT automation are procedural downscaling, in-system preconcentration of the analyte by SPE, gain in information compared to large-scale studies performed in batch, and, often, online coupling to automated analysis by atomic spectrometry or HPLC for higher detection sensitivity, time efficiency, and convenience [154]. In the same sense, flow analyzers have been used for process monitoring, vigilance, and control. They are valuable tools to observe a studied process (environmental medium, technical process, or research experiments, to gain information on the kinetics and concentration levels. This includes for instance the study of single layer cell membranes and related transport processes [155,156], bioprocess monitoring [47,129,158], dissolution processes of drug formulations [159], or ship-board application for ocean surface mapping [160].

The previous list of basic operations could be further extended but it covers many of the most important operations performed *in-flow* yet with two critical exceptions namely solid phase and liquid phase extractions. In the following section, a more detailed view should be given to flow automation of these methodologies, which surely deserve a chapter for their own by the spectrum of approaches and relation to the commented articles and the discussion chapter 0 of this habilitation thesis.

3.3. Methodologies for automated analyte extraction in-flow

3.3.1. Liquid phase (micro)extraction approaches

3.3.1.1. Principles, solvent systems, and basic methodologies of liquid phase (micro)extraction

Liquid-liquid extraction (LLE) and liquid phase microextraction (LPME): The methodologies as well as the modes to perform them automatically are highly diverse, moreover, the number of reporting publications is considerable so the current section will be limited to the description of the basic approaches that were automated also by LIS, existing concepts for automation in-flow, and the advantages and limitations of these approaches.

A general differentiation between LLE and the many LPME approaches is in the phase ratio between the organic extractant and aqueous sample that can be up to 1:1000 as well as the utilization of only several hundreds to few milliliters of sample. The common principle of LLE and LPME is the distribution of an analyte between two immiscible phases described by the distribution coefficient or partition ratio in [mol/L]:[mol/L]. Standardized values that describe the hydro- or lipophilicity of a substance refer to octanol (that shows similar solvation capacity as human body fat) and water and most often, the decadic logarithm of this value is stated.

Classical LLE is based on shaking the extraction solvent with the sample until phase equilibrium is obtained, typically in a separation funnel, with spontaneous phase separation thereafter. After isolation of the organic phase, the process can be repeated to yield a higher extraction yield with pooling of the organic extractions thereafter and concentration by partial or complete solvent evaporation and reconstitution. The process can be mechanized as well as used for analyte fractionation. The most powerful instrumental tool for this purpose is counter-current chromatography enabling chromatographic separation [161]. A significant advantage of LLE and related miniatured techniques are procedural simplicity and low costs for material, straightforward upscaling in industry, and robustness towards particulate matter in the sample.

Further extraction approaches differ in how the constituents are transferred from one phase to another (through head-space or phase contact), how this process is promoted, or phases brought into contact (dynamic: agitation, droplet formation, film renovation, or static: passive diffusion), the state of the phases involved (solid, liquid, gaseous), kind of used solvent (miscible, immiscible, tailored, switchable, surfactants, polymers,), and solvent support (free droplets, hanging drop, plug, film, or impregnated porous media). Three review publications included in this thesis have classified, overviewed, and discussed these varieties in the light of their automation by autosampler and robotic systems and FT with a contrasting juxtaposition and comprehensive list of publication applications [17,111,144].

The extractability of an analyte, or solute, is defined by its affinity to the acceptor phase, generally, the organic solvent. So, the main consideration is on the choice of solvent, in particular its polarity, and ability to undergo further interaction with the analytes, e.g., hydrogen bonding (protic solvents) or pairing with dissociable or charged functional groups. Practical considerations include also viscosity,

surface tension, costs, difference in density towards donor, volatility, and toxicity and flammability in terms of work safety.

Classical solvents for LLE and LPME include in order of increasing polarity: decan, toluene, chloroform, diethyl ether, and hexanol but are unable to extract polar compounds and are, with exception of GC, generally not compatible with advanced instrumental techniques. Typical miscible extraction solvents include methyl tert-butyl ether, butanol, or acetonitrile (ACN). This procedure starts with a homogeneous mixture of solvent and sample. By the addition of a significant quantity of a kosmotropic salt, i.e., a salt composed of ions able to arrange water molecules deep into the bulk solution as multilayer hydration shells, the solubility of the solvent is critically diminished, which leads to phase separation [163]. This corresponding extraction methodology (Figure 7A) is called homogeneous LLE (HLLE). The capability of ions to achieve the explained *salting-out* effect is described by the Hoffmeister series [164,165]. Polyalcohols and sugars can be used in the same way, the procedure is then denoted *sugaring-out* [166].

HLLE can be used for extract also moderately polar compounds which makes this methodology interesting for pharmaceutic or pesticide analysis; however, also more of the sample matrix is co-extracted. The HLLE approach is therefore ideally combined with a second cleanup or preconcentration by SPE. This idea is implemented in the manual methodology of QuEChERS (standing, a bit boastfully, for Quick Easy Cheap Effective Rugged Safe) [167,168]. Here, HLLE is done generally with semi-solid samples (e.g., homogenized vegetables) followed by SPE of matrix compounds – not the analyte – for further extract purification. This indeed highly efficient method is applied to semi-polar analytes, above all pesticides but with significant financial effort for sorbents, solvents, and consumables. In the presence of surface-active substances, the use of immiscible solvents comes with the risk of the formation of stable emulsions. With miscible solvents, this risk is minimal, in fact, they are efficient for emulsion breaking. On the other hand, the achievable preconcentration factors arelow as phase separation is not achieved for high ratios. This thesis comments on LIS-automated QuEChERS-like sample preparation, i.e., HLLE followed by SPE but concentrating in the secondary SPE step the analyte and not matrix remains [169].
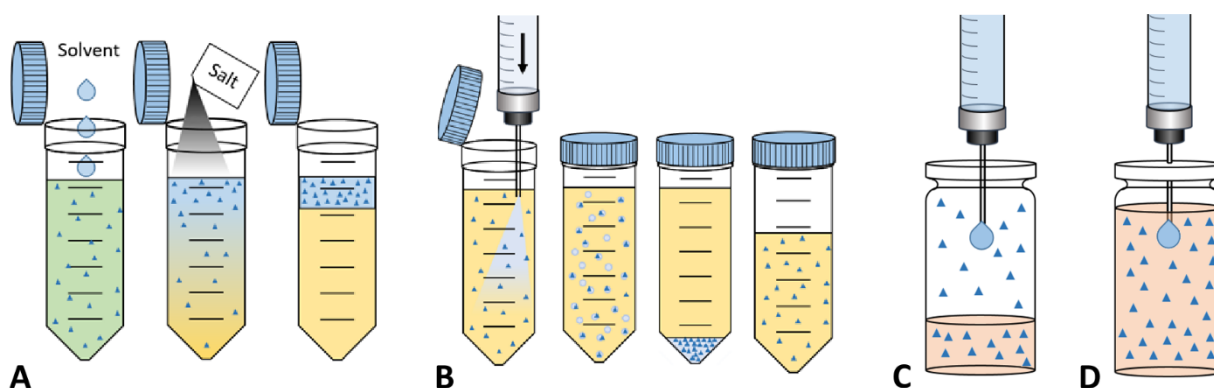
Figure 7: Schematical representation of commonly used sample preparation methods in manual performance. A: salting out assisted homogeneous liquid-liquid microextraction, B: dispersive liquid-liquid microextraction, C: headspace single drop microextraction, D: directly immersed single drop microextraction.

In HLLE, extraction efficiency and speed are high as in the moment of oversaturation, i.e., induction of phase separation, the solvent forms tiny droplets with an enormous surface before they coalesce. A

similar extraction speed can be achieved by an approach termed dispersive liquid-liquid microextraction (DLLME) [170,171], first proposed by REZAEE ET AL. in 2006 [172]. It is based on the rapid injection of a mixture of a miscible dispersion solvent and a far smaller part of immiscible extraction solvent into the aqueous sample (see Figure 7B). By practically instantaneous dissolution of the dispersion solvent, small extractant droplets are formed, which can rapidly extract the analyte in question by the high surface area available. The extraction solvent is recovered after spontaneous droplet coalescence or centrifugation. The DLLME technique has been rapidly adopted for sample preparation with >200 publications counting only reviews.

For floating solvents, this can be aided by solidification of the extractant drop or sample by freezing or using special extraction vessels with narrow necks to confine the solvent [170,171]. Most practical has been, however, the use of a disposable larger plastic syringe [173] into which the sample is aspirated first and then the solvent mixture is injected. By its size adaptability, the extractant solvent was confined in the syringe inlet enabling simple recovery and transfer to the used analytical instrumentation.

This was the starting point for two techniques that are, to increase the confusion, equally termed *Lab-In-Syringe*. The manual approach uses disposable syringes and has been used meanwhile to mechanize many different sample preparation approaches that were recently overviewed by [174,175]. The second approach bases on the utilization of an automatic syringe pump [176,177] and is the topic of this thesis.

DLLME is especially prone to the formation of stable emulsions or loss of solvent droplets on particulate matter. It is therefore less practicable for dirty samples but can be for example used instead of SPE to extract and concentrate the analytes after HLLE after the extract dilution with loading buffer.

Various solvent systems are considered green alternatives to the above classical but rather toxic ones: Switchable solvents are organic molecules with 4 to 8 carbons with dissociable groups, thus amines and carboxylic acids, that are either charged, i.e., water soluble, or neutral, i.e., water immiscible depending on the extraction pH. These solvents can be used for HLLE procedures with the advantage of being less toxic and addition of salts is not required. Instead, a volatile agent (NH_3, formic acid) can be used to induce phase separation, this way making the procedure is compatible with mass spectrometric detection (MS). Moreover, a higher preconcentration factor can be achieved since the polarity of the solvent, not their solubility in neutral stage is decreased by the chemical switch [178].

Ionic liquids are composed of two bulky ions with at least the cation being organic. By the choice of the components, hydrophobicity, and other properties, e.g., viscosity, water solubility, and capability to undergo further interaction with the analyte, apart from obvious hydrophobic and ionic interactions, can be tuned. Therefore, ILs belong to what is known as tailored or designable solvents. ILs are non-volatile, show low electrical resistance, and generally have a high viscosity and density, which can be both boon and bane for the intended analytical technique, i.e., suitable for CE and HPLC but rather not for GC. Suitability for HLLE is given by adding the second component of the ILs to the homogeneous sample solution containing the first one. Not all ILs are environmentally benign, moreover, due to their high price they are more attractive for LPME, i.e., miniaturized approaches to LLE.

Another type of tailorable solvents are deep eutectic solvents (DESs) in which the interaction of the components is hydrogen bond formation, i.e., they consist of at least one hydrogen bond donor and one hydrogen bond acceptor. This leads to a eutectic decrease in melting point compared to the single

components. A subgroup are natural DESs (NADES) that are based on naturally occurring components such as organic acids, phenolic compounds, or amines. As ILs, DES can interact in various ways with the analyte including hydrophobic, π- π, dipole, hydrogen bonding and ionic interactions, so that they are capable, as ILs, of extracting moderately polar and even ionic compounds. Moreover, they are less toxic and more economic than ILs while similarly compatible with HPLC. Hydrophobic DES, reported firstly in 2015 [179], are usable for LLE and LPME methodologies but a certain water solubility remains.

Finally, solvent-like media based on surfactants should be shortly addressed. Nonionic surfactants, if added to the sample in weight permille concentrations, form above a critical temperature, the cloud point, a separate and denser phase. The analytes that have been extracted into or interacted with the micelles formed by the detergent are now found in the precipitated phase that can be diluted with methanol (MeOH) and injected into HPLC. The related extraction methodology is called cloud point extraction (CPE) and was firstly described in 1987 [180]. Meanwhile, it has become an established sample preparation technique mainly for the preconcentration of metals and organic contaminants [181]. Supramolecular solvents (SUPRAS) are the second type of surfactant-related solvents and nanostructures liquids that are formed by aggregation of existing micelles or vesicles in the aqueous solution with the aid of temperature, salts, or solvent. They are very interesting media for the extraction of organic contaminants from highly complex samples given that the solvent can size-exclude macromolecules with the advantages over restricted access materials to be soluble and state-reversable [182,183] that makes them injectable to HPLC. Utilization of SUPRAS seems not to be spread broadly most likely due to a more intricate theory and availability of better characterized materials but show great potential for highly complex samples.

Further approaches of manual LPME related to this thesis are head-space single drop microextraction (HS-SDME) and directly immersed single drop microextraction (DI-SDME), proposed firstly in 1995 and 1996, respectively (see schemes in Figure 7 C and D) [184,185]. Interesting enough, both methodologies were invented on FT analyzers before usage in batch [186-188]. They are based on the formation of miniuscule drops of a few microliters down to sub-microliter volumes, e.g., at the tip of an injection syringe inside the gas phase above the sample, the headspace, or inside the sample. In both cases, very high concentration factors are achievable with just the amount of solvent needed for injection to GC. However, times to achieve phase equilibrium are long due to the minimal contact area so extractions are generally not performed quantitatively but time-controlled/time-limited.

In HS-SDME, the analyte must be volatile to pass first into the gas phase before it can be extracted into the drop, which further adds to prolonged extraction times. Mathematical models of both approaches are described in specialized recommended literature [189] and will not be explained here further. However, the advantage of HS-SDME is that it is applicable even to dirty matrices as sample contact is completely avoided. Solvents of moderately high surface tension and low volatility are ideal for this methodology while DESs and ILs have been used. For DI-SDME, the limitation is solvent solubility in the solution so the use of DESs and ILs is limited to highly hydrophobic solvent compositions. Both extractions can be promoted by magnetic stirring of the sample and salting out of the analyte. In DI-SDME, any dissolution of the drop solvent in the sample must be avoided while in HS-SDME, the evaporation of the drop is potential risk. Moreover, vacuum application and heating can promote

analyte volatilization in HS-SDME. A drawback of both techniques is the risk of drop detachment, especially in stirring assisted DI-SDME.

An alternative to HS-SDME is head-space extraction (HSE), i.e., the gas phase enrichment without using a solvent, so a methodology out of scope of this chapter is yet mentioned as it has been automated by LIS. In procedures carried out manually or via autosampler systems, a part of the enriched gas phase is transferred by a heated gas-tight injection syringe to GC, the instrumental technique ideally suited for HSE. In an application included in this thesis, we have proposed LIS-automated HSE with pressure-assisted gas transfer to online coupled GC via transfer line [190]. This proved similarly effective as dynamic enrichment approaches to HSE based on sample bubbling and gas transfer to programmable temperature injectors.

Before overviewing FT automation of LLE and LPME approaches, one final LPME approach should be briefly explained, namely Supported Liquid Extraction (SLME). Here, the extraction solvent is used to impregnate a porous and hydrophobic support being either a sorbent, hydrophobic foam, membrane, or hollow membrane fiber (HF) tube. This way, the solvent is protected from mechanical abrasion while still exposed to the aqueous sample. The membrane acts further as a filter medium inhibiting the passage of particles while the hydrophobic solvent in the pores inhibits the passage of proteins due to their intrinsic charge. This approach, therefore, has been used for samples of particulate load and biological samples such as blood plasma and serum. The small available contact surface causes slow extraction kinetics. However, by the movement of the donor and accept phase, transfer speed and extraction kinetics are enhanced.

The approach is highly useful to simultaneously extract and backextract polar or dissociable analytes from the sample into an aqueous acceptor on the other side of the membrane or inside the HF lumen. The pH values of the donor and acceptor phases are adjusted so that there is a constant concentration gradient for the neutral form of the analyte, i.e., it penetrates the membrane in a neutral state and becomes and remains ionized in the acceptor. The process can be promoted for ionic analytes by the application of a strong electrical field over the membrane, known as electro-membrane extraction [191]. For this, the solvent must be capable of dissolving the analyte, either by using an appropriate DES or by a mediator additive in the solvent. Both SLME and ECE present significant advantages over the formerly explained LPME approaches, which are compatible with advanced instrumental techniques only by diminishing the injected volume of solvent, thus loosing sensitivity, using separation-friendly solvents that co-extracts matrix components, or by prolonging the procedure by secondary back-extraction.

Other LPME methodologies could be listed in this section but have not been, to my knowledge, automated by FT yet. The corresponding concepts of automation *in-flow* are explained in the following.

3.3.1.2. Principles and approaches for flow automation of liquid phase (micro)extraction

A general advantage of LLE and LPME in terms of automation is the fact that it is based on liquids that can be easily handled in a flow system or also pipetted and transferred by autosamplers. On the other hand, whatever material is used for the manifold lines, mixing chamber, or detection cell, it will be affine to either sample or extractant so that system cleaning with both water and a miscible solvent is regularly

required. Significant advantages of flow automation towards manual processing of LLE and LPME are procedural miniaturization so that far less extraction solvent is needed, reduction of consumables and solvent evaporation with related benefits for expenditure, staff health, and environment, and generally an increase in processing speed and procedural reproducibility. Also, automation by FT can be easily coupled to advanced detection techniques and is economic compared to other automation approaches.

The topic of this chapter has been addressed in three extensive and comprehensive reviews contemplated in this thesis in section 4.5 [17,111,162], which have described in detail the different concepts and published applications. Therefore, only the methodological concepts of flow-automated LLE and LPME and publications reporting on new principles or technical milestones will be discussed here. The commented publications in chapter 4 give an overview of the majority of sample preparation methodologies that have been automated by LIS. Moreover, section 3.4 gives an overview of automation principles and automated methodologies of sample preparation using LIS by other researchers. In the light of this premise and considering that extensive descriptions are given as commentaries in chapter 4, this section overviews only LLE and LPME automated by FTs other than LIS.

A graphical summary of the different concepts of FT automation of LLE and LPME is given in Figure 8 and Figure 9. A clear differentiation between LLE and LPME in FT automation is only possible by comparing the automated approach with existing manually performed procedures but by solvent consumption, in most cases, miniaturization to microliter consumption of solvent was achieved. Moreover, for simplicity, the word "reverse" will be used for extractions, where the sample is the organic immiscible phase and the acceptor is an aqueous extractant.

Flow segmentation

The earliest works for the automation of LLE were applying the concept of flow segmentation of the sample flow but using segments of an immiscible extraction solvent instead of air bubbles (Figure 8A). The first article reporting this concept was by KARLBERG AND THELANDER in 1978 and is registered in the online database as no. 19 of about 25.000 more publications to come related to "FIA & Sons" [192]. Used instrumentation and operation in this so-called flow injection extraction (FIE) thus assembled both FIA (injection of the sample) as well as SFA (segmentation and phase separation). The liquid convection inside the segments of both phases leads to the extraction of the analyte during the transport to the detector. For prolongation of the extraction time, longer tubes were used. The preconcentration factor was adjustable by the volume ratio of the segments [193].

A phase separator was required before the detector to yield reliable reading. It was based either on sedimentation or flotation of the different solution segments, i.e., only on the difference in solution density, or aided by difference in wettability of the two parts of the separator, hydrophobic membrane through which only the organic phase could penetrate [194]. Related advantages were a lower dispersion and higher reliability of operation. The concept has been extended to automation by SFA [195], applying recycling of the segments to prolong the extraction time [196], and mono-segmentation using air segmentation to encapsulate each segment of solvent and sample [197] introduced by a linear valve as shown schematically in Figure 6. The late applications utilizing this approach omitted phase separation or air segmentation and operated with a single solvent drop and the more flexible MCFIA [198] demonstrating a drastic reduction in solvent consumption and improved robustness. Apart from the fact that the approach was the first one invented to automate liquid phase extraction in FT, the

advantages of the approach were simplicity of system and operation. Disadvantages surely include the achievable preconcentration factor since the feasible phase ratio can be scaled only up to some degree given the small contact area between liquid segments.
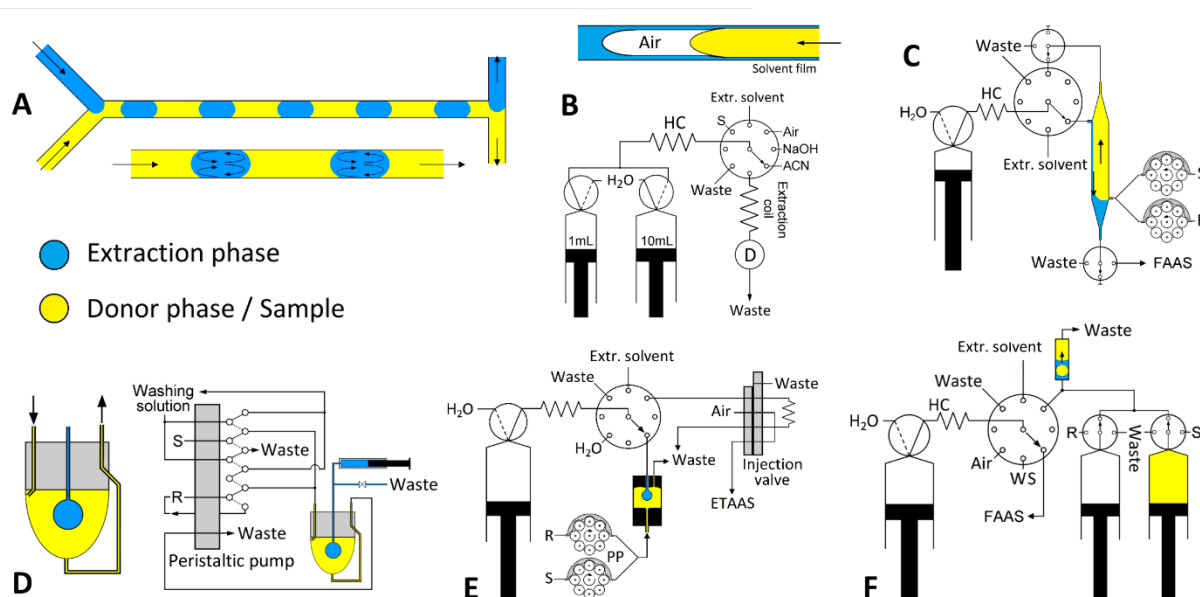
Figure 8: Schematic overview of concepts and systems used for flow automation of segmented flow, film formation, drop formation, and plug-flow. Modified from [17].

Wetting film formation

The approach is based on the formation of a solvent film on the inner walls of non-porous hydrophobic PTFE tubing that was discussed firstly by NORD & KARLBERG (1984) as a side effect in FIE [199]. Film formation contributed negatively to peak dispersion and was thus further investigated by LUCY and co-workers (1994-1995) leading to a *co-current chromatography* denoted approach [200-202]. In 1995, preconcentration of analytes by a solvent film was firstly described using an FIE system with a double injection of sample and eluent [202]. A year later, the concept was applied in SIA where film formation was done not via a continuous water-solvent segmented flow but by aspirating subsequently a single segment of solvent and an air bubble that aided to spread the sticky solvent on the inner walls of the HC [203]. After its use, the wetting film was dissolved with a miscible solvent and passed through the detector for quantification of the enriched analyte. The approach was improved in subsequent works, in particular by moving the detector and location for the formation of the solvent film from HC to an extraction coil located on a lateral port of the SV in the SIA system (see the system in Figure 8B) [204] as well as by enhancing sample throughput by performing extraction and analyte backextraction into an aqueous acceptor in parallel to wetting film formation using two extraction coils operated alternating in a MSFIA system [205].

Moreover, counter-current wetting film formation was reported using a special extraction chamber in which the solvent was allowed to flow downwards by gravity while the sample was passed in counter-direction (Figure 8C). Apart from at least one additional pump needed, the larger dead volume of the extraction presents a drawback in terms of system cleaning yet, the approach is applicable to continuously provided sample and a film formation is more reliable than by active film spreading [206].

Advances of the wetting film approach are, if performed in SIA, operational simplicity, and possibility for backextraction, and a large surface created in comparison with using for instance a single drop or flowing solvent segment, as well as the possibility to increase the phase ratio and thus feasible preconcentration factor even more by passing a larger sample volume through the extraction coil. On the downside is the instability of the film, limiting in effect the possible sample volume and thus only moderate reliability and reproducibility. Moreover, the extraction solvent must be sticky and of low water solubility, and a more miscible solvent is needed for solvent renewal.

Drop formation

There are only a few applications based on FT related to drop formation, above all, the works from Dasgupta and co-workers. In 1995 they proposed the use of a drop for gas sampling for the very first time in analytical chemistry and applied the new concept to the determination of NH_3 and SO_2 [184]. A drop was created at the tip of a tube leading to the SIA system that provided the respective aqueous reagent and after extraction retracted the drop for either photometric or conductimetric detection of the converted gaseous analytes. The drop was created inside a chamber where the sample gas passed through. Hereafter, the drop was also used as an optical detection cell and on-drop sensing of Cl_2 was demonstrated [190]. In this case, drop retraction was not necessary but instead discarded inside the chamber to create a new one. A year later, the first work on DI-SDME was accomplished by MCFIA where a drop of chloroform was created inside another drop of a constantly replenished sample (Figure 8D) that acted as a wall-less extraction chamber. The determination of anionic surfactants as a sum parameter of methylene blue active substances was carried out with on-drop determination of the extracted blue ionpair by photometry [207].

Although these works were ground-breaking for proposing for the first time the extraction into immersed or gas-phase located drops of reagent, they were proof of concepts. Using SIA and a purpose-designed extraction chamber, extraction form a continuous flow of sample and complexant diethyldithiophosphate for the extraction of metal into an immersed drop of diisobutylketone was possible (Figure 8E). After the extraction, the drop was retracted and injected to electrothermal atomic absorption spectrometry (ETAAS) [208]. Although reported for a solvent of higher density than water, there is no reason why this principle could not be applied to lighter solvents or to extract analytes. In another work, the autosampler of an ETAAS system was connected to an LAV analyzer to create a drop of toluene inside a mixing chamber, also operated from the SIA analyzer to extract Cr(VI) with ammonium pyrrolidinedithiocarbamate as complexant [209]. This way, automation of DI-SDME was feasible but the tricky task of drop transfer was done by the coupled instrument itself.

Another approach to perform a DI-SDME-like procedure is by passing the sample slowly and dropwise though a small plug of solvent in a flow-through chamber on a SIA system (Figure 8F). This approach was effective for the extraction of Pb^{2+} as pyrrolidine dithiocarbamate complex into 80 µL of chloroform [210] with subsequent analysis by flame atomic absorption spectrometry (FAAS). LAV was also used for the automation of HS-SDME of NH_3 but with drop creation inside the sample vial. The drop solution was provided by the analyzer and used to create a 5 µL drop at the tip of a tube inside the sample vial. After extraction, it was retracted into the system and let react in a heated mixing chamber using the Berthelot chromogenic assay followed by detection of the reaction product [211]. The mixing chamber was of high utility in method optimization to adapt the volumes of the three needed reagents easily.

Solvent impregnated membranes
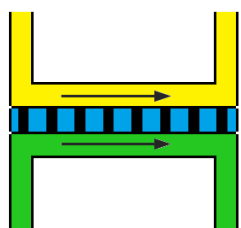
The principle of SLME was already explained in the previous section. It is based on using a microporous membrane impregnated with an immiscible extraction solvent for either bi-phasic extraction where the membrane separates the sample from the extraction solvent [212] or simultaneous extraction and back-extraction of the analyte into an aqueous acceptor on the other side of the impregnated membrane [213], both proposed in 1986. Ideally, the acceptor phase is kept static while passing the sample along the membrane achieving a higher enrichment. The system and required membrane device are comparable with the ones used for gas diffusion (section 3.2.4). The approach has been used FIA, CFA, or SIA [111]. Specific reviews on the topic have discussed the benefits of flow-automated SLME such as high enrichment factors [144,145,214]. Selectivity is increased in tri-phasic systems by the additional backextraction while the bi-phasic mode suits for extracting analytes from oily samples, i.e., the organic sample itself impregnates the membrane.
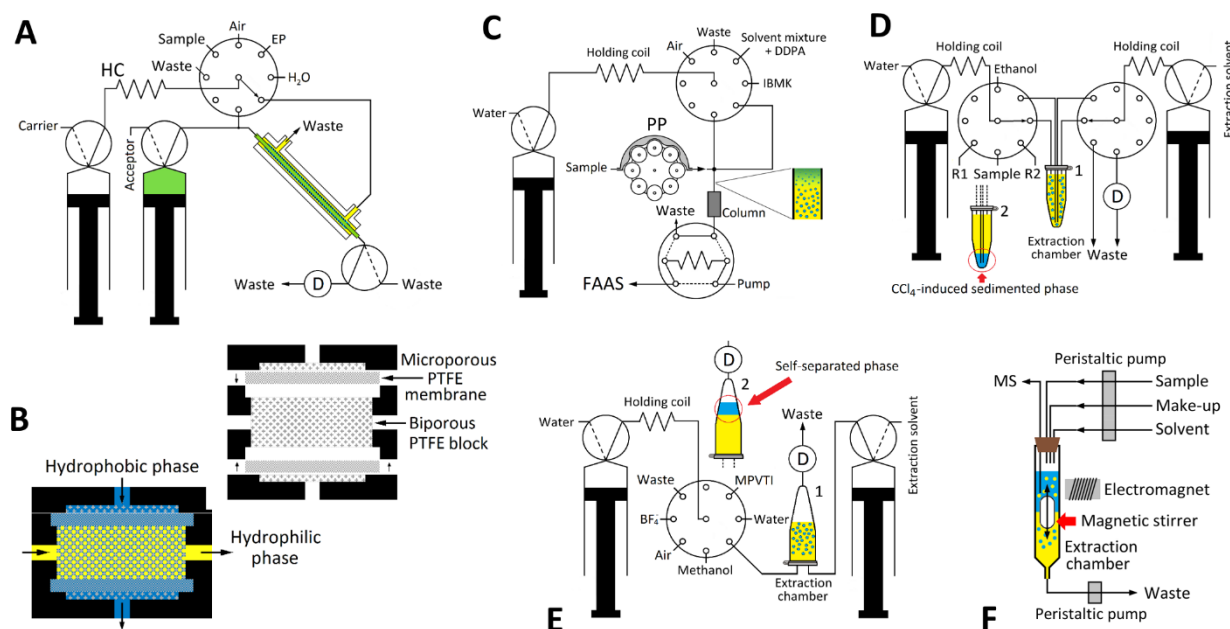
Figure 9: Schematic overview of different concepts and systems used for the automation of LPME and LLE based on support and impregnation of porous media: supported liquid membrane, hollow fiber membrane and chromatomembrane cell. Modified from [111].

Hollow membrane extraction
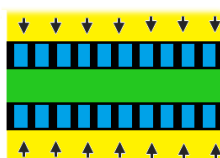
Instead of a solvent-impregnated membrane, also other porous supports can be used, such as packed sorbent particles [215] as well as an impregnated HF [216-218]. Even though HF are tubes, most works with HF have been carried out as manual approaches or using simple microsyringe delivery pumps [111]. The first FT-related use of HF for liquid phase extractions was reported in 1996 **Error! Reference source not found.** for analyte extraction in samples as complex as plasma. Since then, the device used for the housing of a HF, shown in Figure 9A in a flow system has changed little.

It consists basically of two t-connectors on the end of a polymer tube, in which the HF is located. On each end, one port of the T-connector is connected to the lumen of the HF to pass the acceptor solution

of a solvent for renewal of the solvent film, and the other one is connected to the ring-gap outside the HF to pass the sample. As for SLME, both bi-and tri-phasic extractions are possible. Most applications were done with FT relying on continuous flow with one application automated in MSFIA (see Figure 9A) [220]. The difficulty with all tri-phasic SLME applications is the reproducible renewal of the solvent impregnation while analytical problems requiring reverse LLE, i.e., ideal for bi-phasic SLME, are not often confronted in FT automation. This, and the slow extraction kinetics in particular for the relatively thick tubing walls of a HF are likely the reasons why a only few FT applications can be found.

Chromatomembrane extraction
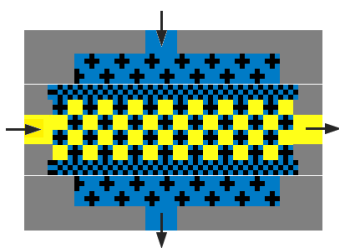
 The chromatomembrane device was introduced by MOSKVIN AND SIMON in 1994 as an ingenious interface to both liquid-liquid and gas-liquid extractions [221,222]. It is based on sandwiching two kinds of porous foam of hydrophobic PTFE. The top and the bottom layer only show micropores (< 1 μm) that are accessible only for the immiscible organic phase (or air) and thus present barriers for an aqueous phase. The middle layer also shows macropores (> 200 μm) where the aqueous phase can pass through. The assemble is closed in a stainless-steel housing featuring each two perpendicular flow inlets and outlets (Figure 9B). The main difficulty in performing chromatomembrane-based extractions is related to reliable flow control and precise adjustment of pressure. If done right, the simultaneous but orthogonal passage of both phases is possible with high interfacial area and extraction efficiency achieved in a small device. It is also possible to keep one phase stationary while the second one is passed through the device. An anticipated limitation is that the samples must be particle-free, since even small particles will block the pores of the device over the time. The chromatomembrane cell has been proven versatile by applications to LLE, reverse LLE, gas-into-liquid as well as to liquid-into-gas extractions [111,223].

DLLME in confluence flow
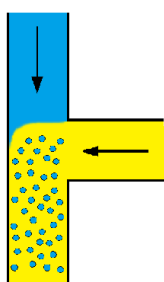
 The first possibility to automate DLLME using FT is the injection, i.e., a confluence of the mixture of dispersion and extraction solvent into the sample flow followed by the formation of fine droplets of the extraction solvent that extract the analyte rapidly, given the creation of an enormous surface. To recover the extraction solvents, the droplets are retained on a hydrophobic sorbent. Afterward, the retained droplets are eluted with a second solvent towards the detector. This automation approach for DLLME was proposed by ANTHEMIDIS AND IOANNOU in 2009 for the preconcentration of Cu^{2+} and Pb^{2+} for determination in FAAS. Exemplarily, the used system setup based on SIA is shown in (Figure 9C) [224]. Advantages such as rapidness, efficient recovery of the extraction solvent, and applicability to large volumes of the sample can be named for this approach; however, also solvent mixture has to be added continuously to the sample. On the other hand, the elution of the solvent drops implies a dilution, i.e., a loss of sensitivity, which can be compensated only partly by a higher phase ratio between the solvent mixture and sample to start with. Moreover, the relatively complex system with many experimental parameters to optimize requires significant time for setup and fine-tuning.

DLLME in flow-batch
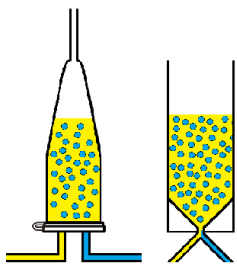
The second possibility is to mix the sample and solvent mixture in a FT-operated extraction chamber. This was first reported by SWEILEH ET AL. in 1988 [105] using a mixing chamber that was operated by a MCFIA system based on air-pressure [104]. Extraction of methylene blue active substances was aided by computer-controlled stirring followed by spontaneous phase separation, droplet coalescence, and in-chamber spectrophotometric detection. Instead of magnetic stirring, HSIEH ET AL. proposed in 2015 [225] using a vertically positioned, loosely fitted magnetic stir bar inside a miniature glass chamber actuated by an electromagnet so that the extraction solvent butanol was dispersed by the strokes of the stir bar to extract (Figure 9D).

A variety of SIA-based flow-batch applications were reported, with first one by our group [226-229] with solvent dispersion assisted by air-flow, flow pressure, or dispersion solvent. Two SIA systems were connected to the conical extraction chamber via PTFE tubing for provision of aqueous solutions, cleaning, and extraction solvent by the first one, and extract collection and submission to spectrophotometric detection by the second (Figure 9E). This was done to avoid problems due to the unintentional introduction of water into the system connected to the detection flow cell. Adjusting the depth of immersion of the tubing used for solvent aspiration, the approach was usable for both solvents of higher and lower density than water. In one case, the solvent density was adjusted by tetrachloromethane and dispersion aided by ACN as a miscible solvent [228]. A simpler approach to utilize of a floating extraction solvent was the use of a closed extraction chamber with a conical top outlet towards the detector in which DLLME was accomplished by vigorous injection of sample and solvent (Figure 9F) [230].Multiple applications have been reported on the use of the flow-batch concept for the automation of DLLME and the concept has also been transferred to other batch-FT, e.g., based on MPFA [231] with the advantage that the lasting back-and-forth operation for the handling of all solutions in SIA is replaced by a unidirectional, faster solution provision.

Advantages of flow-batch operation of DLLME are, independently for the specific approach and its efficiency for solvent dispersion, straightforward implementation of rection steps before the extraction, moderate simplicity of instrumentation, and a plannable procedure: chamber filling with sample and required reagents, injection of solvent for droplet formation, phase separation and droplet coalescence, solvent re-aspiration, and submission to the detection flow cell. What is more, flow-batch systems can easily be used for the automation of HLLE without any modification needed as well as to CPE if chamber heating can be done [232] as well as to the use of green solvents, e.g., switchable solvents in combination with effervescence-assisted mixing [233] or DES [234], where a benefit is taken from the fact that solutions can be added to the chamber stepwise.

On the other hand, the design of the laboratory-made chambers have a significant influence on the applicable solvent and dispersion means as well as on feasible solvent collection. Phase ratios, in theory easily adaptable, are limited by the possibility to recover enough solvent for subsequent detection, which is simplified by a settling solvent and in-chamber detection. Finally, extraction chambers must be cleaned thoroughly before re-use implying more time for the analytical procedure, and also a significant consumption of miscible solvents to remove remains of the extraction solvent. Moreover, the extraction chamber is typically open, which implies a risk of contamination, solvent evaporation, and solution

splashing by stirring, while a low stirring rate also decreases extraction efficiency. It is especially these three issues that are elegantly overcome by using the void of a syringe as a size-adaptable yet sealed chamber. However, taking advantage of effervescence for solvent dispersion and solution mixing, as it has been possible to demonstrate for flow-batch applications [233,235], is unlikely to work in LIS. Mixing the effervescence solutions in a sealed glass syringe is surely risky to try; moreover, volumes could not be precisely aspiration, even might shot out of the syringe, by the forming gas pressure.

In conclusion, there is no perfect liquid phase extraction methodology, the choice will depend on the required preconcentration factor, sample complexity or "dirtiness", the needed robustness, throughput, and detection technique. There is also no automation approach that is capable to automate all extraction methodologies yet to my believe, LIS is among the most versatile ones. In general, complex matrices will require some manual work, e.g., filtration, both for automation in-batch and in-flow. Dealing with matrix precipitation due to the used solvent and achieving compatibility with the advanced instrumental techniques remain important challenges were the combination of liquid and solid phase extraction could bring new possibilities, e.g., as used by the QuEChERS principle, and similarly demonstrated by two applications of LIS contemplated in this thesis, HLLE as a first step for matrix removal with SPE for preconcentration. Another possibility could be solvent exchange, e.g., backextraction into a separation technique friendly IL to achieve higher sensitivity. In chapter 4, published application of the LIS technique to a variety of analytical problems mostly based on liquid phase extraction approaches, will be discussed.

3.3.2. Solid phase (micro)extraction approaches

Solid phase extraction: SPE belongs to the most widely used sample preparation methodologies and frequently automated operations in FT. Generally, a prefilled sorbent cartridge is integrated into a flow line to preconcentrate the analyte of interest and eliminate the interfering matrix. Alternatively, the prior explained BI concept can be used to automate in-system packing of a renewable SPE microcolumn that is generally done in a LOV conduit (see section 3.2.3.3.2). After preconcentration, the analyte is eluted towards a selective detector, e.g., electrochemical detection or atomic spectrometry or a chromogenic reagent is added to yield a product that can be easier or more selectively quantified than the original substance by optical detection techniques. Moreover, FT-automated SPE is often done for analyte enrichment to online coupled separation techniques, benefiting for an additional boost in sensitivity and procedural efficiency. Examples can be found for HPLC [169], SIC [124], and CE [236] with selected application cited. Here, the matrix elimination by SPE is of the same importance as the achieved gain in sensitivity by analyte preconcentration since damaging the connected instrument or affection the separation must be avoided.

An SPE column packed with small particles exerts a significant backpressure that increases to the second power of the inverse diameter of the articles. Therefore, FT based on syringe pumps present a significant advantage regarding the robustness and reliability of methods that involve SPE. The standard manual procedure of SPE comprises several steps: sorbent conditioning, i.e., for hydrophobic sorbents a sorbent solvation and equilibration with loading buffer or water, sample loading, washing of the sorbent to remove remains of unretained or weekly bonded matrix components, and elution of the

analyte. At times, even drying steps are demanded. Therefore, using a FT capable of switching between different solutions is of high interest as well as the possibility to mix the sample with an appropriate buffer before loading to assure that dissociable analytes can be retained in their most appropriate form. The most often used sorbents rely on hydrophobic (C18, polymeric resins), including hydrogen bonding (hydrophilic-lipophilic balanced sorbents) or ionic interaction (weak and strong cationic and anionic exchangers) while all other sorbent materials are in principle usable. For instance, restricted access materials [237] and molecularly imprinted polymers [238,239] have been used for matrix clean-up as a highly selective alternative to classical materials, respectively. A special modality is passing the sample in turbulent flow conditions, i.e., at very high flow rates, though the SPE column inhibiting the retention and unspecific sorbent interaction of macromolecules characterized by large hydrodynamic diameters and short diffusion lengths but under the premise of high-pressure robustness of the used pump [240].

FT automation of SPE typically comes with a gain in reproducibility, and sample throughput and SPE cartridges can generally be utilized over several working days depending on the matrix in question. FT also enables procedural downscaling, i.e., straightforward application for smaller sample volumes or higher achievable preconcentration factors. The filling of a single commercial SPE cartridge of 100 mg resin can suffice in BI for an entire working day and dozens of samples. BI (see section 3.2.3.3.2) is an ideal approach for the automation of SPE given that the costs for resin per analysis are greatly reduced. What is more, no accumulation of matrix components, as possible by repeated use of cartridges, must be confronted since the microcolumn is discarded and packed from fresh sorbent after each use. For BI, soft sorbent particles of cellulose, sepharose, sepadex, and of spherical shape, e.g., styrenedivinylbenzene-based polymers, are preferred over irregularly-shaped silica or alumina sorbents to avoid damaging the SV during switching and to facilitate their handling. Nonetheless, approaches for their handling have been proposed [241].

Two possibilities exist for coupling FT-automated SPE online to HPLC. The first is performing the SPE procedure on the FT system with the cartridge integrated, e.g., in the transfer line to the injection valve of the HPLC. In consequence, the eluate containing the formerly preconcentrated analytes is loaded into the injection loop followed by injection for analyte separation. Commercial packed needle devices, known as MEPS (Microextraction by packed sorbent), are miniatured cartridges that can be easily integrated into the flow system with elution volumes that are perfectly harmonized for what is needed for SIC [124,242].

In case that both SPE and separation rely on hydrophobic interaction of analyte and sorbent, an eluent with high content of organic solvent, typically MeOH or ACN, must be used as eluent and consequently be injected. Analyte retention on the separation column can be improved by using a significantly lower initial percentage of organic solvent in the HPLC mobile phase than the eluent for SPE [243,244]. The second possibility is to use the SPE cartridge integrated into the injection loop, a mode termed online SPE. Here, the use of orthogonal retention mechanisms between SPE and HPLC has shown to be of advantage since the initial mobile phase composition can elute all analytes at once [149].

The sorbents for FT-automated SPE can is further be created as a sorbent layer on an appropriate solid support as exemplarily demonstrated in the enrichment of Ra^{2+} from water samples. Automated formation of a MnO_2 coating on cotton was done for each analysis. This layer was then dissolved by a

reductant to regenerate the column and release the analyte and co-precipitate with $BaSO_4$. The achieved clean-up enabled offline determination of [226]Ra activity by alpha emission count [245].

Another sorbent type for SPE that has only recently been explored is polymeric nanofibers. They can be used in packed cartridges for online SPE [246] where the porosity of the packing depends significantly on the user while common sorbents show far higher mechanical strength and intrinsic porosity that is not affected by compaction [247]. For the same purpose, nanofiber tissues can be used as sorbent membranes, which yields high reproducibility of usage and enables loading of larger volumes than possible in HPLC from a low-pressure flow system [248]. Advantages of nanofibers include a simpler production compared to particulate sorbents, high surface-to-volume ratio, and property tuning, e.g., mixing different polymers as well as using additives and coatings with sorptive properties. Their use for turbulent chromatography and matrix elimination already during loading was recently demonstrated [249].

A final operation mode for SPE that should be addressed is the use of the sorbent in free flow, also described as dispersive SPE [250,251], or, in case of nanofibers, as tissue fabric. The advantages involve that no pressure is needed for loading and larger sample volume can be treated per time unit [252]. On the other hand, sorbent recovery must be ensured by either spontaneous sedimentation, centrifugation, filtration, or, using magnetic susceptible sorbents, by a strong magnet [253]. Recently, automation of dispersive SPE has been demonstrated by the LIS technique for the first time, where the magnetic nanoparticles used were captured on the magnetic stirrer inside the syringe. In this thesis we report on an application that combines the BI concept with in-syringe dispersive SPE [254] and on a simple methodology to yield magnetic-susceptible polymer sorbents from commercial SPE particles [244].

3.4. The automation technique Lab-In-Syringe

3.4.1. Main principle, instrumentation, and operation modalities

The automation technique Lab-In-Syringe (LIS) was first proposed by MAYA ET AL. (2012) [176] as a not specifically designated approach to automate a manual procedure of DLLME in a disposable plastic syringe by CRUZ-VERA ET AL. (2009) [173]. The denotation Lab-In-Syringe was established for this new automation technique by a later publication when in-syringe spectrophotometric detection was firstly used [177]. The use of disposable syringes developed further into a manual approach equally denominated Lab-In-Syringe to process samples in manual fashion [174,175]. Although the approach has proven ingenious, effective, and versatile, with applications based on a variety of LPME approaches and SPE methodologies based on dispersed sorbent, foam, or membranes, the topic cannot be further discussed in this thesis.

The key principle of the automation technique LIS is to make use of the void of an automatic syringe pump for solution mixing, gas phase confinement, or vigorous dispersion of immiscible phases by stirring. The system (Figure 10) consists of an automatic syringe pump[10] equipped with glass syringes of typically 1, 2.5, or 5 mL and a rotary SV that can be either a separate device or integrated into the syringe instrument as a multiposition head valve. The system elements are thus identical to SIA, but the operation is as in flow-batch, so it can be seen as a hybrid of both techniques. Most often, a magnetic stir bar is used inside the syringe void to aid solution mixing. The concept of in-syringe stirring was proposed about one year of the existence of the technique by HORSTKOTTE ET AL (2013) [255].
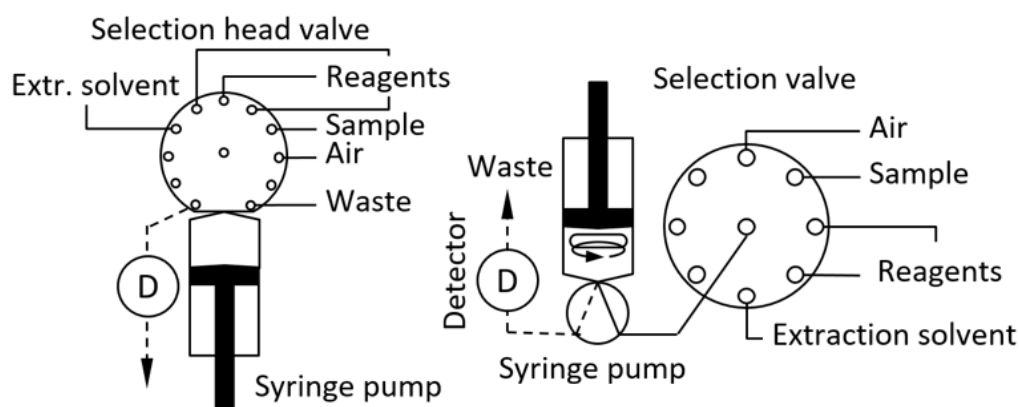
Figure 10: Schemes of typical manifolds of the automation technique Lab-In-Syringe with the syringe pump with in upright or upside-down orientation, using either a separate or an integrated selection valve and applying either in-syringe stirring or not. All combinations of these options proved effective.

The main applications of this technique to this point have been the automation of liquid-phase extraction procedures aimed for analyte enrichment and clean-up, that benefit from a large ratio in volume between sample and extraction solvent, or for gas-phase separations and gas-liquid extraction methodologies.

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[10] In my work, I have used multisyringe modules from Crison Instruments s.a. (Alella, Spain) or Cavro pumps from Tecan Trading AG (Männedorf, Switzerland) with quite significant differences regarding syringe design.

To minimize the system dead volume, i.e., outside the syringe, the typical HC of the SIA system was generally shortened as much as possible, if not possible to omit by the integrated syringe pumps. Solution mixing inside the syringe instead a tubing, e.g., the HC, was a fundamental violation of the principle of FT where a carrier solution typically carries out all used solutions (self-cleaning principle). In lack of a carrier, the carrier reservoir, in SIA typically connected to one of the ports of the syringe head valve can be used for waste disposal and so increase the efficiency of syringe cleaning. This operation was needed due to the large dead volume of the syringe void, probably the main drawback of the technique. For this, about 20-30% of the syringe void volume of cleaning solution is aspirated, potentially mixed by the stir bar, and discarded. In terms of system configuration, it proved further effective to locate the detector or interface to another instrumental technique not on a lateral position of the SV but to connect it directly to one of the ports of the syringe head valve, optionally integrated into the tubing line used for waste disposal.

A curious difference between LIS and SIA is that an autosampler can easily be used as an alternative solution selector in LIS, mainly because the detection is carried out either *in-syringe* by using an optical fiber connector to the transparent syringe barrel or in the outflow of the syringe after solution mixing, possibly by a coupled secondary instrumental technique. In both cases, the analyte is quantified in a homogenous solution. In contrast, detection in SIA is performed in-flow and gradient formation is very often intended. Therefore, an autosampler can still be used as a versatile addition to the SV of an SI-Analyzer but not as a replacement. Such compatibility of LIS with an autosampler was shown in one of the works commented in section 4.3 dealing with a LIS-automated QuEChERS-like procedure [169]

The advantages of using the syringe void over using an open mixing chamber or a tubing network are shortly discussed in section 0. The general advantage of using a syringe as an extraction chamber, which also explains the success of its use in manual sample preparation, is natural thanks to its size adaptability while being also a sealed container. The automatic syringe pump does not serve liquid containing, as a mixing chamber in flow-batch, but does also the pumping and can be used to exert pressure or confine gas. The wiping of the inner walls by the syringe piston and the narrowing at its neck is ideal for recovery and confining the droplets formed in a DLLME procedure, manual or automated.

The stir bar has been proven a key element to achieve operational versatility with an overview given in section 3.4.2. However, it inhibited emptying the syringe completely and with a diameter of 2-4 mm it consequently causes an increase in the dead volume of 5-10 % of the syringe volume. Concerning operational modalities to confront this problem are thus the following options: First, omitting in-syringe stirring and, if required solution mixing outside the syringe. Second, filling the dead volume "with something that does not matter". Using the syringe in normal, i.e., upright orientation, the best option was to perform after syringe cleaning an additional cleaning step with the sample that was intended to be aspirated in a larger amount anyway, so that no available space for the sample would be lost. Third, turning the syringe upside-down so that air remains like a cushion in contact with the syringe piston that can expulse practically all liquid from the syringe void if there is a smooth transition from the barrel of the syringe to its inlet. On the other hand, this caused a delay in liquid movement, so a delay time of 1-2 s is required after each solution aspirating or expulsion. The fourth option is a different approach to mixing the solution content. The only two reports to my knowledge on a promising tactic are discussed in section 3.4.2. An alternative could be diminishing the dead volume by replacing the stir bar with a stir

disk or ring or bubbling air through the piston channel, yet this approach is likely to be sufficient only for mixing and not for solvent dispersion. In the following two sections, an overview is given on so far automated sample preparation methodologies with an overview on developments and applications reported by other researchers.

3.4.2. Challenges and achieved improvements in operation and instrumentation

The achievement of reliable in-syringe stirring, support of the required motor, stir driver, and other necessary materials represent the main challenges in the work with LIS that are discussed in this chapter with the achieved improvement.

3.4.2.1. In-syringe stirring and operational issues

Stirring rates in manual stirring-assisted DLLME approaches are generally not faster than 1000 rpm [162]. The stirring rate is defined partially by the limitation of the used stir plate, yet a higher stirring rate would be unfeasible since the attraction between the stir bar and the rotating magnet of a commercial stir plate is not strong enough to overcome the liquid inertia inside the stirred vessel. This is also the reason why modern stir plates start slow. Moreover, there is the risk of solution splashing so in manual laboratory work, more violent vortexing of solutions in closed falcon tubes or glass vials is preferred when vigorous mixing is needed.
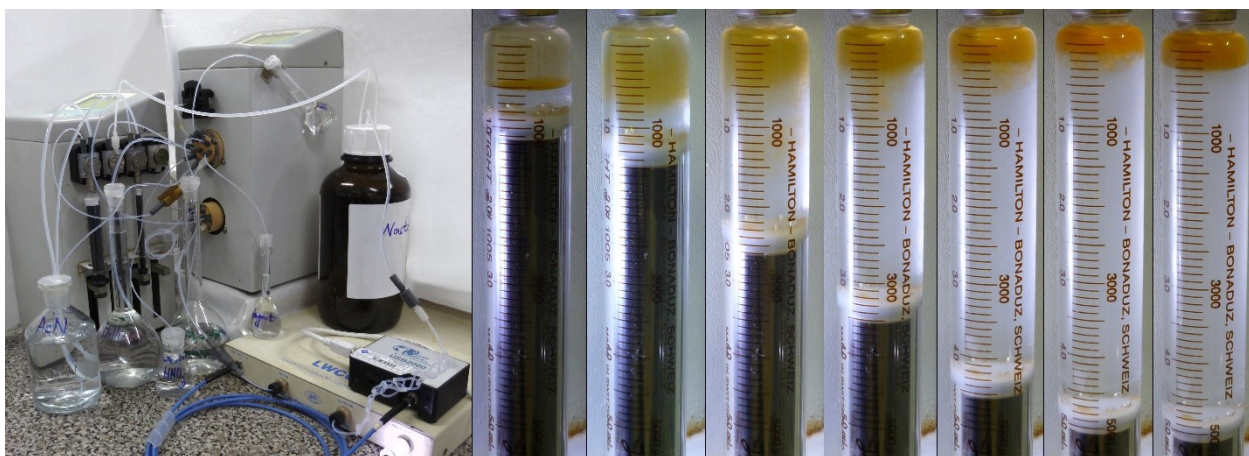
Figure 11: LIS system used for first experimental work of the author for solvent-assisted DLLME of Cu^{2+} as bathocuproine complex (Left) and photographic documentation of the extraction process (Right).

Apart from works that originated from the first year of working with LIS, I have always used in-syringe stirring and the approach has been also adopted by other researchers. In the beginning, an additional mixing chamber on one of the lateral positions of the SV was required in the early years for mixing sample and reagent to obtain an extractable product, e.g., complexation of copper with bathocuproine, [256]. Not only did this occupy one SV port, the cleaning, although possible, required time and a significant amount of a miscible solvent. Naturally, the complex formed in the reaction before the extraction was hydrophobic and using a mixing chamber of polypropylene implied that part of it could adsorb to the inner surface of the mixing chamber. In effect MeOH was found insufficient to dissolve the remains. Finally, we observed that part of the extraction solvent stuck to the piston head and could

be removed only by the aspiration of miscible solvent at a high flow rate. Due to the battered neck shape of the syringes, which I used in combination with a multisyringe pump, it was impossible to completely empty the syringe, so the cleaning solvent was diluted with the water still contained in the syringe and in the HC.

Figure 12 shows the used system and operation of DLLME without stirring for a highly concentrated standard used for process visualization. The proximity of the valve and syringe module was of importance here to keep the HC as short as possible as well as the stuck solvent droplets and consequent air bubbles on the piston head that was chosen of polyethylene since it performed better than the one of PTFE.
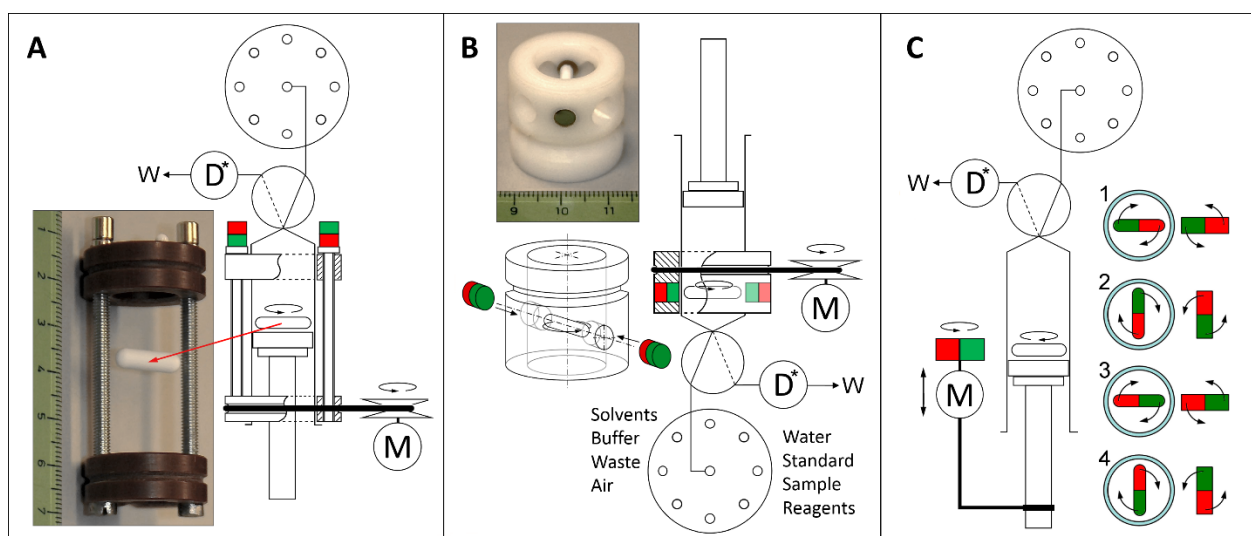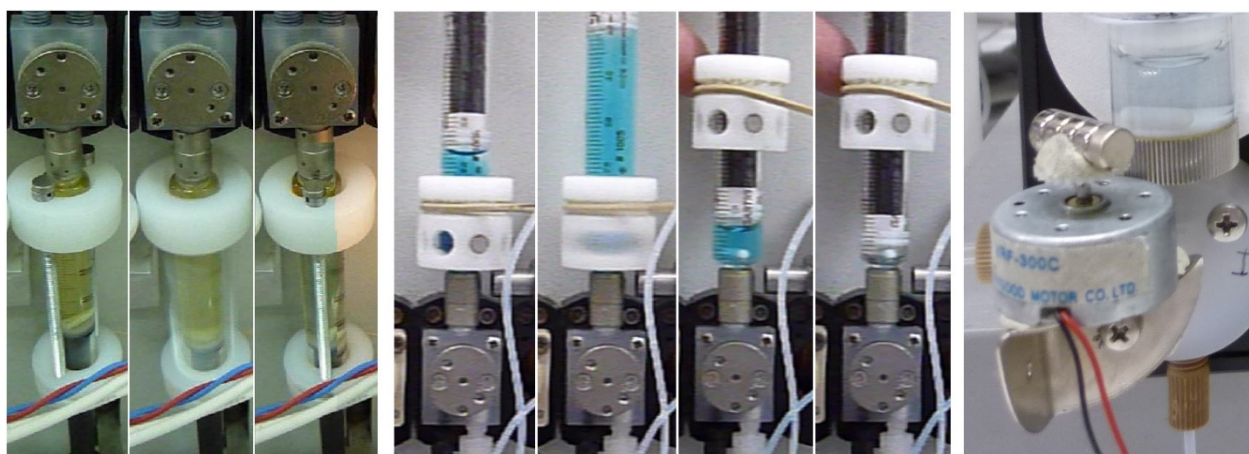
Figure 12: Schematics and photographic documentation of stirring operation and stir bar driver devices developed for the LIS technique. A: two steel rods, magnetized by two NdFeB magnets and held by two polymer rings create a rotating magnetic field all along the entire syringe barrel [255], B: simple driver ring with two NdFeB magnets to align the stir bar in the same location [108], C: motor NdFeB magnets fixed to the axis of a slow stirring motor, for upright syringe orientation fixed to the syringe piston. In detail photographies in A and B, a magnetic stir bar is held and elevated between the magnetized rods and magnets, respectively [258].

Using a stir bar inside the syringe, a rotating magnetic field had to be created to force the stirrer to turn, independent of the position of the syringe piston on which it rested and the inertia of the solution content. The first design of a device that could accomplish this is shown in Figure 12A together with

photographic documentation of its operation. Explanatory videos on the Youtube channel "In-Syringe Analysis/Lab-In-Syringe", prepared to explain the first experimental works with LIS are highlighted [259].

As it is shown, it was assembled from two 8 cm long steel screws, two plastics rings, one with ring groove, and two NdFeB magnets, and was driven via a standard rubber ring by a DC motor for 5 Euros that could be relay controlled by the syringe pump and for speed regulation, a simple potentiometer was used [255]. In-syringe stirring worked from the first second, but it was as loud as it was efficient, and the stir bar added to the dead volume inside the syringe. However, cleaning the syringe was required anyway, which could be done with more efficiency now that the syringe content was possible to mix.

It was found that both mixing and solvent dispersion in DLLME were significant improved when using a floating solvent by the aspiration of air about 5% of the syringe volume of air to create an open liquid surface inside the syringe which enabled vortex formation during in-syringe stirring.

An improvement concerning system simplicity and the sound level was the use of a ring-like *driver* as shown in Figure 12B instead of the above-described design. The motivation came from using the syringe upside-down to facilitate working with chloroform as a solvent of higher density than water. Turning the syringe meant that the solvent would leave the syringe first plus the issue of dead volume was almost overcome. The solvent was required for two standard sum parameters in water analysis for anionic and cationic surfactants [108,260]. Since then, I have used only this simpler design and rather lifted the driver ring with the piston, for which supports described in section 3.4.2.2, were needed, or did not use a driver at all. Placing a motor close enough to the syringe with a few NdFeB magnets fixed to its axis, allowed a stir bar inside the syringe to rotate (see Figure 12C). This approach was effective for relatively low rotation speeds (the alignment of the stir bar and NdFeB magnetics was far less strong), and for short stir bars and even more for stirring crosses. To achieve smooth rotation for a long stir bar, it was important to sand them down to a length just to fit smoothly inside the syringe and to adjust both the distance of the motor and the number of magnets on its top.

Regarding the motor, the carbon brushes worn out over time and the motor had to be replaced for each new experimental work. Very soon after initiating my research at my current institution, I started to use brushless motors, which I produced from standard computer fans. The main problem was the strength of these motors, in particular if not operated at maximal speed or if no stir driver was used, the attraction of the stir bar inside the syringe stopped the motor from rotation start. Using a higher supply voltage, the motor would start but would be hindered in catching up with the fast rotation of the motor due to the inertia of the solution content. The problem was solved by using laboratory-made analog control circuits that enabled reliable operation by first providing the current needed for motor start but then enabling a slow turn-on, i.e., a gradual increase to the final rotation speed [261][11]. Moreover, reducing the required momentum by size decrease of the pulley wheel for the rubber band that connected the stir bar driver and the motor was a simple but necessary modification.

A further improvement was therefore the use of pulse-width-modulated (PWM) brushless motors, made from PWM computer fans, with laboratory made control circuits. These motors receive the

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[11] At this point I thank Dr. David J. Cocoví-Solberg for his help with this problem.

maximum supply voltage while motor speed is controlled by a modulation of the control frequency. Finally, this allowed us to software control the motor speed via Trinket M0 (Adafruit) microcontrollers[12] since 2022.

3.4.2.2. Supports and required auxiliary materials

During my work in Spain, I was able to use a mechanical tool shop, in particular a lathe, to produce the material for the experiments. This included also a laboratory-made fluorimetric detection used in two works with LIS. In the Czech Republic, I had to rely on the tool shop of the faculty, which was soon cut for cost reduction, but it allowed me to develop a modified syringe piston with a flow-channel that was done by drilling a through-hole and using silicone to permanently seal a PEEK tube into it as shown in Figure 15. During the first 3 years of my time at my current institution, I used mainly driver-less in-syringe stirring leading to the observations explained above. Soon after, we got in contact with the 3D printing company Trilab in Hradec Králové, which allowed me to design, fabricate, and improve supporting materials.

The purchase of a fused material deposition 3D printer by our department was an important step further as well as the earlier purchase of syringe pumps from the company Tecan that featured head valves of 9 and 12 ports, as if purpose-designed for LIS. Examples of such auxiliary materials are given and explained in Figure 13 that also shows the addressed syringe pumps with multiposition head valves.

In conclusion, the reliability of the stirring approach has been constantly improved over the years. While in the beginning material from hardware stores was assembled for support, the progressive improvement of the auxiliary material by 3D printing now allows preparing all that is needed for system setup of-the-shelf in a few hours, so that we have achieved a certain material standardization even without direct support by companies. Microcontrollers are now an intrinsic element of our LIS system setup to control the motor speed or trigger an online connected analytical instrument. Further improvements can be expected, in particular stir bars that further reduce the dead volume of the syringe, or integration of functionalities into the head valve that are known from the LOV technique.
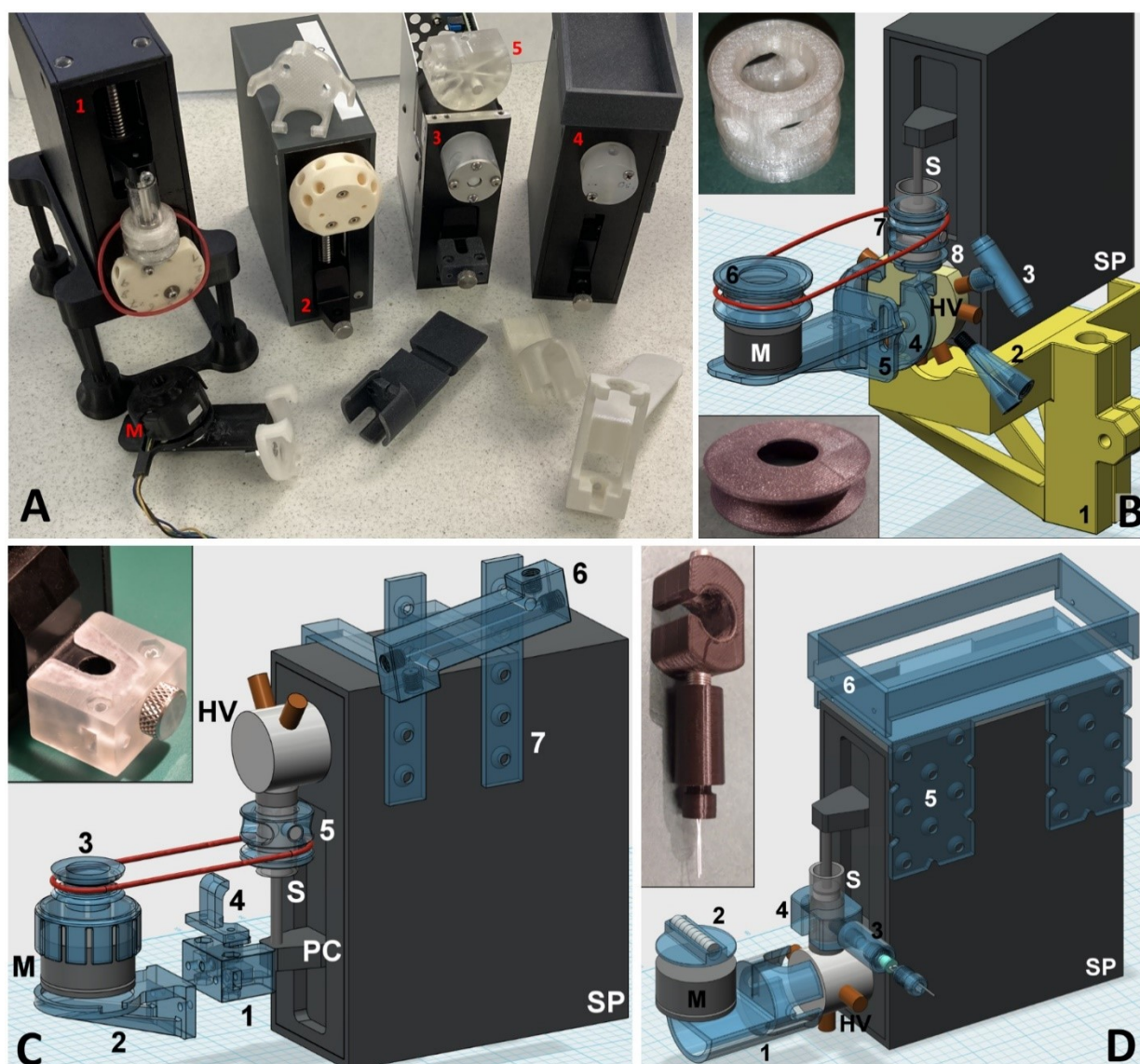
Figure 13: Examples of auxiliary material produced by 3D printing for the assembly of a LIS system [116]**.**

A: Cavro syringe pumps from the company Tecan with different multiposition head valves and purpose designed adapters to support a motor on the different head valves (No 1-4 correspond to syringe with 9, 12, 2, and 3 positions, respectively, 5: stereolithographic printed LOV-like monolithic head valve manifold to be used in future works).

B: Laboratory stand-compatible support (1) for syringe pump (SP), connectors to the head valve to add flow-through port functionality (2) and for drain tube connection enabling to be used on port for both waste disposal as well as air aspiration (3). Head valve adapter (4) to mount board (5) to position and level stirring motor (M). The motor is equipped with a pulley-wheel (6, photography below) connecting the motor via a rubber band to a simple stir bar driver ring (7, photography above) that is placed on the syringe (S) and hold in place by a supporting ring (8).

C: Stir bar driver (5) pulley wheel motor head (3) adapter (1, photography) for the piston carriage (PC) to fix a holder for the motor (2) and a lifting lug (4) to move stir bar driver and motor upwards with syringe piston. SLA-printed 5 cm detection flow cell (6) mounted on a universal adaptor (7).

D: Adaptor for the head valve (1), holder for NdFeB magnets (2) glued onto the motor, LED holder (3) and fiber-optic adaptor (4, photography) for in-syringe photometric measurements, head tray and support for deposition of tools (fittings, screwdriver, etc.) or to attach further items, e.g., the relay board for motor control (5,6).

3.4.3. Automatable procedures and reported applications and developments

FT are unconventional tools in the laboratory in their concept and operation, they show their best side where "hu-man-ual" work can simply not do the trick, e.g., carrying out every 2 min, 24 h a day, 7 days a week, reproducible sample preparation and quantification with assays based on fast reactions without much need in instrumentation, consumables, or reagents. Procedures can readily be downscaled without the need for the lasting development of microfluidics [83-85]. Moreover, they are capable of carrying out practically all tasks required to yield chemical information from continuous process streams. In these cases, they are the ideal tool and superior to batch automation.

In contrast, LIS is, as the flow-batch approach, a simple but versatile tool performing operations that a laboratory worker can do, and might do every day, sample preparation applied to liquid samples. These procedures are composed of simple operations such as of stepwise addition of reagents or solvents, mixing, heating, and liquid withdraw. Thus, the workflow is predictable and so it the planning of the procedure to automate when these unit operations can be done "as by hand". Problems arise first when aiming for more complex steps like precipitation, centrifugation, or solvent evaporation [116].

Figure 14 gives an explanatory overview of the so far automated sample preparation methodologies and possible operations modes with LIS. So far, DLLME with solvents denser and lighter than water, DLLME with dispersive backextraction, continuous DLLME, HLLE, HSE, HS-SDME, DI-SDME, gas-stripping, extraction into a floating drop, dispersive SPE, and CPE have been automated. Moreover, LIS systems were combined with BI, SPE based on sorbent membranes, in-syringe extraction on fabric tissue sorbents, online SPE on HPLC injection valve, and sample deproteination that are not schematically shown. Moreover, LIS was coupled to spectrophotometry, fluorimetry, HPLC with spectrophotometric, fluorescence spectrometric, and MS detection, as well as FAAS, ETAAS, ICP-AES, and GC.

The publications contemplated in this thesis report on the automation of most of the former listed sample preparation methodologies with detailed comments on operation, challenges, solutions, and outcomes in sections 0, 4.3, and 4.4. Therefore, hereafter, the experimental works and reported applications on using LIS for automated analysis are discussed that were reported by other research groups. A description of the milestones of technical developments in LIS, an overview of published applications until 2020, and tips and tricks regarding system setup and operation have been published as an open-source tutorial, included in section 0. A more recent overviewing article on LIS application can be found in the same journal [262].

A significant part of the publications commented in chapter 4 are related to my postdoctoral studies at the University of the Balearic Islands (Spain) in the group of Prof. Cerdà. The group continued in using this techniques mainly applying the in-syringe magnetic stirring concept with in-syringe DLLME. The exception was the development of in-syringe CPE based on Triton X-114 for the enrichment of antimony as SbI_4^- complex enabling detection by spectrophotometry. In-syringe heating was accomplished by mixing the aqueous sample in-syringe with 2.9 mol/L H_2SO_4 while speciation was achieved by mixing the sample in-syringe with ascorbic acid [263]. Further applications reported on DLLME in combination with spectrophotometric detection of caffeine [264] and lead as dithizone complex [265].
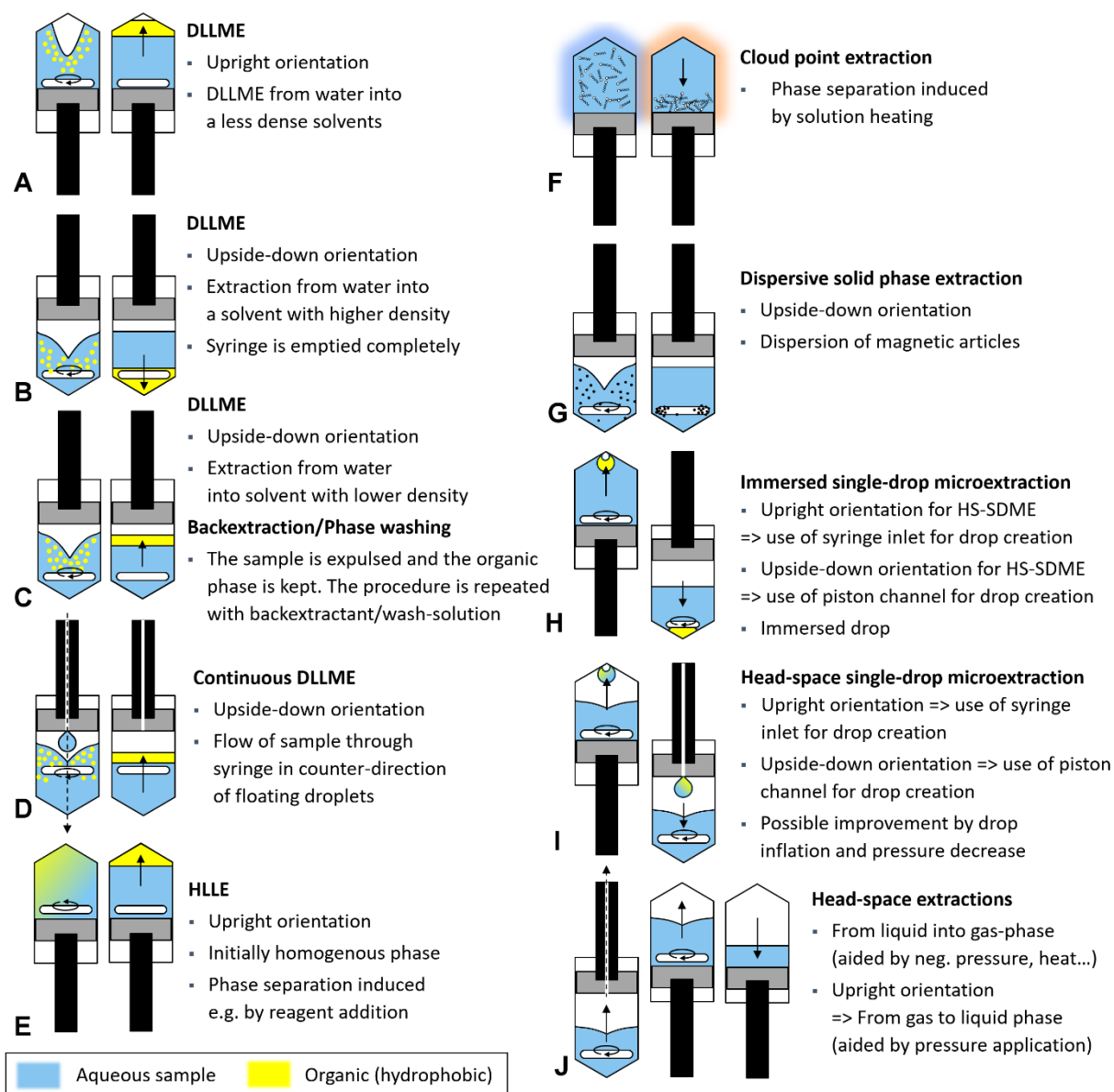
Figure 14: Selection of sample preparation approaches automatable by the Lab-In-Syringe technique with listed system characteristics, and operational features [116].

The coupling of LIS-automated DLLME of phthalate esters to GC with MS detection is to be highlighted [266]. A modified injection valve enabling the injection of 3 µL solvent to an air flow towards the GC injector was used as an interface between the sample preparation part and GC. The same system was used then for the enrichment of UV filters as well as herbicides in environmental waters including in-syringe silanization of the analytes [267,268]. Moreover, a preconcentration method for 99Tc from biological samples and hospital residues by in-syringe DLLME was developed, and used for automated sample preparation for analyte quantification by offline scintillation counting [269]. Moreover, online coupling of LIS-automated DLLME to HPLC was done for the determination of phenolic water contaminants by a rather tedious procedure requiring intermediate backextraction into an alkaline acceptor followed by its neutralization [270. Moreover, UV-filters in surface seawater and swimming pool water were analyzed after LIS-automated IL-based DLLME on online coupled HPLC [271. Of special interest are further the combination of LIS with robotic handling of organic phase solidification and drying for which a Peltier cooled 3D printed cold trap was employed. The system was applied to the

analysis of parabens in water samples and personal care products with analysis of the collected extract offline by HPLC [272. Finally, two applications were developed based on dispersive SPE using magnetic metal organic frameworks as well as magnetic carbon microparticles for the enrichment of malachite green by spectrophotometry and estrogens offline by GC-MS as model contaminants in water samples, respectively [273,274]. This concept was later further developed by our group with a publication explained in section 4.4 reporting on magnetization of HLB sorbents [244].

A research work from the Zhejijang University of Water Resources and Electrical Power (China) reports on LIS application for automated magnetic stirring assisted DLLME of arsenic species as As(V) chelates with molybdate into IL. The extract is analyzed by online connected ETAAS and speciation was done by offline oxidation of As(III) to As (V) before the automatic procedure [275].

Significant work has been done by the research group of Prof. Bulatov from the Saint Petersburg State University (Russia), in particular exploring green alternatives to classical extraction solvents. Sugaring out assisted HLLE was automated for the extraction of pesticides with posterior separation on online coupled HPLC [276]. Furthermore, in-syringe stirring assisted DLLME of sulfonamide antibiotics by a switchable solvent was studied. The syringe was used upside-down for easy removal of the aqueous phase before offline dilution of the extract with methanol and determination by HPLC [277]. Moreover, CPE based on the nonionic detergent Triton X-114 was automated for the enrichment of a condensation product of the analyte epinephrine with o-phenylenediamine. This was produced in the heated chamber of a flow-batch analyzer following the SWIA principle. The void of an automated syringe pump was connected to one lateral port of the SV and used for phase separation and the extract was submitted to fluorimetric quantification [278]. Finally, the use of DES in LIS extractions was studied in three experimental works. Sudan dyes were extracted by stirring assisted DLLME and extracts were analyzed offline by isocratic HPLC [279]. Spectrophotometric determination of sulfonamide antibiotics in urine samples as a sum parameter was done by extraction with a vanillin-based DES that formed colored Schiff's bases [280]. The last work explored the use of DES as a water soluble and alternative disperser of octanol in stirring assisted DLLME. The extracted analyte was the complex formed upon the reaction of diphenylcarbazide with chromate that was determined by spectrophotometry [281]. Recently, LIS automated stirring assisted DLLME was used for the first time for SUPRAS-based extraction, applied to the determination of 13 polycyclic aromatic hydrocarbons in tea infusion and determination by liquid chromatography with fluorescence detection [282].

The research group of Prof. Anthemidis from the Aristotle University Thessaloniki (Greece) has developed a variety of new approaches and applications to LIS that were directed mostly to the enrichment of metallic contaminants and new extraction modes for LIS related to gas-liquid and liquid-gas extractions. The first application of an automated syringe pump with a multiposition head valve in LIS was from this group and was used for the automation of cold vapor generation inside the syringe and gas-liquid separation in an added chamber that enabled argon to carry elementary volatile mercury towards the coupled AAS instrument yielding a compact, as stated, "*all-in-one-platform*" [283]. In another work for mercury determination, formed elementary mercury vapors were collected in a single drop containing palladium nanoparticle undergoing amalgam formation. The drop was created inside the syringe and after the extraction transferred to online coupled ETAAS for detection [284]. The work showed high similarities with a work from our group that has been presented at a flow conference the

year and published at the same time [285] before but admittedly improved our approach by the usage of a second syringe for the provision of the nanoparticle suspension for drop creation. This concept was then adopted by us to perform HS-SDME of ammonia combined with on-drop sensing [258].

Another automated concept with LIS was extraction in a floating drop of diisobutylketone, thus omitting any solvent support. The work was applied to the enrichment of silver as dithiocarbamate complex and determination by online coupled ETAAS [286]. Moreover, the researchers used for the first time syringe heating as well as a double LIF system for the determination of ammonium. Alkaline generated ammonia was volatilized in one LIS system, aided by heating and negative pressure application. Then, the gas was transferred into a second syringe where positive pressure and heating were applied to favor analyte dissolution in a reagent solution that contained orthophthaldialdehyde for subsequent fluorimetric analyte quantification [287]. While the system and operation were complex, the versatility of the LIS approach for gas-phase extractions was demonstrated. In related work, the formation of ammonia was done after in-syringe mixing of all solutions in a connected heated separation chamber and the analyte was purged by a flow of argon gas into a trapping solution with subsequent fluorogenic reaction [288].

Recently, automation of emulsion breaking for the determination of Cu in edible oil samples was reported. The procedure is based on the formation of an acid-sample emulsion for the increase of surface area and extraction speed followed by emulsion breaking through heating. The emulsion formation inside the syringe was aided by magnetic stirring and surfactant. A heated chamber on top of the multiposition head valve of the syringe pump served for emulsion breaking. Subsequently, the aqueous phase with the extracted analytes was submitted to analyte quantification by FAAS [289]. Finally, a double LIS system was used to automate a double sample preparation consisting of in-syringe complexation of Cd^{2+}, Cu^{2+}, and Pb^{2+} by diethyldithiophosphate complexes with extraction onto sol-gel modified polymer fiber tissue discs that were inserted into the syringe. This step could be repeated as needed to reach the required preconcentration factor. The second step was the elution of the retained complexes into solvent in the second syringe. In the third step, oxidate backextraction as previously reported by our group [261] was done to obtain an aqueous extract that could be quantified by online coupled ICP-AES [290].

In only a few applications, the usefulness of LIS for the automation of chromogenic assays has been demonstrated, the most originating from the research group of Prof. Ma at the Xiamen University (China). The developed applications were with a LIS system but proposed as "Integrated Syringe-Pump-Based Environmental-Water Analyzer" (iSEA). It comprised an automatic syringe pump with multiposition head valve and solution mixing was achieved by external mixing coils or chambers. It was applied in field campaigns to the analysis of nutrients phosphate [291292], silicate [291,293] nitrite and nitrate [294], and ammonia [295,296] in fresh, estuarine, and seawaters where advantage was taken from system compactness for field work and homogeneous mixing to enable analyte quantification by spectrophotometry without Schlieren effect and to increase the path length of the detection flow cell. For the determination of chromate, even a 2.5 m long liquid waveguide capillary cell (LWCC)12 was

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[12] In a LWCC, the sample passes a tube of Teflon AF, a polymer with lower refraction index than water. The sample therefore acts as an optical fiber, which makes extremely long pathlength possible. However, detection is

possible to use without drawbacks to the homogeneity of the solution [297] Two further examples were from the group of Prof. Kościelniak at the Jagiellonian University in Krakow (Poland), in-syringe mixing was applied for flexible preparation of sample solutions for iron determination and the study of chemometric tools for reduction of matrix effects, respectively [298,299]. Finally, the group of Prof. Rocha at the University of São Paulo (Brazil) reported on the automated determination of total ester content in biodiesel for which the sample was diluted in-syringe with ethanol to enable the chromogenic assay [300].

Two further publications must be mentioned that report on the development of another mixing concept for LIS. It is based on dividing the syringe piston into two parts that can move interlocked to dispense or aspirate solutions or unlocked so that the inner part can be moved independently up-and-down to mix the solutions contained inside the syringe or disperse immiscible phases for DLLME [301,302]. The purpose of the device is to be used on versatile autosamplers, which are able to do the half turn needed to link both piston parts. So far, the approach has been successfully applied to liquid-liquid extraction of caffeine from tea, and various model analytes from waters including polychlorinated biphenyls and aromatic hydrocarbons from water samples with analysis by GC [302]. In the second work, dioxine from water was extracted as a model analyte [301]. Considering that reported extraction recoveries were in some cases not more than 70% requiring 4 min of extraction, in-syringe appears to be the more effective mixing approach with DLLME seldomly taking more than 2 min. Moreover, a carry-over effect of 4% was observed.

Concerning my work, a photographic documentation is here added to show the operation of LIS for different methodologies to the newcomer of this topic. Figure 15 shows examples for LIS-automated extractions other than DLLME since such examples were already shown in section 3.4.2.2. In photo A, the formation of a drop of hydrophobic DES for DI-SDME of fluroquinolone antibiotics from water samples is shown. Also visible is the formed bubble inside the drop for stabilization and drop inflation. Photo B second photo shows the cloudy state due to droplet formation in salt-assisted HLLE using ACN as extraction solvent and gradual phase separation. Both works are further discussed in section 4.3. Photo C shows in-syringe HS-SDME of ethanol to reduce chromate contained in the drop reagent that was assisted by low pressure. Photos D shows drop formation in HS-SDME using the syringe in an upside-down orientation to create the drop via a flow channel featured in the syringe piston (E). A fiber optic adapter for on-drop spectrophotometric measurement NH_3 is shown in photo D, too. Both works are discussed in section 0.

Figure 16 shows examples of handling particulate matter inside the syringe void. Photo A shows the in-syringe suspension of hydrophilic-lipophilic balanced sorbent particles, modified with magnetite particles to provide magnetic susceptibility. The so functionalized sorbents were used for dispersive SPE using a specially made stir bar from NdFeB magnets sealed into a 3D-printed polypropylene casket to yield efficient sorbent recovery. The work was applied to the extractions of 5 water contaminants, the

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impossible in case that the light path is broken by air bubble, and seriously affected by changing refraction index or smallest quantity of suspended particles. Other issues such as absorption of organic material to the polymer are prevented by using a fused silica capillary inside the Teflon AF tube that acts as a hydrophilic cladding of the inner walls as well as by regular cleaning.

published work is discussed in section 4.4. The other photos originate from in-syringe deproteination of milk (C and D) and serum (E and F). One part of the respective sample was mixed in-syringe with two parts of ACN to denaturate the contained proteins followed by the aspiration of concentrated salt solution to induce phase separation. As can be seen, under optimized conditions, compact protein layers and clear organic supernatants containing the extracted analytes were obtained without the need for centrifugation. For serum samples, the system was downscaled by factor 5. Both works are discussed in detail in section 4.3.
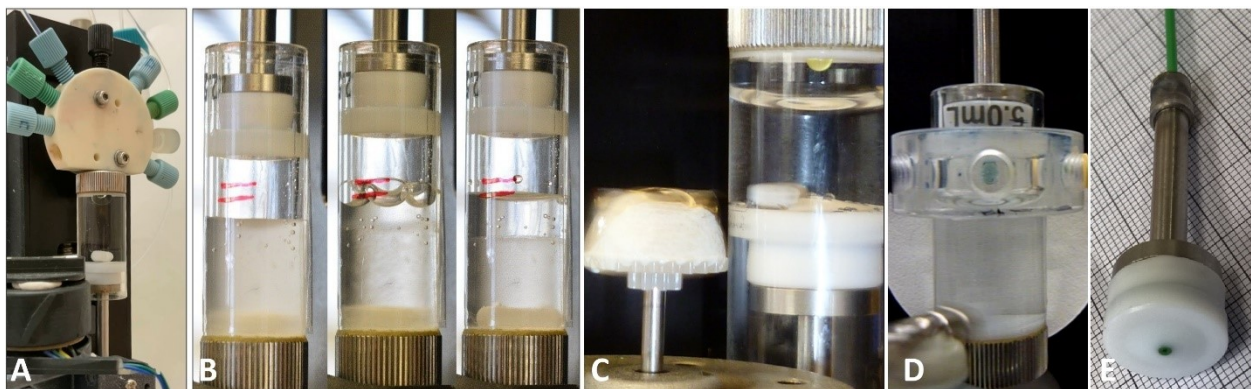
Figure 15: Photos documenting liquid phase microextractions other than DLLME. A: DI-SDME applying bubble-in-drop for stabilization of DES drop [303], B: HLLE before, during, and after phase separation [169], C: HS-SDME with drop creation (chromate) in the syringe inlet for determination of ethanol [285] D: HS-SDME for DI-SDME with drop creation (indicator) via piston channel for determination of NH_4^+ as NH_3 [258] E: Piston with drilled-through channel for drop creation in DI-SDME, gas transfer in HSE, sample passage for continuous DLLME [190].

To give a critical estimation of the value of the LIS system for potential use in commercial laboratories, Figure 17 finally shows three examples of commercial developments that show proximity to the concepts applied in the automation technique LIS. The company Cetoni GmbH (Korbußen, Germany) has in their portfolio, to the best of my knowledge not earlier than 2014, syringe pump modules that are capable of slow in-syringe magnetic stirring. As in LIS, stirring is induced by a driver ring holding NdFeB magnets. These pumps are used for the provision of suspension in the industry.
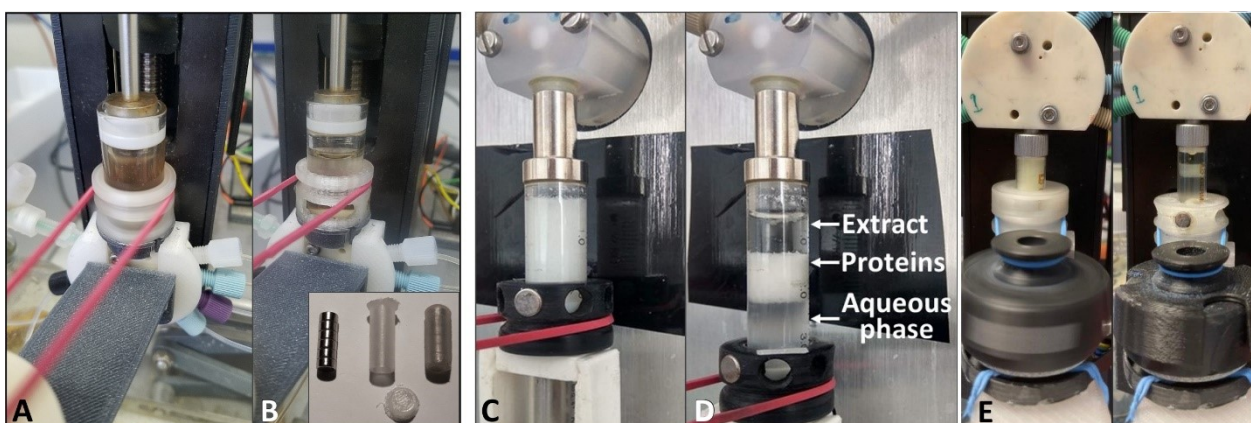
Figure 16: Photos documenting solid handling in LIS. Left: magnetized polymer beads as suspension for automated dispersive SPE during the extraction (A) and after sorbent capture on the magnetic stirring bar (B) specially made from NdFeB magnets (detail photo) [244]**. Middle: automated sample deproteination of milk based on salting-out assisted HLLE before (C) and after phase separation (D)** [149]**, Right: Automated sample deproteination by salting-out assisted to lyophilized serum before (E) and after phase separation (F)** [150]**.**

The second example is an autosampler system from the company Parker Hannifin Cooperation (NH, USA) with an upside-down turned syringe pump with available sized of up to 1 mL. The sampler is used for microtiter automation, handling one position at a time and mixing aspirated solutions in-syringe. The final example is a syringe pump with a multiposition head-valve with practically zero dead volume by the company Advanced Microfluidics (Ecublens, Switzerland), used in customized systems, e.g., to carry out analytical and bioanalytical applications including chromogenic reactions or bioassays such as cell staining. So far, no in-syringe extractions have been developed. A collaboration with this company initiated in 2021 will hopefully benefit both sides in terms of method and technical further developments of LIS-technique similars and concepts.
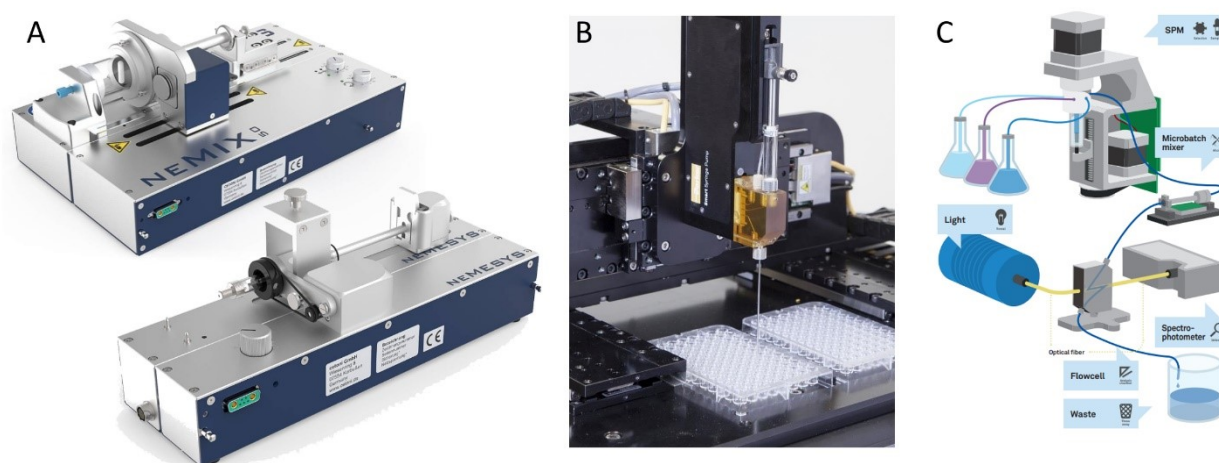
Figure 17: Three examples of company development applying similar concepts or showing significant overlap with the here presented LIS technique and operation procedures. A: Syringe pumps from the company Cetoni GmbH (Korbußen, Germany) used for the provision of suspensions adopted likewise in-syringe stirring, B: Combination of the syringe pump with a microtiter plate robotics from Parker Hannifin Cooperation (NH, USA) for in-syringe solution mixing, C: Syringe pump from company Advanced Microfluidics, here shown for chromogenic assays.

3.4.4. Evaluation of LIS and comparison to other flow techniques

This chapter has shown insides to the initial difficulties of using LIS and to the variety of automatable laboratory procedures. It is safe to say that it is a versatile automation tool with significant improvements in instrumentation and reliability. These do not originate only from academic work but to a significant agree are owned to market availability of specific instrumentation, most about all, 3D printing and automated syringe pumps featuring multiposition head valves. This is the same in other FTs: without the availability of linear operating syringe pumps, solenoid valves and pumps, or monolithic columns, SIA, multicommutated FTs, or SIC would not have been thinkable.

LIS has broken with an unwritten rule of FT automation, i.e., using a tubing manifold for solution mixing with a gradual change in analyte concentration to produce transient peak signals. In consequence, a "concentration gradient in space" cannot be produced using LIS while it is in fact possible to aspirate solutions slowly into the syringe to produce a "gradient in time" [116]. Moreover, in-syringe mixing of solutions is, by the very high stirring rates applicable, instantaneous, so reaction kinetics can be followed despite not using laminar flow via in-syringe detection. Admittingly though, tube-based FTs are surely better suited for this task.

In terms of sample throughput, LIS is limited in particular by the need to clean the syringe pump. This step can take about 1 min, which is often enough for the entire analysis performed by other FTs. Its use for automation of chromogenic assays will be advantageous if homogenous mixing is needed, e.g. for automated standard preparation, sample dilution, or when sample viscosity varies or is an issue by being different from the used standard solutions [304], if a procedure requires multiple reagents and reaction times, e.g., for automated derivatization [267,268], if a precipitate is formed [149,150], or if a detector is used that operates better when provided with a homogenous sample or relatively large volume such as spectrophotometry with long pathlength detection cells [291]. Where these conditions are not fulfilled, other FTs will enable faster analysis with lower solution consumption.

Other FTs will also perform better when solution heating or radiation is required, which are more efficiently done using polymer or steel capillaries and membrane separations are not feasible but possible to replace by heated aspiration tubes or head-space extractions and gas transfer [255,287]. In contrast, the LIS technique is ideally suited for mixing solutions of very different volumes and in particular for dispersive liquid phase extraction methodologies [116,262].

LIS shows characteristics that are very similar to those of flow-batch automation, with a few differences to point out. LIS enables positive and negative pressure application, e.g., to promote sample degassing or stripping of gaseous analytes into an acceptor solution. Moreover, stirring can be done with far greater efficiency and cleaning times are reduced since the volume to be cleaned is, by the piston movement, reduced to what is used for the performed extraction procedure. On the other hand, a standard or screen-printed electrodes can easily be used in flow-batch or integrated into a tubing manifold. In conclusion, the overlap of LIS capabilities to those of other FTs is only partial so it can be said it presents one completing puzzle piece for the automation of laboratory procedures added to the existing techniques.

Recently, there is a growing interest in greener methodologies including the ones used for sample preparation. Applications of FT and LIS as automation tools for such tasks fulfill the proposed 10 principles of green sample preparation to a high degree [305,306]:

1st Portability: FT are ideally suited for monitoring applications and allow application where the sample is generated whereas autosampler systems and robotics will need lasting setup and configuration

2nd Safer solvent and reagents: Modern FT increasingly explore alternative solvents for sample preparation, in particular deep eutectic solvent (DES), ionic liquids (IL), and water-soluble solvents.

3rd Reusable materials: Flow-automated SPE is often based on reusable material and consumables such as cartridges and vials and pipette tips are not required to run the flow analyzer

4th and 5th Minimal waste, sample, and material: Flow-automated methods generally require only a fraction of what is used in manual procedures because of downscaling. This is especially of interest for immiscible solvents or costly regents with a consequent reduction in waste. BI enables renewable SPE with only a few mg sorbent and miniaturization of DLLME in LIS requires about 50-200 µL solvent only.

6th High sample throughput: FT automation generally achieves higher sample throughput than the manual performance of analytical or preparative procedures but works sequentially, so that just-in-time preparation for a coupled separation technique is typically achieved.

7th Integration of steps and automation: It has been proven that FT in general and LIS specifically perform all steps of the sample preparation, with the possible exception of required sample filtration or decanting form sediments particles, automatically. Moreover, online coupling enables downscaling the automated procedure so that a significantly larger fraction of the extract is de facto used for the analysis compared to manual sample preparation.

8th Minimal consumption of energy: LIS is based on automatic syringe pumps that have power sources of 2 A, 24 V, which is significantly less wattage than required by laboratory centrifuges. Moreover, de facto operation time of the syringe pump is estimated to be below 70% of the method run time while the stirring motor consumes in the range of a tenth of this power.

9th Greenest possible detection: In my work, I used most often USB-powered spectrophotometers and sometimes LEDs as light sources. However, the tendency goes towards the use of HPLC to correspond to analytical tasks such as the analysis of organic contaminants. Due to analyte preconcentration, highly power-consuming instrumental techniques are not always required but principal compatibility with ICP-AES, GC, and LC-MS was studied.

10th Safety of operator: By design, the flow practitioner is exposed to harmful reagents, if used, only during solution preparation or handling waste containers, while the automated sample preparation is encapsulated in the tubing manifold or syringe void. Moreover, only a minimum of such chemicals is required due to procedural downscaling.

In conclusion, FT and LIS also play and important part in making laboratory chemistry greener.

3.4.5. Future trends in flow technique application and development

Two recent and coincidental trend articles have commented on the future of FT automation, one by the thesis author [41,307]. They both agree on the main premises summarized here together with recent impressions and feedback on the market situation for FT from representatives of the companies FIAlab Inc. and GlobalFIA Inc. A critical discussion that is strongly related to LIS is given in chapter 5.

- With the development of better instrumentation for advanced instrumental techniques and increasing interest in trace analysis of organic contaminants in more complex samples, FT are used more and more for niche applications. These are foremost the automation of chromogenic assays based on specific reagents for mostly inorganic analytes as well as total indices. They also are ideal tools for the monitoring and vigilance of technical, environmental, and biological processes with achieved advantages for field work of portability, robustness, simplicity, and related costs. In this sense, use in oceanography, analysis at-side rivers, wastewater effluents, or industrial plants, is a long distant runner in FT. Moreover, FT are unlevelled solutions to reliably carry out chemiluminescent, enzymatic, and catalytic assay as well as for kinetic studies such as dynamic leaching or membrane processes.

- FT are increasingly used for the automation of sample preparation coupled to advanced instrumental techniques and for studying and characterizing novel sorbent materials, separation-friendly extraction approaches, and using of greener solvents.

- FT are tools of inventive science with influence on microfluidics or sample preparation methodologies They are also wonderful platforms for teaching analytical thinking, novel chemistry, and procedural automation at universities, among others for being fast in setup, use, and optimization, by carrying out analytical assays in transparent conduits, and training of searching technical solution.

- Research money flows to life science, point-of-care, global change research, and new technologies including 3D printing, nanomaterials, and green chemistry approaches. In this sense, microfluidics sells better as related research is directed to bioassays, single cell analysis, or organ-simulating screening platforms. Flow practitioners will have to seek overlaps with these areas to receive better funding, e.g., downscaling and aiming for a "lab-in-a pocket", automation of sample preparation for biological materials, or reawakening flow-automated immunoassays.

- The advantages of combining the most suitable flow automation approaches for sample preparation, in my opinion Bead Injection on Lab-On-Valve and Lab-In-Syringe, and with autosampler systems could contribute to improvements in the versatility and applicability of flow techniques.

- The market demand for FT is stable with a strong focus still on environmental analysis. Some market shares have been lost to other analytical techniques or automation approaches during the last two decades mainly to discrete analyzers but also ion chromatography and ICP-atomic spectrometry. A shift is perceivable seeing combinations with autosamplers, instrumentation sold for flow chemistry (synthesis *in-flow*) [32], and hyphenation with batch concepts. Demands are mainly, with growing shares, from academia, private agricultural and environmental laboratories, governmental institutions including wastewater treatment plants, and, large industries, e.g., pharmaceutical companies.

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4. Commentary on included publications

4.1. Remarks on the origin, time, and classification of the publications

The publications presented in this habilitation thesis correspond to my main research line being the instrumental and methodological advances of the flow-batch technique *Lab-in-Syringe,* its application to environmental, food, and pharmaceutical analysis, and the automation of sample preparation approaches. In consequence, only a part of my publication record is included in this thesis. On the other hand, being a co-developer of the LIS technique, which has been used until this day, 11 years after its invention, in 9 research groups related to flow technique automation in 7 different countries, I consider these papers to be my most significant contributions to my area of research. The publications originate from research that I have carried out between 2012 and 2023 and correspond mostly to two working periods as postdoctoral researcher at the Mediterranean Institute for Advanced Studies, Esporles, Balearic Islands, Spain, and at the Charles University, Faculty of Pharmacy in Hradec Králové, Czech Republic, as well as to my current employment as an assistant professor at the latter institution.

Most experimental works of the first postdoc period listed in sections 4.2.1 and 4.2.2 were carried out in the Group of Analytical Chemistry, Automation, and Environment at the Department of Chemistry of the University of the Balearic Islands, Spain, where I had also done my dissertation studies. Later works were carried out mostly at the Department of Analytical Chemistry of the Faculty of Pharmacy in Hradec Králové, often in collaboration with foreign researchers, and during research stays in the research groups and laboratories of Prof. Manuel Miró Lladó, Ph.D. (FI-Trace group) at the Department of Chemistry, University of the Balearic Islands, Spain, of Prof. Jose-Luis Todolí, Ph.D., at the Department of Analytical Chemistry, Nutrition, and Food Science, University of Alicante, Spain, and of Prof. Spas D. Kolev, Ph.D., at the School of Chemistry, University of Melbourne, Australia (Victoria).

Considering the purpose and focus of this habilitation thesis, the included works were divided into thematic areas according to the automated sample preparation procedure and application. Comments on the aims, instrumental development, and achieved implementation and application are listed in the following sections. The publications to each section are added, color separated, to the printed work.

4.2 Lab-In-Syringe for the automation of liquid-phase microextraction approaches
without analyte separation
4.2.1. Solvent-assisted DLLME with spectrophotometric and fluorimetric detection
4.2.2. Stirring-assisted DLLME with spectrophotometric and fluorimetric detection

4.2.3. Stirring-assisted DLLME coupled to ICP-AES

4.2.4. Headspace and Direct Immersion SDME

4.3. Lab-in-Syringe for the automation of liquid-phase microextractions
coupled to liquid and gas chromatography

4.4.Lab-in-Syringe for the automation of solid phase extraction approaches
coupled to liquid chromatography

4 5. Laboratory automation and the potential and contributions of Lab-In-Syringe to this field

Given that the commented publications also report on the used instrumental configurations which are not easily explained and that a significant part of the novelty of each work is related to methodological, operational, and instrumental developments, configuration of the syringe pump and stirring modes are summarized here in Figure 18.
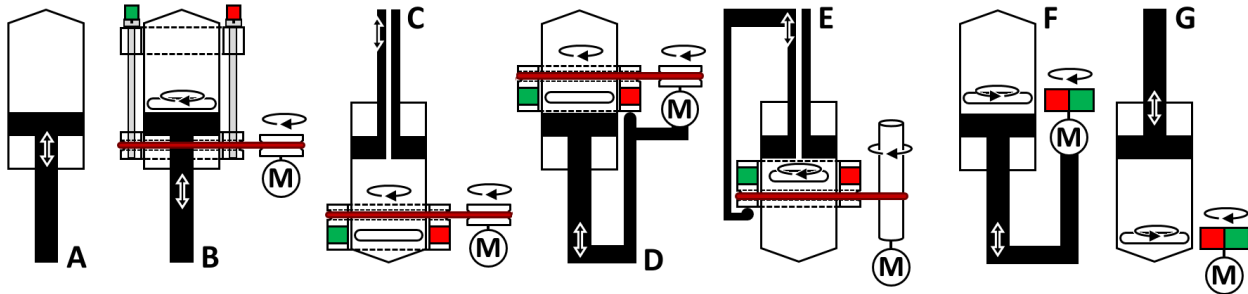
Figure 18: Stirring configurations used in experimental works related to the commented publications. A: no stirring, B: stir driver to create a rotating magnetic field along the entire barrel length, C: driver ring remaining at the bottom of the upside-down turned syringe pump, D: driver ring that moves up and down with the piston in the upright-positioned syringe, E: driver ring that moves up and down with the piston in the upside-down-positioned syringe pump, F & G: rotating magnetic field created by a motor close to the syringe with magnets on top with different syringe pump orientations (only slow rotation possible using short stir bars).

4.2. Lab-In-Syringe for the automation of liquid-phase microextraction approaches without analyte separation

This chapter comments on those applications where the LIS technique was coupled to optical detection methodologies including spectrophotometry, fluorimetry, and ICP-AES. The chapter also contains the earliest works describing and using LIS as a tool for chromogenic assays and automation of liquid phase microextraction approaches except for the very first report published by Dr. Fernando Maya, who proposed this methodology in 2012 [176][13]. In consequence, this chapter also describes many of the technical developments of the LIS technique which have enabled the high operational versatility of LIS despite using simple and compact instrumentation.

I consider myself lucky that my colleague and friend Fernando, from the Group of Analytical Chemistry, Automation, and Environment at the University of the Balearic Islands in Spain, was working on the first LIS experiments right next to me so I got induced and inspired by his shared enthusiasm of the outcomes of the first LIS work. In the following years, we collaborated on experimental works[14] and a later review article. This continued even when Fernando left for a postdoctoral research stay at the Lawrence Berkeley National Laboratory (USA) in the group of Prof. F. Švec while I was continuing the development of the LIS technique in Spain and when afterwards I had the possibility to continue this research at the Faculty of Pharmacy in Hradec Králové of the Charles University (Czech Republic). The LIS concept has enthralled me ever since.

4.2.1. Solvent-assisted DLLME with spectrophotometric and fluorimetric detection

The first works with LIS listed in this section reported on the automation of DLLME procedures based on floating extraction solvents and using analyte-selective reactions that enabled spectrophotometric or fluorimetric determination of the reaction product in the organic phase. The word "fluorimetric" was used purposely as the detector was laboratory-made and spectrum acquisition was not feasible.

In these early works, no stir bar was used inside the void of the automatic syringe pump (Figure 18 A). To achieve droplet formation, the extraction solvent had to be mixed with a dispersion solvent in volumetric ratios of 1:5 to 1:10 just, less than used in offline DLLME procedures. While manual DLLME protocols are based on the rapid injection of a small volume of the solvent mixture into the sample, in LIS-automated DLLME, the solvent mixture was first brought into the syringe followed by the aspiration of the sample at high flow rates (15-30 mL/min). In both cases, phase mixing occurs due to high turbulence, upon which instantaneous dissolution of the dispersion solvent in the aqueous sample leads to the formation of extraction solvent droplets. Floatation and spontaneous coalescence of the droplets in the conic syringe inlet allowed to collect the solvent efficiently and propel it to the detector.

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[13] with the stirring part added by me in 2013 [255]

[14] In this time, the techniques denomination Lab-In-Syringe was excogitated. I opted for *In-Syringe Analysis*, which would not have coincided with the equally termed preparative and analytical approach used for manual procedures based on disposable plastic syringes but was outvoted.

In manual procedures, the extraction time can be increased by shaking the extraction container, whereas in LIS, the available time for analyte extraction without stirring was limited to the time required for sample aspiration and droplet floatation. Given the enormous increase in surface area due to solvent dispersion, the improvement in sensitivity of the first work with the LIS technique justified further investigation. However, extraction efficiencies were below the theoretically achievable maximum, e.g., 8-12.5 instead of 40 in the first work by Maya et al. (2012) [176]. Moreover, homogeneous mixing of the sample with possible extraction buffers or reagents was not feasible in this first instrumental setup of LIS, yet also not necessary as no pH adjustment was needed for the chosen model analyte benzo[a]pyrene. Instead of a selective reaction, method selectivity was achieved by in-system low pressure chromatography after confluent mixing of the extract with 9:1 ACN:water.

The first article included in this thesis is also the second publication using the LIS automation technique. It is entitled "**Automatic determination of copper by in-syringe dispersive liquid-liquid microextraction of its bathocuproine-complex using long path-length spectrophotometric detection**" and was published in 2012 in the journal Talanta [**256**]. Bathocuproine was used as a selective reagent for Cu^+ forming orange-colored complexes that can be readily extracted into an organic solvent. For this work, it was necessary to mix the sample with hydroxylamine and acetate buffer to reduce Cu^{2+} to Cu^+ before DLLME. This was accomplished by the aspiration of the aqueous solutions into the syringe followed by their expulsion into a 5 mL pipette tip that was connected to one lateral port of the SV of the analyzer system as an atmospherically open chamber. After the aspiration of the solvent mixture containing xylene as extractant, ACN as dispersion solvent, and bathocuproine and the sample mixture for DLLME, the yielded extract was diluted 1:2 by confluence with ACN, provided by a second syringe of the used multisyringe pump module, and loaded into an injection loop. From here it was passed through a long waveguide capillary detection cell (LWCC) for spectrophotometric detection with high sensitivity. A preconcentration factor of 30 was achieved by the DLLME step with an extraction efficiency of > 90 % and a limit of detection (LOD) of 5 nmol/L. The method including syringe and chamber cleaning required only 220 s. The average repeatability of 7 % relative standard deviation (RSD) and an average recovery of 101% for spiked waters proved its efficiency and applicability.

In a second work published in 2012 in Microchimica Acta, entitled "**Determination of ppb-level phenol index using in-syringe dispersive liquid-liquid micro-extraction and liquid waveguide capillary cell spectrophotometry**" [**308**], the same analyzer setup was used for the automation of the sum parameter of "total phenols", being a standard analytical parameter in water analysis. It is based on the chromogenic reaction of phenols with 4-aminoantipyrine and oxidation with hexacyanoferrate. For homogeneous mixing, double solution transfer between the syringe and the mixing chamber was required. The entire procedure, including color reaction, mixing, DLLME, and extract quantification, required only 200 s, including 30 s for the chromogenic reaction and 30 s for phase separation achieving an LOD of 0.9 ppb. Higher sensitivity could have been enabled by prolonging the reaction time. However, this option was rejected in favor of a higher sample throughput. Other figures of merit include a preconcentration factor of 20 and average RSD of 3.1%, and an average analyte recovery of 101%.

In both former works, the possibility for derivatization of analytes prior to DLLME and measurement was successfully demonstrated. On the downside, two additional syringes were needed for confluent addition of ACN to the extract for homogeneous dilution and for completely filling a 100 cm long LWCC.

In the following, this issue was omitted by measuring the undiluted extract directly inside the syringe or in a self-made detection cell mounted directly onto the syringe outlet, respectively.

The work "**Lab in a syringe - Fully automated dispersive liquid-liquid microextraction with integrated spectrophotometric detection**", published in the journal Analytical and Bioanalytical Chemistry in 2012 [257], reports on performing spectrophotometric determination of the analytes in the extract inside the glass syringe. For this, an adapter ring was fabricated from plastics that enables alignment of two optical fibers at the top of the syringe barrel, one leading to a halogen light source, the second towards a USB-powered fiber DAD spectrophotometer. In this way, the syringe served not only as a mixing and extraction syringe but also as a detection cell. The inclusion of all parts required for the analyzer system inspired the name of "Lab-In-Syringe" that was used hereafter for this technique. To demonstrate the feasibility of the approach, octanol was used as a floating solvent for DLLME of rhodamine B as a coloring dye from waters and soft drink samples that was measured during droplet floatation and coalescence so that a homogeneous organic phase accumulated in the upper part of the syringe. It was found that 120 s were required for signal stabilization. A faster time would be achievable for a solvent with lower density, viscosity, and surface tension; however, a sample throughout of 51 per hour was possible. Moreover, high reproducibility with 3.2 % RSD and a preconcentration factor of 23 were achieved. Most noteworthy was the significant reduction in system size and operation complexity, in other words, a compact and simpler analyzer system was developed.

In the article "**Fully-automated fluorimetric determination of aluminum in seawater by in-syringe dispersive liquid-liquid microextraction using lumogallion**", published 2012 in the journal Analytical Chemistry [309], a LIS-automated DLLME procedure for aluminum quantified as fluorescent complex with lumogallion is described. A detection flow cell was fabricated from a bended glass tube serving as flow channel, a green light emitting diode (LED) as excitation source, a photomultiplier tube as light sensitive detector, a dark plastic casing, a bandpass interference filter of 500 nm ± 10 nm and a long-pass filter with 580 nm cutoff consistent with the optimal excitation and emission wavelengths, respectively. To promote the reaction speed and yield, a laboratory made heater was integrated into the short HC that connected the SV and syringe, using a halogen light bulb as a heat source. In this way, the solutions were warmed during solution passage both at aspiration and at flow reversal to the mixing chamber, and signals for a 0.5 μmol/L standard increased by factor 4. N-hexanol was used as extraction solvent that showed faster droplet coalescence and improved extraction capacity towards n-octanol as anticipated. Fluoride interference was successfully masked by beryllium cations to the buffer solution.

The entire method including syringe cleaning required only 262 s, 120 μL n-hexanol, and 830 μL of ethanol as a dispersion solvent. An LOD of 8 nmol/L and repeatability of < 1.5% RSD at 200 nmol/L level were achieved. Above all, the method was usable for genuine concentrations of the analyte in seawater and recovery values in spiking experiments ranged from 97 to 113 %.

In all works of this section, the used dispersion solvent occupied a significant portion of the syringe volume (always 5 mL) and thus reduced the usable volume of the sample. It also increased the solubility of the reaction product in the sample, which consequently leads to a lower extraction efficiency. Finally, it also increased the solubility of the extraction solvent in the aqueous phase so that more extraction solvent was needed. This issue was solved by replacing the dispersion solvent by mechanical disruption of the extractant as applied in all works contained in the sections 4.2.2 and 4.2.3.

4.2.2. Stirring-assisted DLLME with spectrophotometric and fluorimetric detection

From all further development of the LIS technique, the introduction of a magnetic stir bar into the syringe void is to be highlighted. It enables efficient and nearly instantaneous solution mixing widely independent of their number, volumetric ratio, viscosity, or gas content. Moreover, it increases the efficiency of syringe cleaning, a procedural step that is imperative after each analysis. This is due to the dead volume of the syringe void and to remove solvent droplets accumulating on the piston head (polyethylene or PTFE) with isopropanol or ACN. As a consequence, the mixing chamber on the SV became needless. This was a significant advantage because it always had to be cleaned together with the void volume by repeated and complete filling with an appropriate solution and posterior re-aspiration and discharge. In contrast, the cleaning solution had to fill the syringe only to a fifth of its nominal volume for efficient cleaning given the wiping action of the syringe piston and high turbulence of the stirring action.

On the other hand, the dead volume of syringe increased significantly with the use of the stir bar inside. This foremost and inherent downside if the LIS technique was partly overcome by i) using the sample for the last syringe cleaning so that at least the usable volume of sample is not reduced and ii) using the syringe upside-down so that an air cushion remains inside the syringe that allows the push-out of nearly all liquid from the syringe at the emptying step. The works included in this chapter are the first ones to describe these instrumental developments related to this concept and the resulting pros and cons on six examples in which *in-syringe* stirring-assisted DLLME is carried out for the determination of two inorganic analytes, for automation of two standard methods for total indices, and for three phenolic conformers.

The article "**In-syringe-stirring: A novel approach for magnetic stirring-assisted dispersive liquid-liquid microextraction**" published in 2013 in the journal Analytica Chimica Acta reports, to the best of my knowledge, for the first time the use of a magnetic stir bar inside the void of an automatic syringe pump [255]. In the first works, 5 mL syringes of 6 cm stroke length were used. A driver element was developed that generated a rotating magnetic field around the syringe over the entire stoke length as shown in Figure 18 B and described in the chapters 3.4.2 of this thesis. It consisted of two iron rods that were aligned with the syringe barrel in-between using two plastic rings as spacers that fit smoothly onto the syringe barrel. They were oppositely magnetized by NdFeB magnets. This driver was turned via a rubber ring by a relay-controlled motor and activated by software instruction. It was found that even at rotation speeds of 2000 rpm, the stir bar remained aligned with the driver independently from the piston position. The homogeneity of the syringe content was evaluated for stirring times of 1, 3, 7, and 12 s using 3 mL water and 1 mL aqueous dye solution as well as dye solution with 20%(w/v) glycerol added to increase its viscosity. Full homogenization was achieved after 7 s for both solutions. Much faster homogenization was achieved when stirring was started already during solution aspiration. Nearly instantaneous mixing was possible when air was aspirated into the syringe that allowed vortex formation. Hereafter, the system was used to improve the prior described method for Al^{3+} [308], using the same chemical conditions. Removing the external mixing chamber enabled a 20% faster procedure and omitting the dispersion solvent yielded 25% lower LOD values and 8% higher sensitivity. Repeatability and analyte recovery were comparable with the previous approach while 25% more hexanol was required. Reaction and extraction times were studied by *design of experiment* comparing

also two reaction temperatures (25°C and 45°C). Heating of the extraction solvent was found advantageous in terms of method sensitivity and required shorter extraction time, probably due to the formation of smaller droplets and ongoing complexation reaction. Two parameters were critical for efficient dispersion: air aspiration into the syringe to create an open surface inside for vortex formation that could draw the extraction solvent downwards, and a stirring speed fast enough to create a vortex deep enough to force contact of the extraction solvent with the rotating stir bar.

It might be worth mentioning that the first oral presentation at the International Conference on Flow Analysis XII in 2012 in Thessaloniki, Greece, was strongly criticized by the co-inventor of FIA and SIA, Prof. Růžička, Ph.D. for the issue of additional dead volume inside the syringe. However, the use of *in-syringe* stirring also by other researchers has proven the usefulness of the approach. For instance, the linear range can be enlarged at will simply by dilution of the sample with water before the addition of the other reagents, including the extraction solvent, as demonstrated in the following work.

In the article "**In-syringe magnetic-stirring assisted liquid-liquid microextraction for the spectro-photometric determination of Cr(VI) in waters**" published in 2014 in the journal Analytical and Bioanalytical Chemistry [304], reports on the LIS-automation of the diphenylcarbazide assay for the colorimetric determination of chromate that oxidizes the reagent to diphenylcarbazone in strong acidic medium forming a purple complex with chromium. Acetate was proposed as the ion-pair reagent for the cationic complex to achieve stirring-assisted DLLME into n-hexanol. Multivariante optimization of the reaction time, extraction time, and quantities of reagent, the acid HNO_3 chosen, and buffer was done. The versatility of LIS was proven by performing a step-by-step procedure in the closed system consisting of mixing the sample with acid for the color reaction, followed by buffer addition for pH adjustment, followed by the aspiration of the extraction solvent for DLLME, and finally droplet collection and propelling the organic phase to a 1 cm path length flow cell for spectrophotometric determination of the extracted complex. Moreover, in-system preparation of standard solutions was demonstrated, which was done by the mixing of appropriate volumes of a chromate stock solution and water at the beginning of the procedure. Comparing calibrations with in-system and manually prepared standards did not result in a significant difference. A study of interferences was carried out and EDTA was successfully used to suppress interferences from Fe^{3+} and Cu^{2+}. A 28-fold pre-concentration of the analyte and an LOD of 0.27 µg/L were achieved. This is five times lower than achieved with a reference method used for comparison of sample analysis. This way, the accuracy of the LIS-method was proven. The procedural time including all cleaning was 4.5 min only.

The following two articles report on the new approach of using the syringe pump in the LIS system upside-down so that the stir bar remains in the same position at the syringe outlet. In upright orientation, the stir bar inhibited complete emptying of the syringe for its liquid content. In upside-down orientation, air accumulated inside the syringe, which was used to great advantage to propel all liquid from the syringe void at emptying. The new configuration also allowed using extracts of higher density than that of water. As downside, the content of the syringe became compressible, and all liquid movements were delayed since the pressure exerted by the piston first acts on the air cushion inside the syringe and only secondary on the liquid. However, a simpler stir bar driver was possible to use that consisted of a plastic ring featuring a groove for the rubber ring and two opposed NdFeB magnets (Figure 18C). The stronger attraction with the stir bar enabled even higher rotation speeds.

The article "**In-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction for automation and downscaling of methylene blue active substances assay**", published in the journal Talanta in 2014 [260], reports on the automation of the standard procedure for the total index *methylene blue active substances* (MBAS) as an analytical parameter for waste and effluent waters [260]. MBAS are commonly equated with the total sum of anionic detergents which form hydrophobic complexes with the cationic dye methylene blue. These can be extracted into chloroform, carried out at acidic pH to suppress the interference from organic carboxylates. After cleaning the syringe, the procedure consisted of mixing the sample with all aqueous solutions for the formation of the ion-pair followed by the aspiration of chloroform for subsequent DLLME, thereafter droplet sedimentation at the downwards oriented syringe inlet, and propelling the extract through a spectrophotometric flow cell. After two-level screening, four of six variables were chosen for optimization by *design of experiment* being the stirring time corresponding to the extraction time, and solution volumes of methylene blue, sulfuric acid, and NaH_2PO_4. Two supply ports of the syringe pump were used to relay control a change in stirring speed with 2000 rpm used for solvent dispersion and 1000 rpm to assist droplet coalescence and for extract washing with water to avoid droplet formation. This was needed by the low viscosity of chloroform and a significant difference in density towards that one of water. A study of interferents including humic acid and cationic surfactants proved selectivity similar or better than the reference method even omitting extract washing and the determination of the sample using the reference and LIS automatic method did not reveal significant differences. Compared to reference method that required 10 mL chloroform (for 25 mL sample), a 50-fold reduction in solvent consumption was achieved. Moreover, the entire procedure was automated and required only 345 s with an LOD of 7 µg/L (standard substance sodium dodecyl benzene sulphonate) with an RSD of 3% at 100 µg/L level.

The article "**In-syringe magnetic stirring assisted dispersive liquid-liquid micro-extraction with solvent washing for fully automated determination of cationic surfactants**", published in Analytical Methods in 2014 [108], reports on a similar LIS-system and operation procedure but was applied to the sum of cationic surfactants in water based on the ion-pair formation with the anionic dye disulfine blue that was likewise extracted into chloroform. The absorbance of disulfine blue in the extract is measured against extracts using cetyltrimethylammonium bromide (CTAB) as a reference standard.

The initially observed difficulties of analyte loss on the walls of the volumetric flasks and bad wettability of the detection cell for the extract were solved by silanization of the glass surfaces. Furthermore, the addition of 5%(v/v) hexanol to the chloroform improved the signal reproducibility. The simple extraction procedure analogous to the previous work required only 220 µL chloroform and was completed in 240 s. However, interferences were observed for the anionic surfactant sodium dodecylsulfonate (SDS), which competed with the dye in ion-pair formation with the analytes. Weaker interferences were found for different salts. Extract washing was investigated, for which we proposed $BaCl_2$ as efficient interference suppression of SDS. Washing was done twice before the extract was finally propelled towards the detector. For the optimization, signals obtained from standards with and without added interferent were compared. Double extract washing required additional 40 µL chloroform and a prolongation of the procedural time by 305 s. On the other hand, extract washing significantly reduced the interference level and allowed quantitative analyte recovery in spiking experiments for lixiviate and well waters. For the complete elimination the interference from SDS, use of a less hydrophilic anionic dye was discussed as improvement. The procedure required only a fraction of the amount needed by

the manual standard procedure and user exposure to chloroform was avoided. Furthermore, an LOD of 12 nmol/L CTAB and RSD values of 3.5% were obtained.

The article "**Automated continuous-flow in-syringe dispersive liquid-liquid microextraction of mono-nitrophenols from large sample volumes using a novel approach to multivariate spectral analysis**", published 2019 in Talanta [310], reports on the first continuous flow DLLME as proof-of-concept allowing rapid LLE from large sample volumes. The idea follows fluidized bed SPE where the sorbent is elevated by the sample flow but remains in its container by gravity or a filter medium.

Mono-nitrophenols in surface waters were chosen to be used as model analytes and matrix, respectively. The 5 mL syringe of the LIS system is used upside-down as shown in Figure 18E. A floating extraction solvent was constantly dispersed into droplets by magnetic stirring in the upper part of the syringe void. Using a piston with a drilled through channel, a second syringe pump was connected to a head valve port to force a sample flow through the syringe in downward direction at a speed just low enough so that the floating droplets would not escape. After the extraction, the aqueous phase was discharged, and the remains were eliminated by washing with water. Then, the nitrophenols were backextracted into a minimal volume of alkalinized aqueous acceptor phase, taking advantage of the void of the syringe acting as an extraction chamber of adaptable size. This was followed by passing the backextract through a 1 cm flow cell to register three absorbance spectra between 270 and 470 nm. Simultaneous quantification of the three conformers was done by multivariate spectral analysis. In addition to a linear combination of the spectra of the pure nitrophenols, a fourth-order polynomial function was added to compensate for the signal background. The background was due to light refraction and humic substances that were co- and backextracted into the aqueous acceptor.

Testing different solvents and mixtures, n-octanol was chosen as extraction solvent for showing low water solubility, highest extraction and back-extraction efficiency, and significantly lower density than water. The position of the stir bar, the stirring speed, and the sample flow rate through the syringe showed great effect. The stir bar was elevated with the driver ring so that it was at the boundary layer of the organic and aqueous phase. For the highest stirring rate of 1130 rpm, the extraction efficiency was virtually independent of the flow rate tested in the range of 10 to 40 µL/s. In contrast, for slower stirring, i.e., less effective solvent dispersion, sensitivity decreased with increasing flow rate. As the highest flow rate that did not lead to loss of solvent drops, 30 µL/s was chosen with an octanol volume of 400 µL. Surfactants were studied as possible interferences finding that CTAB lowered the sensitivity significantly, most-likely due to ion-pair formation with the analytes. This could be compensated by adding SDS as a competing anionic surfactant that itself did not show to affect the analyte recovery.

The influence of humic acid on the signal that has been shown to be a drawback of previous analytical methods for these analytes was reduced by factor six. Modeling the background spectral matrix in the applied multivariate spectrum analysis proved highly efficient and allowed a reliable determination of o-, m- and p-nitrophenol with LOD values of 0.14, 0.26, and 0.02 µmol/L and average recoveries of 94, 82% and 92%, respectively, but with lower values for samples with higher humin acid content. The RSD was generally < 5% and the achieved enrichment factors 19, 25, and 21, respectively, were high considering the values typical for back-extraction procedures. These results were obtained with 24 mL of sample (re-filling the second syringe 9-times) but at a cost of 20 min for the entire procedure. The graphical abstract was used for the Outside Front Cover of the corresponding volume 202 of Talanta.

4.2.3. Stirring assisted dispersive liquid-liquid microextraction coupled to inductively coupled plasma - atomic emission spectrometry

In this section, LIS automation of DLLME of metallic analytes coupled online to ICP-AES is described. Two approaches were developed to achieve extract compatibility with the used detection technique: back-extraction into an aqueous solution of an oxidant and heating the nebulizing chamber of the ICP-AES. Online coupling was done through a low-pressure injection valve loaded with the extract by the LIS system. The works were carried out in collaboration with Spanish research groups from the University of the Balearic Islands and the University of Alicante.

The article "**Online coupling of fully automatic in-syringe dispersive liquid-liquid microextraction with oxidative back-extraction to inductively coupled plasma spectrometry for sample clean-up in elemental analysis: A Proof of Concept**", published in the journal Talanta in 2017 [261], reports on DLLME of transition metals cadmium, copper, and lead as pyrrolidine dithiocarbamate complex in toluene and dispersive back-extraction into 1 mol/L nitric acid with added 20 mmol/L potassium iodate. The article reports for the first time on the oxidative decomposition of this complexant to promote metal back-extraction.

A stirring configuration as shown in Figure 18 G was used and the distance of the motor to the syringe and the strength of the bar magnet on top of the motor were adapted to allow a rotation speed of 1600 rpm which was sufficient to achieve *in-syringe* solvent dispersion. The final method consisted of aspiration of the sample, complexant, buffer, and extraction solvent under activated stirring. After an optimized time of 300 s for DLLME and phase separation that took not more than 15 s, the aqueous phase was discharged and the residues were removed by washing the extraction solvent with water. The acceptor phase was then aspirated and dispersive back-extraction was carried out for 100 s. After phase separation, 200 µL aqueous extract was loaded into the loop of an injection valve that was integrated into the feeding line of the ICP-AES instrument. Triggering of ICP-AES started transient signal registration and injection into an acidic carrier that transported the backextract to the ICP-AES.

The optimization parameters included the extraction and back-extraction time, the pH of the buffer and the type and volumes of extraction solvent and complexant. Methodological challenges were the adhesion of toluene droplets to the PTFE stir bar, which inhibited solvent recovery and incomplete back-extraction of the extracted analytes even using 2.85 mol/L HNO_3. The first issue was solved by aspiration of 30 µL plugs of isopropanol into the syringe during phase separation that allowed the solvent droplets to float and coalesce. Concerning back-extraction, replacement of analytes from their complex by Pd^{2+} was tested, but still yielded poor recovery for Cu^{2+}. It was known from the literature that dithiocarbamate can be oxidized by iodate, which deteriorates its complexing ability, yet oxidative back-extraction has not been used before. We observed an immediate and quantitative release of the analytes with an 8-fold surplus of KIO_3 compared to the complexant.

Peak heights were found to yield better linearity and repeatability compared to peak areas, probably due to tailing effects due to residual toluene in the backextracts with RSD values of typically 4% for three determinations. Average repeatabilities for Cd, Cu, and Pb were 2.9%, 3.5%, and 3.5% RSD with LOD values of 1.9, 1.4, and 5.6 µg/L, respectively. The method, which took 11 min in total, proved reliable in

the determination of the analyte in coastal seawater, surrogate digestive fluids, and soil leachate with recoveries of 90% to 118%, 68% to 104%, and 86% to 112%, respectively.

The article "**Fully automatic in-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction hyphenated to high-temperature torch integrated sample introduction system-inductively coupled plasma spectrometer with direct injection of the organic phase**", published in Analytical Chemistry in 2017 [311], reports on LIS-automated DLLME of cadmium, copper, lead, and silver as diethyldithiophosphate complexes into xylene and direct injection of the organic phase into ICP-AES, i.e., without the need for back-extraction into an aqueous solution. This was enabled by i) injecting only 12 µL of extract, transported by an air flow to the nebulizer, ii) using a micro-nebulizer to produce smaller droplets, and iii) using a spray chamber heated to 350°C that was a development of the group of Prof. Todolí from the University of Alicante, where this work was carried out.

A significant challenge during method optimization was the elimination of the carry-over effect by an optimized cleaning protocol. The reason for not having this problem in the previous work was that diethyldithiophosphate is able to form more stable complexes at lower pH values than the formerly used dithiocarbamate. The procedure started by cleaning the syringe, used in upright configuration as shown in Figure 18 F, with isopropanol to remove residues of the extraction solvent from the previous extraction, 15% (v/v) HNO_3 and two times with 2% (v/v) HNO_3 to remove metal traces, and finally with the sample acidified with 2% (v/v) HNO_3. Then these agents were aspirated: air to promote vortex formation, xylene, sample, an air plug to avoid contact between sample and chelating reagent in the HC, the complexant solution, and final air to empty the HC into the syringe. Stirring was started directly before aspiration of the solvent and remained activity for 120 s of DLLME. During 30 s for phase separation and droplet coalescence (stirring stopped), stuck xylene droplets were removed from the stir bar by activation of stirring eight times for less than 1s. The organic phase was then pushed through a transfer line to an injection valve as interface between the LIS and the ICP-AES instruments. The first part of the solvent served for the cleaning of the transfer line and injection loop from the previous injection. Then, the extract was used for three consecutive injections to ICP-AES before emptying the aqueous syringe content to waste. The order of aspiration and the univariate optimization of the volume of extraction solvent, the quantity of complexant, the nebulizer gas flow rate, the nebulizer chamber temperature and the extraction time were studied. Using a stirring rate of 800 rpm throughout, quantitative extraction was achieved within 100 s. The automated procedure achieved analyte preconcentration at trace levels in seawater, salt, and juices with enrichment factors of about 13 using 270 µL of organic solvent. The recoveries from the spiking experiments ranged from 92 to 103% for all analytes. Moreover, two certified reference materials (serum) were analyzed finding no significant differences at 95% level to the certified concentrations. LOD values for Ag, Cd, Cu, and Pb were 0.05, 0.04, 0.04, and 0.06 ppb, respectively. Extraction efficiencies close to 100% allowed using organic standards prepared with xylene and a certified reference material. Repeatabilities were below 5% RSD at 25 ppb level. Interday precisions in the range of 4% to 8% for calibration curve slopes of the four target analytes were obtained.

4.2.4. Headspace and direct immersion single drop microextraction

In this section, LIS applications are reviewed that describe the automation of headspace and directly immersed SDME using spectrophotometric analyte determinations and, in one case, the measurement of the analyte during extraction *on-drop*.

The article "**Automated in-syringe single-drop head-space micro-extraction applied to the determination of ethanol in wine samples**" is the first work to report a gas-liquid separation using the LIS technique. It was published in the journal Analytica Chimica Acta in 2014 [285]. It reports on a proof-on-concept for SDME of volatile oxidizable substances in the syringe headspace. The acceptor drop was chromate dissolved in 8 mol/L sulfuric acid, which was reduced from the yellow to greenish chromium. The method was successfully applied to the determination of ethanol in wines.

Permanent in-syringe stirring at 300 rpm was done using a configuration as shown in Figure 18 F. This demonstrated that computer control was not needed for the simple task of syringe content mixing. The efficiency of HS-SDME was enhanced by the inflation of the reagent drop by an air bubble, increasing the surface of the drop, an approach known as *bubble-in-drop* [312] and by decreasing the pressure inside the syringe to approximately 0.67 bar. For this purpose, the head valve was turned to a permanently closed position and the piston was lowered, corresponding to half of the volume of air forming the headspace inside the syringe. After returning to the initial position of the piston, the drop was propelled to the detector. Main challenges to solve were avoiding drop detachment in the moment of low-pressure formation and pressure equilibration before measurement to enable a controlled slow passage of the drop solution through the detector. Another issue was that for the aspiration of the drop reagent, it had to pass the HC as before the sample. Small segments of water were aspirated between both solutions to clean the HC from oxidizable remains of the sample. However, application to beer samples was unpractical due to the adsorption of lipophilic proteins on the inner tubing walls, which would have caused the reduction of the chromate of the drop reagent before exposing it to the headspace inside the syringe. The interference of organic volatile acids was suppressed by *in syringe* addition of alkaline phosphate buffer to the sample. Using a drop of only 20 µL reagent, high repeatability of typically < 4% RSD was achieved. The entire procedure required only 5 min and was very sensitive with an achieved LOD of 0.025%(v/v) ethanol and wine samples had actually to be diluted tenfold. The results fitted a nonlinear calibration, which corresponded a depleting behavior given the limited and steadily decreasing amount of chromate and H_2SO_4 in the drop. Upon application of the method for wine analysis, the ethanol contents were found to be in good agreement with a reference method by GC as well as with the declared ethanol contents of the tested samples. The work proved the feasibility of in-syringe HS-SDME using a simple and compact analyzer.

The article "**A novel approach to Lab-In-Syringe Head-Space Single Drop Microextraction and on-drop sensing of ammonia**", published in 2016 in Analytica Chimica Acta [**258**], reports on the further development of LIS-automated HS-SDME using ammonium in surface waters as a model analyte. The syringe pump was used upside down and the stirring system follows the scheme of Figure 18 G by applying slow stirring at 400 rpm. A novel instrumental element was developed for the LIS system being a piston with a drilled through-hole into which a short PEEK tube of 0.5 mm i.d. was glued. This enabled a second access to the syringe void, which was used to create a drop of 16 µL of an aqueous solution of bromothymol blue indicator inside the head space over the sample, which was alkalinized by in-syringe

addition of NaOH. The color change of the reagent drop due to the analyte was observed *in-syringe* over 30 s using a white LED used as a light source and an adapter for an optical fiber leading to a USB-powered spectrophotometer. The drop reagent was provided by a second syringe pump, and air inlet to the reagent reservoir was introduced via a wash-bottle to avoid its contamination with ambient ammonia. The difference of the absorbance maxima of the basic and acidic forms of the indicator was calculated for each data point, and the signal slope was used as the analytical signal.

In addition to indicator concentration and initial pH, the creation, positioning, and size of the drop were critical parameters of optimization and were carefully studied also with the aid of a fluorescence dye to observe the light path inside the drop. Reduction of pressure inside the syringe by 50% as explained for the previous publication increased method sensitivity by 55%. The study of interferences showed moderate cross-sensitivity for volatile amines. The precipitation of earth-alkaline and heavy-metal cations was suppressed by adding Na_2-EDTA to the alkalinization reagent. The method lasted only 208 s, RSD were typically < 8%, and an LOD of 1.8 µmol/L was achieved. The method was applicable to seawater and river waters and spiking resulted in recoveries between 96 % and 110 %. It should be noted that drop formation via the piston channel was a significant improvement with respect to method robustness compared to the earlier work that reported drop creation in the syringe inlet.

The article "**Direct-Immersion Single-Drop Microextraction and In-Drop Stirring Microextraction for the Determination of Nanomolar Concentrations of Lead Using Automated Lab-In-Syringe Technique**", published in Talanta in 2018 [313], reports on two DI-SDME methodologies for the determination of lead in waters based on the formation of a red complex with dithizone that was extracted into a drop of organic solvent. The aim was to automate DI-SDME as a sample preparation methodology that does not risk the formation of stable emulsion and achieves higher enrichment factors than those in DLLME. In Method A, the syringe pump was used upright with a stirring configuration as given in Figure 18 F and DI-SDME was in a drop of immersed solvent being a mixture of toluene and hexanol. The bubble-in-drop concept was applied for drop stabilization and to increase its surface area by partial inflation for extraction enhancement. In Method B, the syringe pump was used upside down with a stirring configuration as given in Figure 18 G. In drop stirring was proposed as a new approach to DI-SDME. For this, a commercial stirring cross was used instead of the usage stir bar which proved to stabilize the position of a drop of chloroform in the inlet of the syringe. By agitation at a low stirring rate, it also budged the drop surface and this way promoted analyte extraction yet without causing droplet dispersion. In both system configurations and methods, the drop solvent was aspirated last and pushed out of the syringe first after the extraction through a 1 cm flow cell made of PEEK of 5 µL inner volume, connected to a diode array spectrophotometer via optical fibers.

At first, both methodologies were optimized with aqueous standards regarding extraction time and pH, quantity of dithizone, drop volume, and, for Method A, solvent composition. With the use of a 60 µL drop and 80:20% toluene-hexanol, Method A achieved incomplete extraction even for times over 10 min. Another finding was that free dithizone did not dissolve in solvent above pH 8.5 while it dissolved in chloroform used in Method B to a significant degree. Therefore, it was mixed with chloroform from the start while in Method A it was used as separate reagent. The high absorbance of the free dithizone was further compensated by shifting the reference wavelength used in Method A from 700 to 660 nm where the free DTZ showed similar absorbance as at the detection wavelength.

Using also 60 µL chloroform, Method B achieved quantitative extraction in 3 min, which proved the high efficiency of the approach and superiority over Method A, which was consequently discontinued.

Fe^{3+}, Cd^{2+}, and Zn^{2+} interfered significantly due to absorbance at the analytical wavelength and Cu^{2+} due to absorbance at the reference wavelength. Therefore, use of recommended masking reagents hydroxylamine, tartrate, and cyanide was explored. The first one reduces interfering Cu^{2+} to non-interfering Cu^+ while reduction of Fe^{3+} to Fe^{2+} resulted in inacceptable absorbance at the reference wavelength and its use was omitted. Fe^{3+} interference was then masked with tartrate, while the remaining interferents were suppressed to an acceptable degree using cyanide.

A total time of analysis of 6 min for method A and 5 min for method B were achieved with LOD of 75 nmol L^{-1} and 23 nmol L^{-1} with repeatabilities of 5% and 4% RSD at 400 nmol L^{-1} level, respectively. In the analysis of spiked well and tap waters, recovery values were between 92 and 116% for method A and between 93% to 107 for method B. Due to the small volume of solvent, an enrichment factor of about 50 was achieved with method B and 18 in method A because of slower enrichment kinetics. The LIS system was compact and economical, and the proposed methods were, in principle, combinable with ETAAS to yield higher sensitivity and selectivity. The required interference masking is the main drawback of the dithizone assay, but its consumption compared to the manual extraction procedure was greatly reduced. Fe^{2+} was added to the waste container to safely complex the discarded cyanide.

4.3. Lab-In-Syringe for the automation of liquid phase microextractions coupled to liquid and gas chromatography

This chapter comments on publications that deal with LIS-automated sample preparation based on liquid-liquid and, in one case, liquid-gas microextractions coupled online to GC and HPLC including the combination with matrix precipitation. The issues to optimize were finding extraction approaches that were compatible with the coupled separation technique. In this sense, solventless headspace extraction, DES-based DI-SDME, and HLLE were explored, once in combination of LPME and SPE aiming for a QuEChERS-like automated protocol, and twice to accomplish simultaneous in-syringe matrix precipitation. Method development also included optimizing the used separation methods.

In all works, online coupling of the LIS system with the separation instrumentation was done and separations were performed in parallel with the LIS-automated preparation of the next sample to maximize the sample throughput. For coupling to HPLC, a high-pressure injection valve served as an interface and it was loaded via a transfer line from the LIS. This implied optimization of the transfer volume, feasible injection volume, and required extract dilution.

The article "**Lab-In-Syringe automation of stirring-assisted room-temperature headspace extraction coupled online to GC with flame ionization detection for determination of benzene, toluene, ethylbenzene, and xylenes in surface waters**", published in the Journal of Chromatography A in 2018 [190], reports on the enrichment of benzene, toluene, ethylbenzene, and xylenes (BTEX) as model volatile analytes for online coupled GC. The LIS system consisted of an upside-down turned automatic syringe pump with a 5 mL syringe connected to a SV as given in Figure 18 G. For hyphenation, a fused silica capillary was used to connect a channel drilled into the syringe piston to a metallic hollow needle that was inserted permanently into the injection port of the gas chromatograph. On-demand opening of this connection was done by a software-controlled pinch valve. Initial tests aiming for in-syringe HS-SDME revealed that reliable drop transfer, and, even more so, transfer line cleaning without GC overload were impractical. Thus, LIS-automated solvent-less head space extraction was developed. The method consisted of the aspiration of air and sample into the syringe and gas phase equilibration aided by slow stirring. Thereafter, the head space gas was rapidly compressed by lowering the piston just above the liquid surface with the head valve turned to a permanently closed position allowing the gas to pass the transfer line for pressure injection to GC.

Generally, head space extraction is done by heating the sample and using a small headspace-to-sample ratio followed by the transfer of a fraction of the head space gas with a heated injection syringe to the GC or by analyte trapping from a gas stream bubbling through the sample. In contrast, this article shows that higher sensitivity can be achieved by increasing the headspace-to-sample ratio, also deriving a mathematical model of the process. No sample or transfer capillary heating was required, while similar or better sensitivity was achieved compared to previous reports with the highest influence of the headspace-to-sample ratio and gas compression. The interferences found were compensated by using chlorobenzene as the internal standard, resulting in an average repeatability of the peak area of 2% RSD. Analyte recoveries for spiked samples were 99 ± 9 % with detection limits between 1 and 2 µg/L. Separation conditions in GC were optimized using the SIMPLEX method.

The article "**Lab-In-Syringe automation of deep eutectic solvent-based direct immersion single drop microextraction coupled online to high-performance liquid chromatography for the determination of fluoroquinolones**" published in Talanta in 2022 [303], reports for the first time on LIS-automation of SDME using a hydrophobic deep eutectic solvent (DES) as a green alternative. The LIS system was connected online to HLPC with fluorescence detection, finding that a fused silica capillary worked more reliably than a narrow PTFE tubing. A method for the extraction of five fluoroquinolones as permanently charged model analytes was developed to demonstrate the capacity of DES to also extract moderately hydrophilic analytes. The syringe pump was used in upright position and the DES drop formed in the inlet of the syringe. To avoid drop detachment, a simple stirring system as shown in Figure 18 F was used that allowed the stir bar to sit on-top of the piston head, i.e., the lowest possible position, and a stirring rate not higher than 400 rpm was applied. The bubble-in-drop approach as described in section 4.2.4 was applied for drop stabilization and increase of surface. Parameters for optimization included the choice of separation column and gradient condition, choice of DES components, and molar ratio in terms of extraction capacity and compatibility with analyte separation, extraction time, pH, and applied stirring speed, and volumes of DES and air. Drops smaller than 40 µL were not practical due to partial dissolution of the DES in the sample and finally 60 µL was chosen to ensure complete and reproducible filling of the injection loop of the HPLC instrument with the solvent.

Analyte recovery was affected by the sample matrix, e.g., by complex formation with divalent metal cations and due to analyte absorption to glass surfaces. In consequence, surrogate very hard water was used for matrix-matched calibration. A comparison with recently reported HPLC methods using optical detection showed similar or better performance in most parameters, in particular, enrichment factors of 35-44 and LOD values of 6 to 9 ng/L with RSD values typically below 3%. Extraction efficiencies of 53 to 66 % were obtained due to permanent charge of the analytes and accuracies of 85 to 117% were found for wastewater treatment plant effluent, lake, and river waters that were spiked at 0.5 µg/L. Analyte separation that was running in parallel to the LIS-automated DI-SDME. Nonetheless, the main disadvantage was a sample throughout of only 3 per hour.

The article "**Lab-In-Syringe for automated double-stage sample preparation by coupling salting out liquid-liquid extraction with online solid-phase extraction and liquid chromatographic separation for sulfonamide antibiotics from urine**", published in Talanta in 2021 [169], reports, to the best of our knowledge, for the first time on double-stage clean-up and analyte preconcentration aiming at complete automation of a QuEChERS-like preparation procedure. As model analytes and matrix, five sulfonamides, one used as the internal standard, in urine, were selected. The method consisted of in-syringe HLLE at acidic pH using ACN as a water-miscible extraction solvent and 80% saturated salt solution to induce phase separation. This step was followed by in-syringe dilution of the extract at a ratio of 1:5 with alkaline loading buffer and preconcentration of the analytes on an anionic exchanger. The corresponding SPE cartridge was integrated into the injection loop of an online connected HPLC. The droplets were formed by induction of phase separation so that a simple stirring system as shown in Figure 18 G was sufficient. Low pressure load of the obtained HLL-extract was enabled by using an anion exchange column prepared by filling an emptied monolithic guard column with Strata-X-A resin. The combination of the LIS technique with autosampler-based automation was successfully demonstrated by replacing the typical multiposition valve for solution selection with an AIM3000 autosampler to aspirate extraction solvent, salt solution, standards, sample, buffers, and cleaning solutions, including

water. A mixed solution of 2 mol/L $MgSO_4$ and 1 mol/L NaCl was ideal with the observation that both salts, also used in many QuEChERS protocols, acted in complementing ways: $MgSO_4$ showed to produce a solvent-depleted aqueous phase, while NaCl yielded a water-depleted organic phase. The volumes of solvent and salt solutions were optimized by design-of-experiment and both high extraction efficiency and high volumetric recovery of both phases were achieved using a combination of 400 µL sample : 650 µL ACN : 550 µL salt solution. Significantly cleaner chromatograms were found for urine samples, in particular with respect to early eluting compounds, for the proposed double cleanup consisting of HLLE with subsequent online anion exchange SPE compared to the application of online SPE only. Moreover, the SPE step enabled preconcentration factors of 7.2 to 8.0 yielding LOD values in the range of 5.0 to 7.5 µg/L. The average recovery of analytes from four spiked urine samples was 103 ± 7% with RSD values of typically < 5%.

The last two works in this section report on the LIS-automation of matrix precipitation. The idea for the article "**Automated centrifugation-less milk deproteinization and homogenous liquid-liquid extraction of sulfonamides for online liquid chromatography**", published in Analytica Chimica Acta in 2022 [149], originated from the finding that milk protein, denaturated by the addition of ACN, accumulates as a third compact and intermediate layer between the organic and aqueous phase after the addition of salt solution. Benefiting from the experience of the previous work, this article reports on the first and successful automation of sample deproteination in combination with salting out HLLE without the need for centrifugation. The method was applied to sulfonamides in milk samples and beautifully demonstrated the operation versatility of the LIS technique.

Milk and ACN were first mixed *in-syringe* in a phase ratio of 1:2 that is usual for protein denaturation. In addition, formic acid was used for pH adjustment. Afterwards, a concentrated salt solution was added to induce phase separation. A part of the organic layer was stored in the transfer line to the online connected HPLC instrument with spectrophotometric detection, the syringe was cleaned from sample remains, and the extract was re-aspirated to be mixed with water and injected to HPLC for analyte separation. A syringe and stirring configuration as given in Figure 18 D was used. It was found that the stirring speed, together with the volume of salt solution, had a significant impact on the compactness of the formed protein layer. Stirring at 1000 rpm proved ideal, while faster speed decreased the size of the protein flakes, which inhibited layer formation. On the other hand, homogeneous mixing of syringe content was hindered by the formation of the protein layer between phases at slower stirring. When searching for an efficient cleaning solvent that could also remove the remains of milk fat from the PTFE surfaces of syringe and stir bar, isopropanol with a 5% of 28%(v/v) NH_4OH solution was found effective. The previously successful mixture of $MgSO_4$ and NaCl was replaced by $(NH_4)_2SO_4$ that yielded a cleaner baseline of the chromatogram. The most efficient formation of a compact protein layer, with separation times under 1 min, was a 1:2:1 ratio of sample, ACN, and salt solution. LOD values from 25 to 36 ppb with RSD of less than 5.5%, and a sample throughput of 5.7 per hour were achieved. Average analyte recovery, evaluated with spiked milk samples, was 81.5 ± 9.2% and 86.3 ± 6.9% at concentration levels of 0.3 ppb and 3 ppb, respectively, with a tendency to lower recovery for milk samples with higher fat content. The use of an internal standard and the combination with online SPE as in the previous work are envisaged improvements for future experimental works. Fat remains were detected at 200 nm and MS detection would be desirable for higher selectivity. However, this did not interfere with the analyte detection nor did it cause permanent backpressure increase.

The article "**Lab-In-Syringe automated protein precipitation and salting-out homogenous liquid-liquid extraction coupled online to UHPLC-MS/MS for the determination of beta-blockers in serum**" was only recently accepted in Analytica Chimica Acta [150] and reports on a further exploration of *in-syringe* protein-precipitation approach. It was adapted to the extraction of betablockers betaxolol, metoprolol, pindolol, and propranolol from lyophilized human serum and using acebutolol as internal standard. Furthermore, the LIS technique was coupled online for the first time to LC-MS/MS (ESI in positive ion mode). In this way, the principal compatibility of LIS-automated sample preparation to this front-end instrumental techniques was demonstrated.

Extraction conditions were optimized with the LIS instrument connected to HPLC with spectrophotometric detection. A UHPLC system with triple quadrupole MS in mode of selected reaction monitoring was utilized for method validation and sample measurements. Given the availability and accessibility of the sample material, the LIS system was downscaled using for the first time a 1 mL syringe so that only 100 µL serum sample was required. This was less than in all previous reports used for method comparison. Compared to the prior work, higher stirring rates of 2500 rpm were required for complete mixing of the syringe content at the addition of salt solution. 80% saturated Na_2SO_4 solution with added buffer was used to induce phase separation since at the optimal extraction pH 10, salts based on NH_4^+ or Mg^{2+} cations were impractical. A 1 min isocratic hold of 15% (v/v) ACN and a post-column split valve were used to separate the salt load of the injected sample (5 µL) from the analytes and deviate the column outlet during the first 1.8 min to waste to avoid contamination of the ion source of the MS/MS instrument. In terms of optimization, the main challenge was the method transfer from HPLC to UHPLC-MS/MS regarding injectable volume and possible column overload. The extract had to be diluted seven times to avoid this. Considering this, the LOD values achieved, ranging between 0.4 and 1.4 ppb, were satisfactory as they would allow analyte monitoring at a therapeutical serum level. Analyte recoveries were quantitative for betaxolol and pindolol but reduced for metoprolol (64%) and even more for propranolol (23 %). The interday accuracy ranged from 86 to 105% with an interday precision between 5 and 11% RSD. The LIS procedure required only 8.5 min, which was faster than most previous methodologies. Finally, the method was rated according to the Analytical Greenness Metric for Sample Preparation [306] with a score of 0.69 indicating that the automated sample preparation methodology is at least "greenish".

4.4. Lab-In-Syringe for the automation of solid phase extraction approaches coupled to liquid chromatography

This chapter comments on publications reporting on the use of LIS for the automation of sample preparation based on SPE coupled to liquid chromatography and covering three different SPE approaches: in-system packed sorbent microcolumns, sorbent membranes, and dispersed sorbent. In all the publications, the expected and achieved important advantage of using LIS was the increase in sample volume to which the SPE approach was applicable compared to other FTs. As for the experimental works commented in the previous section, online coupling of LIS to HPLC with spectrophotometric detection was done via a transfer line that allowed loading of the sample or SPE eluate into the injection loop of an injection valve of the chromatograph and implied study of loading conditions. Then, HPLC separation was done carried out while the next sample was being prepared.

The article "**Lab-In-Syringe with Bead Injection Coupled Online to High-Performance Liquid Chromatography as Versatile Tool for Determination of Nonsteroidal Anti-Inflammatory Drugs in Surface Waters**", published in the journal Molecules in 2021 [243], reports on the hyphenation of LOV with LIS in a simple configuration (Figure 18D) to study the advantages of renewable microcolumn SPE via *bead injection*, with *in-syringe* automation of sample modification, e.g., mixing with loading buffer.

The method was applied to the nonsteroidal anti-inflammatory drugs ketoprofen, naproxen, flurbiprofen, diclofenac, and ibuprofen and consisted of *in-syringe* mixing of the sample with HCl and loading it onto a SPE microcolumn that was newly packed from 4.4 mg Oasis HLB® sorbent slurry for each analysis. The LOV was modified to enable a larger microcolumn diameter allowing for a higher loading flow rate without overpassing the acceptable backpressure. Analytes eluted with 50% ACN were injected into online coupled HPLC and separated on a RP Symmetry C18 column (4.6 mm i.d. × 150 mm, particle size 5 μm) in isocratic mode while the used SPE microcolumn was discarded. Method optimization included the bead slurry volume, i.e., the sorbent amount, the loading flow rate, the type of solution for sorbent washing before elution, the eluent volume and composition, and the transfer volume to the injection valve as well as the conditions of the HPLC method itself. A large injection volume of 220 μL was used to omit the need to optimize the transfer volume for ideal heart cutting for each evaluated elution condition.

This first combination of the operation principles of the LIS and LOV techniques for the first time enabled straightforward handling of milliliter volumes of the sample with on-demand and in-system preparation of microcolumns from sorbent suspensions. Significant signal enhancement and applicability to water samples with analyte recoveries ranging from 91 to 109% were achieved. Repeatability and inter-day precision were evaluated yielding RSD values from 3.2 to 7.6 % and 5.2 to 9.2 %, respectively and LOD values from 0.06 to 2 μg/L. In comparison, sensitivity and repeatability were similar to those of previously reported methods for the given analytes. Moreover, full automation including sorbent exchange was achieved. The short time of analysis could be further improved by using HPLC in gradient elution mode.

The article "**Renewable sorbent dispersive solid phase extraction automated by Lab-In-Syringe using magnetite-functionalized hydrophilic-lipophilic balanced sorbent coupled online to HPLC for determination of surface water contaminants**", published in the journal Analytica Chimica Acta in 2022 [244], reports on i) the functionalization of commercial hydrophilic modified styrene polymer resin (HLB) with magnetite nanoparticles including their cleanup, characterization, and study of handling and storage conditions and ii) use of the so-prepared particles for a dispersive SPE with procedure that was using the LIS technique, iii) analyte separation and determination by online coupled HPLC-UV. The method was applied to model contaminants of current concern selected from analyte groups of pharmaceuticals, plasticizers, cosmetic additives, and disinfectants. The analytes, in order of retention times, corresponding to increasing log p values, were mebendazole, bisphenol A, benzyl 4-hydroxybenzoate, diclofenac, and triclosan.

A simple and versatile protocol for modification of a polymeric SPE sorbent was developed. It was based on soaking the HLB resin in a mixed Fe^{3+} and Fe^{2+} solution followed by precipitation magnetite nanoparticles inside the sorbent matrix upon the addition of ammonia solution. Cleaning of the particle or *beads* from loose nanoparticles formed on their surface, in pores, or liquid remains between the beads was done by repeated washing and sorbent filtration. Photos from light microscopy documented that magnetite oxidation and related volume increase over time led to bead breakage which was solved by dipping them briefly into diluted phosphoric acid for nanoparticle passivation.

A LIS system and stirring configuration as given in Figure 18 C was used. The automated method started by the aspiration of air, loading buffer, sample, and an aliquot of the bead suspension. The beads were dispersed by magnetic stirring for 90 s to extract the analytes. Stopping in-syringe stirring for 60 s thereafter allowed the beads to be captured by the magnetic forces of the stir bar and the driver ring. To enhance this process, an especially strong stir bar was produced by heat-sealing several NdFeB magnets into a 3D printed casing of polypropylene. The sample was then discharged, the bead washed with 1 mL of water while stirring and after renewed bead recovery and discharge of the cleaning solution, 600 μL of 60% ACN was aspirated to elute the analytes during 80 s of stirring. After the bead capture, the eluent was passed through a transfer line to online coupled HPLC and 75 μL loaded into the injection loop. After triggering the HPLC for analyte separation on a Symmetry C18 column (4.6 mm i.d. x 150 mm, particle size 5 μm) in gradient mode, the used beads were resuspended into water and discharged during a stirring that was stopped only for the last millimeters of piston movement to enable disposure of the full liquid content.

Extraction and elution/desorption times, volume of sorbent suspension, composition and volume of eluent, feasible injection volume to HPLC, and sensitivity enhancement by using the sorbent for repeated load with sample before elution were studied, as well as the operation of bead-renewal, hence *bead injection* performed *in-syringe*. A membrane filter was integrated into the transfer line to hinder escaped sorbent beads entering the HPLC system; however, bead recovery was found to be highly effective. The method was applied to analyte determination in surface waters finding LOD values between 1.2 and 6.5 μg/L for 7 mL sample and method repeatability of 2.7 to 6.7 % RSD at 25 μg/L level. Recoveries obtained by comparing the found concentrations of the original and spiked samples ranged between 78.4% and 105.6% which can be further improved by the use of an internal standard.

The article "**Nanofibrous Online Solid-Phase Extraction Coupled with Liquid Chromatography for the Determination of Neonicotinoid Pesticides in River Waters**", published in the journal Membranes in 2022 [248], reports on the use of polymeric nano- and microfibers mats as sorbent membranes for online SPE in an HPLC system. Low pressure loading of milliliters of sample from an LIS system to mix the sample *in-syringe* with loading buffer was possible. The method was applied to preconcentration and HPLC separation of neonicotinoid pesticides acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam, and herein presents the first automated sample preparation method in combination with HPLC-UV and the first use of a nanofibrous sorbents for these analytes.

A commercial solvent filter holder of approximately 350 mm^2 cross area was used to house six layers of polyimide nanofiber mats and was integrated into the injection valve of an HPLC system. A LIS system with an upside-down placed syringe pump and a stirring system as in Figure 18 G was used in combination with a SV and an autosampler. For online SPE, a 2 mL sample was mixed *in-syringe* with tris buffer, pH 8, before loading the mixture onto the sorbent through a transfer line connected to the injection valve. Switching the valve to position *Inject* allowed the mobile phase of the HPLC to elute the retained analytes onto the separation column. A drawback was that the frit integrated in the solvent filter holder exhibited significant backpressure. To allow low-pressure loading, the frit was replaced by a commercial felt pad in combination with a 3D printed polypropylene holder as a rigid support of low flow resistance. To minimize the effect of the dead volume, elution was done in counterflow, i.e., the mobile phase passed first the felt pad and then the active sorbent.

Several polymer sorbents including nanofibers, microfibers, and nano- and microfiber conjugates were tested, and polyimide nanofibers were selected for showing the highest extraction efficiencies. Optimization also included the number of fiber mats, the flow rate for loading, the number of sorbent mat layers, and the loading pH. Furthermore, washing of the nanofibers was implemented for matrix removal finding that using 1 mL of 2.5% (v/v) MeOH with 2.5 mmol/L buffer did not significantly alter the sensitivity of the method. The extraction capacity of the fibers was sufficient to be loaded with 1 ppm mixed standards up to three times, corresponding to a 6 mL sample with an extraction efficiency achieved from 68.8 to 83.4%. For loading a 2 mL sample, preconcentration factors ranged from 70 to 82. LOD values ranged between 0.4 and 1.7 µg/L with RSD values typically below 5%. The optimized method was applied to paper-filtered river and lake waters that yielded analyte recoveries of 62.8% to 119.8% for 20 µg/L spike concentration. Finally, a comprehensive overview of other methods for neonicotinoids using optical HPLC was given for comparison. The developed method stands out for its simplicity, high preconcentration factors, and for being the only one reporting automation of sample preparation for neonicotinoids.

4.5. Laboratory automation and the potential and contributions of Lab-In-Syringe to this field

This chapter comments on four review publications related to the automation of sample preparation, of which one specifically describes the first LIS applications, an invited trend article that puts the LIS technique in perspective to other flow approaches and discusses the role of FTs in analytical chemistry, and, finally, the only tutorial written on the automation technique *Lab-In-Syringe*.

The review article "**Automated In-syringe Dispersive Liquid-Liquid Microextraction**", published in the journal Trends in Analytical Chemistry in 2014 [177], reviews the first nine applications and developments of the LIS technique and puts them in perspective with other methodologies automating LPME by means of different FTs addressing the advantages and disadvantages of each approach and application. First, the development of DLLME as a newly developed sample preparation procedure and the first flow methods that report DLLME automation are described. Thereafter, *in-syringe* automation is explained, and intrinsic features are listed and justified, followed by an overview of the reported applications. A final comparison points out the advantages of LIS over other flow-automated approaches, in particular safe handling of volatile organic solvents, in-syringe analyte detection, and in-syringe stirring to omit the dispersion solvent. The dead volume to be cleaned after each analysis and the limitation of the syringe size were already recognized as disadvantages. A final outlook demands the applicability of LIS to extraction solvents denser than water and to alternative, tailored solvents, coupling of LIS to separation techniques, and application of combination with SPE protocols. All these perspective issues have been addressed and solved in the following years, to a significant part by the work of the author of this thesis. It is noteworthy that to the date of publication of this review, the terminology *Lab-In-Syringe* was not yet used for this automation technique.

Three review articles originated from a collaboration with the Pavol Jozef Šafárik University in Košice (Slovakia) that give a critical and comprehensive overview of the automation of liquid phase microextraction approaches by FTs as well as by robotic or autoanalyzer systems. The first review "**Automation of static and dynamic non-dispersive liquid phase microextraction. Part 1: Approaches based on extractant drop-, plug-, film- and microflow-formation**" reports on the automation of such LPME approaches in which the solvent is exposed to the sample by an open surface but not dispersed [17]. The review is structured into sections; the introduction deduces the need for LPME automation, explains conceptual differences of static and dynamic operation of the LPME, i.e., moving the extraction solvent or sample during the extraction to create fresh surfaces, increasing analyte transfer and the concentration gradient, e.g., by counterflow, and classifies the LPME approaches. A second part distinguishes different tools of automation being syringe pumps, complex flow technique analyzers including, for instance, the LIS approach, and versatile autosampler and robotic system with a short discussion of specific pros and cons. Afterwards, the automated LPME approaches are overviewed and classified regarding solvent formatting into approaches using confined solvent (drop, solvent plug) and different modes of solvent film formation. Finally, microchip-based approaches to automated LPME are overviewed. It ends with a connecting passage to the second part.

The second review is entitled "**Automation of static and dynamic non-dispersive liquid phase microextraction. Part 2: Approaches based on impregnated membranes and porous supports**" [111]. This review focuses on automated LPME applications that have the solvent supported and thus are protected from mainly particulate matter of the sample and hinder rapid dissolution with the disadvantages of stricter confinement and slower analyte extraction. An introduction section explains membrane- and pore-based LPME approaches and then explains and overviews flat membrane-based LPME, the chromatomembrane approach and impregnated sorbent-based extractions, and use of HF as solvent support. The review finishes with a critical conclusion and perspective of automated nondispersive LPME. Both reviews show as schematic figures principal instrumental configurations and operation principles. They also give tabular overviews on the included applications listing analytes and sample matrix, extraction mode, automation instrument, detection or separation technique used and type of coupling, extraction solvent and volume, acceptor phase is applied, required times, as well as achieved enrichment, LOD, and RSD values. Moreover, the first review gives a timeline on automated non-dispersive LPME and an explanatory figure classifying these approaches.

The third review article "**Automation of dispersive liquid–liquid microextraction and related techniques. Approaches based on flow, batch, flow-batch and in-syringe modes**", published in Trends in Analytical Chemistry in 2017 [162], represents a continuation of the previous two commented reviews and was likewise prepared in collaboration with the Pavol Jozef Šafárik University in Košice. This review focuses on automated LPME approaches that create solution droplets, i.e., temporary formation of an emulsion, either by dispersion of an immiscible extraction solvent using a dispersion solvent or mechanical means (e.g., stirring) or starting with a homogeneous liquid phase in which phase separation is induced by change of pH, temperature, or addition of an appropriate agent. The introduction describes these different modes of extractions, solvent dispersion, and induction of phase separation and gives a short introduction to the possibilities of in-flow and batch automation and limitation and difficulties, e.g., explaining the issues and possible solutions of emulsion breaking and droplet collection. In three sections, reports on DLLME and HLLE automated in-flow, using autosampler and robotics, and finally flow-batch approaches are overviewed. A separate section is dedicated to *in-syringe* automation of DLLME and HLLE procedures. The review ends with a conclusion and an outlook discussing the need to explore alternative greener solvents, to use the automated DLLME and HLLE approaches in combination with online coupled analyte separation, and to overcome issues related to the matrix, e.g., particulate matter, that limits the use of DLLME for complex samples.

The article "**Where are modern flow techniques heading to?**" published in the journal Analytical and Bioanalytical Chemistry in 2018 [41] was an invited trend article in which a personal evaluation on the past and current role of FTs in analytical chemistry is given including an outlook on current trends and likely future developments. This included an overview of different FT and principles, and evaluations of the characteristics and strongholds of two modern flow approaches: LOV and LIS, compatibility of LIS with autosampler systems and critical comparison of robotic batch and flow automation, past and current fields of applications of FTs, and comparing flow technique with similar analytical techniques such as microfluidics and paper devices. Furthermore, data from the "Web of Science" and a

comprehensive online database of publications related FTs [42] at www.flowinjectiontutorial.com[15], were analyzed to show the total number of publications, as well as the D1 journals "Analytical Chemistry" and "Trends in Analytical Chemistry" related to FTs over the years. The statements made in the article can be summarized as follows: i) FTs are relevant and highly useful for niche applications, e.g., monitoring, kinetic measurements, and sample preparations, while the total number of publications per year has decreased, there is an approximately constant share in high-end journals, FTs have constantly inspired other techniques and shall be considered "tools of innovative science" with emerging new automation approaches, and LOV and LIS are approaches completing each other as they are opposed in their operation and functionality. LIS represents a "back-to-the-beaker" development with characteristics in common from both autosampler and flow automation concepts.

The final article of this chapter is denoted "**The Automation Technique Lab-In-Syringe: A Practical Guide**", published in the journal Molecules in 2020 [116], was written due to an invitation by the leading professor of a collaborating research group. It is divided into an introduction to flow technique automation, the technical milestones of the development of the LIS technique, a critical discussion on the characteristics of the technique as well as its pros and cons, operation modes, tips and tricks related to system setup and characterization, as well as to method design and optimization, troubleshooting, and choice of materials, and finally the use of 3D printing of system elements required for setting up a LIS system. Finally, it gives a comprehensive overview of published works on LIS applications and provides photographic documentation, schematic drawings, and written assistance in designing and fabricating of LIS system elements, stirring modes, syringe orientation, or on expectable dependencies on method variables and calculation of dead volumes and performance parameters.

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15 This database is taken care of by Prof. Elo Hansen, one of the two inventors of FIA. Without the possibility of professional data management, careful proof-reading of this list (containing over 23.000 references at the time of manuscript writing) was needed to use the provided information for data analysis and statistics.

5. Discussion

The ultimate test for an analytical method is not that it can do better what can be done by other means, but that it permits us to do something that we cannot do in any other way.   Elo H. Hansen

This section aims to answer anticipated questions related to the Lab-In-Syringe approach which can be summarized into three inquiries: "What was the point of it all?", "What is the current situation?", and "What future lays in front of us?". These are reflected in the following discussion sections. It is not the objective of this section to make a final advertisement for the LIS technique or FTs in general but to discuss honestly and critically their potentials, uses, and limitations.

Did the Lab-In-Syringe technique meet the expectation of a new automation tool?

This question can also be formulated as: "Does the LIS technique deserve a rightful place between the existing automation and flow methodologies? To answer, we must first consider the limitations of non-separative FTs and the characteristics of different automation approaches. Rewinding, in most FTs, the automated task, often a chromogenic analyte-selective reaction, is performed by mixing the sample with one or several reagents in a tubing network of small cross-section areas in the range of 0.2 to 3 mm². In consequence, the flow regime is generally laminar, and mixing is incomplete. The introduced solution zones into the manifold penetrate each other partially and concentration gradients are formed that result in peak-like signals. Using air segmentation, homogeneous solution mixing can be achieved in fluidic segments but only of a few centimeters. Over many years, inventions in FTs have evolved towards lower solution consumption and ingenious approaches took benefits from concentration gradients. The only exception here is flow-batch approaches, i.e., flow analyzers with an added atmospherically-open mixing chamber to the flow network to enable mixing of milliliter solution volumes, homogeneously, and step-by-step. However, flow-batch can be considered by its nature, like LIS, not a *pure* flow technique.

Every newly developed flow technique has aimed generally i) for simplified and more compact instrumentation, lower costs, and higher efficiency, ii) for simplifying operations while increasing the operational versatility, i.e., flexibility in solution handling and mixing schemes, and iii) for minimizing the need for physical system optimization. In this sense, the LIS technique must be evaluated as a valuable contribution and completion to existing flow automation tools. First, the required instrumentation for LIS is a compact syringe pump with integrated multiposition head valves that weighs about 1 kg, fits into a lunchbox, and can be purchased online as refurbished for <200 Euro. Second, the instrumentation and operation follow the known schemes of SIA, i.e., stepwise aspiration of all solutions and software-instruction of all operational parameters, as well as flow-batch automation, i.e., homogeneous solution mixing. Third, a high versatility has been proved in the automation of most existing LPME approaches as well as other sample preparation methodologies including CPE and dispersive SPE. Moreover, the LIS approach overcomes some disadvantages of flow batch and enables new modalities that are not feasible with another flow technique: size-adaptable yet sealed mixing chamber, its usability as a flow-through reactor, pressure change for gas phase extractions, or stirring-

assisted solvent dispersion. In conclusion, LIS has broadened the operational possibilities for flow automation of analytical protocols.

Syringe pumps are also used as an integral component in other automation approaches including autosamplers, pipetting robotics, or control instrumentation for Lab-On-A-Chip automation. Consequently, the operational concept of LIS can be transferred to these systems and enlarge their versatility. However, LIS as well as all other FTs, cannot compete with parallel processing of multiple samples, e.g., on 96well-plates, in terms of sample throughout. LIS can also not downscale preparative procedures nor yield the processing speed of Lab-On-A-Disc. On the other hand, it does not require disposable vials as consumables nor single-use chips, which require a significant time in development and prototyping and are designed for only one specific analytical task. On the contrary, a substantial characteristic of LIS is its simplicity and versatility; the system and method can be set up rapidly and used for a multitude of analytical tasks. On the other hand, the purpose of microfluidics is typically not the automation of LPME. There is certainly an overlap in the fields of application but the LIS technique and concept are rather to be seen as a completion of the existing automation approaches.

It is to be pointed out that there is in fact a growing interest in the LIS technique by fellow researchers working in the field of FTs and laboratory automation that is reflected in the number of research groups publishing experimental works based on this technique. Including our group, application of LIS or an equivalent concept for the automation of sample preparation approaches has been published from 9 research groups in 7 different countries including Poland, Greece, Spain, Brazil, Russia, China, and the Czech Republic. Moreover, two researchers from Brazil and Turkey who carried out research stays in our group working with LIS, are now continuing to work with this technique at their home institutions and researchers from Australia have developed a different stirring concept [301,302].

Scrutinizing this development would most likely reveal that naturally, a new technical approach is drawing the attention of the peers on the research, being tempted to explore the possibilities of the new tool. Attractiveness is likely added by the relative novelty of the technique, since "novelty sells", i.e., using a new technical approach surely aids in the publication process. A critical assumption is therefore that the LIS technique is currently undergoing the initial rising phase that the Gartner hype cycle model [41] describes as a "technological trigger" or, the following phase denoted "peak of inflated expectations". In other words, it is still too early to answer the above question with confidence.

It can be asserted that there is a current interest in developing and using FT methodologies that can process larger volumes of sample than what can be processed in-tube as inherit concept of most FTs. This is due to a growing interest in developing automated sample preparation methodologies and larger sample-extractant ratios simply enable higher preconcentration factors. In this sense, two very recent articles propose alternative in-syringe automation of extraction procedures using yet another mixing approach than magnetic stirring. In a trend article, I have described this development in FT automation provocatively as "back to the beaker" [41] in a reference to a review published in 2000 by the inventors of FIA: entitled "Flow injection analysis: from beaker to microfluidics" [33] or, citing the author of this publication: *From batch to flow and back, we go..."* [1].

To stir or not to stir?

Many tasks in the analytical laboratory required agitation, mostly to achieve homogeneous mixtures, for cleaning glassware, for preparation of samples, standards, and reagent solutions, mixing solutions to enable chemical reactions, and finally for the solubilization of salts or the dispersion of immiscible phases. Simple mixing is a vital *building block* in analytical work combination with e.g., solution metering, transfer, vessel filling and emptying, tempering, evaporation, phase separations, and waiting. Probably most preparative procedures can be broken down to these *unit operations*. In consequence, the ability of homogeneous solution mixing is also of interest for laboratory automation. In an automation approach where homogeneous solution mixing is feasible, the operations, final reagent concentrations, phase ratios, etc. are predictable and human work is emulated. This might make them more attractive for preparative tasks than dispersion-based concepts. This is surely one of the multiple reasons why autosamplers and robotic systems are preferred over non-separative FTs to automate analytical and preparative procedures in commercial laboratories.

FTs are based on performing mixing tasks in tubing systems *in flow*, mostly by zone dispersion, leading to incomplete solution mixing. Step-by-step procedures, e.g., the addition of several reagents to the sample with intermediate mixing, requires some thinking. Options that come to mind are:

- using a multichannel flow technique, e.g., a complex flow network with a pumping line, confluence, and mixing coil for each required solution, implying tedious system design and optimization.

- using a mono-channel FTs such as SIA, the solutions must be aspirated one after the other and mixed by back-and-forth movement of the mixture or the solutions must be stacked in zones small enough to enable penetration, at least partly, of the outer-situated ones. This can be aided by solution splitting into smaller segments, requiring more time, or mono-segmentation, where the stacked solutions are confined by two segmentation bubbles to promote turbulent mixing. In any case, mixing becomes increasingly difficult for more solutions and larger solution volumes, i.e., when aiming for high phase ratios.

- using programmable flow, i.e., using two independent pumps on a SIA system, both connected via HCs to the central port of the SV. One pump aspirates solution B while the second one provides the previously aspirated solution A at a lower flow rate so that both solutions mix by confluence in the HC leading to pump 1. This procedure can be repeated multiple times but at the cost of procedural time. A limitation of the technique might be the thorough mixing of immiscible phases for LLE, also given that the flow regime is assumably laminar.

- using mixing chambers, i.e., a flow-batch approach. This approach was applied in the first works with LIS. If such a chamber does not comprise a mixing element, cleaning requires filling the chamber multiple times to at least the same level as used during the actual procedure, implying significant time and solution consumption. Using such a chamber in SIA, solution handling is done by only one pump and four steps are needed for cleaning: aspiration of cleaning solution, loading the cleaning solution into the chamber, chamber emptying, and solution discharge.

In this light, the use of the void of a syringe pump as a size-adaptable chamber, i.e., combining the liquid handling with the mixing unit, had some obvious operational advantages. Moreover, the chamber is sealed, so sample contamination during mixing is avoided, solutions do not splash even at violent

stirring, and gas phases are confined and can interact with the liquid phase. However, the introduction of a stir bar into the syringe was surely a game-changer in Lab-In-Syringe development. Now, homogeneous, and nearly instantaneous mixing was feasible, proceedable as long as needed, and widely independent from the solution ratio and efficient cleaning of the size-reduced space is possible.

Therefore, it is my belief that it is in fact *in-syringe* stirring that makes LIS what it has shown to be, a versatile and operationally flexible automation technique. On the other hand, the combination with another mixing element, e.g., an external mixing chamber, requires a simpler setup[16]. The primary drawback of *in-syringe* stirring, the dead volume due to the stir bar, has been discussed in chapter 3.4 as well as solutions for solving this issue. In this context, it should be highlighted that solution mixing in an automated syringe pump has recently been proposed using modifications of the piston rather than in-syringe stirring [301,302]. It is unclear whether the *in-syringe* stirring approach was adopted or has, at least, inspired further developments or whether the listed inventions were completely independent. However, it is clear that *in-syringe* mixing appeals gainful also to others.

Where does Lab-In-Syringe shine, where does it smolder?

This question aims at the fields of application where the LIS technique can be used with an advantage over other automation approaches and where it did not fulfill the authors' initial expectations in terms of performance and competitiveness against already existing methodologies.

I believe that the LIS syringe is indeed a versatile automation technique given the variety of optimized sample preparation methodologies, the instrumental facets, and operational possibilities. Like autosampler systems and in contrast to most other flow automation approaches, handling of vial-sized sample volumes is possible, and standard laboratory tasks are done in similar way as they would be done manually. There has surely been a gain in experience by using the LIS technique for over a decade so when looking back, I consider that some things could have been done more elegantly. Nonetheless, significant improvements were achieved especially regarding system setup and system functionality. For instance, higher operational reliability *in-syringe* stirring was achieved using PWM motors enabling also software control of stirring speed.

If I would have to pick the most convincing experimental works with LIS to which I have contributed, it would be the ones reporting on the automation of extraction methodologies based on a dispersed solvent yield fast extraction kinetics: DLLME, HLLE, and HLLE in combination with matrix precipitation. Here, the possibilities to violently mix all solutions in a closed container with no user exposure to the handled solvent, to confine solvent droplets in the conical inlet of the syringe for easy and automated recovery, to connect sample preparation directly to a detection or separation system with both tasks performed in parallel, and to simultaneously precipitate sample matrix and extract modification before loading, are noteworthy benefits that can hardly be achieved by other techniques. Similarly, solvent-

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[16] Having the possibility to use 3D printing for the fabrication of the few required elements for *in-syringe* magnetic stirring, i.e., the driver ring and motor supports, has been extremely helpful over the last 5 years.

free HSE with pressure infection that was enabled by the size-adaptability of the syringe void has demonstrated beautifully the abilities of the LIS automation technique.

On the other hand, versatile autosampler systems are powerful competitors regarding automation of approaches that require long extraction times and use a supported solvent, for instance HS-SDME and DI-SDME based on classical solvents for these procedures. Here, creation of a microliter-sized drop is far easier than in LIS, not to mention the ease to retract the drop after the extraction and to inject it into a gas chromatograph. Achievable preconcentration factors range an order of magnitude higher than when automating these approaches by LIS or other FTs and solvent consumption for cleaning the used injection needle is highly reduced. An important advantage is the movement of the syringe pump rather than of the liquid samples and reagents so that no connecting lines with implied dead volumes are required. However, the situation changes when a moderately water-soluble solvent is intended for drop creation, e.g., a deep eutectic solvent, as anyway a larger drop volume must be used that cannot be created reliably on the needle of an autosampler syringe. In this case, it would be desirable to combine both automation approaches, i.e., to perform Lab-in-Syringe with a movable syringe pump on an autosampler system. The development and study of the applicability of such a system is a major aim of the candidate for the next future.

In the second, we proposed using a simple LIS system for low pressure loading of a sorbent membrane for online SPE with a milliliter volume of sample; however, such preparative procedure can also be done by some HPLC autosamplers so that the LIS-based approach would be more beneficial if a more complex procedure, e.g., analyte derivatization before loading, would be required.

In any case, all FTs including LIS working on sequential performance, e.g., of a preparative procedure, fall behind parallel execution on 96 well plates. This virtually yields a very high sample throughput even if the procedure itself takes long. However, for analytical procedures applied to the monitoring of any kind of system or process, just-in-time operating FTs are one step ahead since they can be easily coupled to a flowing sample stream. Here, the LIS technique would be the ideal tool for the automation of any procedure requiring or benefiting from larger sample volumes.

Finally, it should be pointed out that each automation approach and each FT still of interest today has its specific pros and cons and applications domains. The techniques LIS and LOV as ideal FTs for the automation of SPE by BI and of LPME procedures, respectively, were compared regarding instrumental and operational characteristics in a trend article included in this thesis [**41**]. It was concluded that they complete each other showing strongly contrasting capabilities. Thus, merging both approaches could open new possibilities such as for the automation of two-step sample preparation.

Which are current constraints and interests to use Lab-in-Syringe for routine analysis?

To my opinion, the robustness and reliability of the LIS technique suffice for routine analysis. However, without the support of a company interested in developing a commercial product, its use will remain mostly as an analytical tool and concept used by other researchers to develop automated preparative procedures for environmental vigilance and monitoring, potentially coupled to separation techniques. The expectable benefit of commercialization might be limited as the customer will not require additional consumables but can set up a LIS system easily without a company support.

The LIS technique has been used foremost for the automation of sample preparation procedures rather than chromogenic assays. It is my belief that there will be a growing interest in laboratory automation, mostly requesting autosampler systems. So far, liquid-liquid extractions in such systems are based on mixing all solutions in individual vials rather than in one common external mixing chamber. This way, lasting tasks such as LLE can be performed in parallel and instead of using a device that enabled shaking one grabbed vial at a time, an entire vial rack can be shaken as long as required. Moreover, vials can be disposed of after the accomplished procedure or larger flasks are cleaned together in a laboratory dishwasher. Cross-contamination and consumption of cleaning solution in the moment of analysis are therefore significantly reduced. On the other hand, such systems are impractical for monitoring and where sample preparation is required for monitoring, LIS can be a powerful tool.

Moreover, the principles of green chemistry will push technical development also towards preparative approaches that re-use material. This is surely a chance for LIS as an operation principle to be applied to autosampler systems or used in combination for more complex preparation. Therefore, I am confident that commercial instrumentation based on the LIS principle will be developed in the future. This does not necessarily mean that in-syringe stirring will be the adopted approach to homogenize the syringe content. In fact, there are commercial solutions that show strong related to the LIS approach from the companies: Parker Hannifin Cooperation (NH, USA), which use a milliliter syringe pump in combination with an autosampler for in-syringe mixing with reagents, Cetoni GmbH (Germany), which do in-syringe stirring to handle viscose suspensions[17], and the collaborating company Advanced MicroFluidics (Switzerland), which mix solutions in-and at-syringe, e.g., for biological assays such as cell staining but without in-syringe stirring.

Moreover, it is remarked that the Tecan Trading AG (Switzerland) started not long before the first publication on LIS to provide syringe pumps with multiposition head valves that turned out to be a perfect match for setting up a LIS system as it combines all instrumentation required but the stirring operation. Meanwhile, other companies produce syringe pumps of similar design, however, I am not aware of any commercial solutions related to LPME approaches performed *in-syringe*.

Company-based development is always motivated by demand, i.e., if academic research proves that the LIS technique can solve problems of interest for industry or as an analytical tool in other research disciplines. It is safe to say that LIS is already becoming an established flow technique with several new applications of LIS presented at the last international conference related to flow automation in 2022

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[17] This development has been shown on the company website since 2015, which proves the usefulness of the in-syringe stirring concept also for other fields of application than automated sample preparation.

(Flow Analysis XV in Krakow, Poland). However, the market for flow technique instruments is, in comparison to other analytical techniques, not large[18] so progress will not happen fast. In terms of providing LIS tools for academics, many research groups are setting up flow systems of their own design and use laboratory-developed control software due to economic restrictions.

Responses to the personal inquiry at GlobalFIA Inc. and FIALab Inc., two leading companies developing FT analyzers and components based mainly on SIA confirmed mostly my considerations summarized in this discussion. As it appears, the market for FTs is stable with interest not only in complete systems but also in components including the request for purpose-adapted developments. Based on the given information, I estimate the total number of analyzer systems sold worldwide less than 100 per year.

In terms of customers in the last 5 years, about a tenth of the demand was by academia and about double by governmental institutions, e.g., for wastewater treatment plant laboratories. The main share of requests is from industry, mainly large pharmaceutical companies, and commercial agriculture and environmental laboratories, but also to private sections, e.g., wine producers. One company stated that due to limited personal capacity, laboratories do not belong to the preferred customers as control software must be adapted to the specific needs and full-time technical support must be an option to provide. It should be highlighted that this is a question of market orientation than the suitability of the respective analyzer systems, and information was also provided that segmented flow analyzers are in fact sold regularly by other companies, e.g., to clinical laboratories. Another focus of FT analyzer applications is still determinations of environmental parameters in waters and for ocean science.

There is a slight shift towards the hyphenation of FTs with batch concepts, i.e., including mixing chambers and autosampler systems into the analyzers, and towards the same preparation for separative analysis, including process sampling, high-precision dilution, and sample pretreatment such as pH adjustment. Regarding trends there is further increasing interest in FT for synthesis – so-denoted *flow chemistry* – and away from custom design of analyzer systems toward standardization.

For those who have reached the level of decision-makers in industry, analysis in flow might be associated with tedious optimization by manual adjustment of tube lengths assuming that they are familiar with early FTs FIA and SFA, not knowing that modern FTs offer computer-based method adaptation[19]. When purchasing an instrument capable of automation of sample preparation procedures, decisions might therefore be biased towards, e.g., versatile autosamplers. As autosamplers are part of many analytical instruments already, the concept is familiar and the adjustment period is short. Due to a larger market, the producing companies have the economic power to constantly perfect instrumentation, control software, and service, and be highly visible, e.g., present on analytical trade shows. On the other hand, academic flow practitioners are used to plugging, tinkering, and adjusting

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[18] The number of publications found in the WOK using "HPLC" or "high performance liquid chromatography" in as search criterium is about 30 times of what can be found for "FIA" or "flow injection analysis", i.e., not considering other flow techniques. The ratio for use in laboratories unrelated to academia is assumably larger.

[19] I see my own impression confirmed by the opinion of the CEO of GlobalFIA Inc. and co-inventor of SIA, G. Marshal, Ph.D.: "The spaghetti tangle of tubing is still a hinderance to the wide acceptance of flow systems."

and do not have the financial possibility or pressure to demand perfect solutions that companies related to FTs development surely aim to provide.

In my experience, the software used for instrumental control and data acquisition and treatment has always been a critical and often limiting factor. For a proof-of-concept publication, a calculation worksheet, e.g. helping in data evaluation, is often enough, but for routine work in an analytical laboratory or even in field work, the demands are far higher, for instance, help option and documentation, data management and project definition, etc. while my wishes would be rather a possibility for using acquired data for feedback action, the option of interaction with the running method, improved tools for data acquisition and treatment, e.g., peak integration, data smoothing, compensation of error peaks due to air bubbles, etc., backward compatibility or easy integration of other instrumentations. Of the three software tools I have used, none fulfills all these items. I see this as one of the constraints for using LIS, and FTs in general, not only for routine analysis but also for use in research and academia. They are unlikely to be solved soon, mainly because the uses and modalities of FTs are too diverse, and the user needs are too individual. In contrast, the main task of HPLC is more clearly outlined – separation – and instrumental adaptation is generally done by using another detector that can be usually integrated by analogous signals, changing the separation column, and adaptation of separation conditions (gradient, mobile phases, column temperature, etc.). In this sense, the flexibility of FTs might be both "boon and bane" and it is an important segment for FT companies to develop methods and analyzers on for personal requests and for specific problems.

In conclusion, the constraints on FTs can be lifted only by a gradual awareness of the potentials of modern FTs to which teaching this subject at universities, workshops, and excellently developed applications and innovative publications need to contribute most significantly. Moreover, the demand for greener chemistry or environmental and process monitoring, and the development of more appealing flow approaches will promote the use of FTs. It must be a self-reinforcing cycle: step-by-step, the excellent work of flow technique producing companies and more visibility also provided by presentation and publication of academic research will lead to positive feedback and will benefit companies, customers, and academic researchers altogether.

To sum up, discussed constraints for FTs include prejudice, visibility, and awareness, as well as supporting software, and a confusing branching of flow approaches and application fields. Apart from these, limitations for spreading the LIS technique were some years ago still related to know-how, *in-syringe* stirring, and company support. A recent tutorial [116] was prepared to promote the LIS technique and to facilitate its usage. Concerning company support of the LIS technique, a good start can be proclaimed by a recently started industrial collaboration with the company AMF Inc. that should result in commercialize-able products and new methodological solutions. I conclude that there are no substantial constrains for routine use of LIS or its commercialization any different from other FTs but rather a higher potential by its similarity to autosampler systems.

What are the likely prospects of LIS and non-separative flow techniques

As graphically shown in a recent trend article [41], it took publication records for both HPLC and FIA more than 20 years to culminate and 10 years for SIA given the already existing community of flow technique practitioners. On the other hand, SIA is still a young technique concerning its use outside the research laboratories. In the beginning of FIA, mostly chromogenic assays, and enzymatic, and electrochemical determination procedures were automated with a focus on inorganic contaminants in environmental compartments. Related problems such as overfertilization and algae blooms have long ceased to hit the headlines in Western countries also due to stricter regulation and wastewater treatment. Today, the focus of analytical chemistry has changed to organic trace contaminants in various matrices, including food and biological materials. This development came along with the establishment of separation methods hyphenated to MS detection as standard analytical tools. Another hot topic is surely the development of sample preparation methodologies including the study of novel sorbent materials or green, tailored solvents. As consequence, many experimental works using FTs are related to monitoring applications, e.g., process vigilance, contaminant screening, or total indices. In the same sense, the study of the automatability of sample preparation methodologies, coupling analyte derivatization and extraction to atomic spectroscopic or separation techniques, and the use of novel sorbents and solvents for these purposes have become of interest to FT practitioners.

I am convinced that FTs are highly useful tools, in particular for niche applications such as environmental vigilance, process monitoring, and at-line sample preparation. For these three fields of application, a growing interest in modern flow approaches can be anticipated but prospering will depend on the contributed benefit and reliability of developed methods.

With more experience, it is always possible to improve already published experimental work. This said, promising concepts should be further developed to increase method effectiveness, reliability, and robustness, and to find solutions for observed difficulties. However, academic researchers get less credit for perfecting an analytical system than developing a new application or coming forwards with a novel technical approach unless they draw their focus from developing a new analytical tool to rather using it for a superordinated multidisciplinary question, e.g., studying the behavior of contaminants in the environment. So, the progress from the proof-of-concept to a mature analyzer is left to industrial developers who benefit from the pool of ideas published. Moreover, companies have the resources to employ specialized craftsmen, designers, engineers, or programmers or to outsource certain tasks while academic developers must be all-rounders and seek collaborations to exploit synergies or will find it difficult to compete with industrial developments. Bringing together commercial companies and academics is an evergreen objective set by ministries of science and education via project tenders.

As beautifully an automated flow system might function, single-use devices or automation approaches based on disposables and consumables are often preferred or even demanded to absolutely prevent any risk of cross-contamination, e.g., in bioanalysis. Several research groups known for their contributions to FTs also work on another flow approach namely paper devices. These have evolved considerably over the last decade and fulfill, more than probably any other analytical approach, the KISS principle – "Keep It simple, stupid!" Characteristics such as little costs, simple fabrication, nearly no waste, mobile phone as a detector, no moveable parts, and sample filtration by the genuine material make it difficult to highlight the advantages of an automation technique based on a computer-

controlled automatic syringe pump at least for chromogenic assays. Manual approaches will generally require less optimization time but typically render also less reliability, reproducibility, and information than instrumental approaches.

The LIS technique has already gained followers among researchers working in laboratory automation as well as in sample pretreatment methodologies. It is honest to accentuate that the flow community is not a big one in analytical chemistry but there has been a growing interest in novel approaches to sample pretreatment that is reflected for instance in the recently initiated Sample Preparation Study Group and Network of the Division of Analytical Chemistry of the European Chemical Society (EuChemS) to which our working group is contributing.

LIS is based on homogeneous mixing resulting in intuitive straightforward operations and high versatility. It has been shown that it can exhibit interesting advantages over other flow technique approaches. It is an ideal tool for a key application field of FTs being also a current focus in analytical chemistry - automation and miniaturization of sample preparation procedures. Therefore, and despite the discussed difficulties, this technique and operation concept is just on the starting blocks and I think that it will find, in the here-presented form or possibly using another *in-syringe* mixing approach, its way also into commercial products.

6. Summary

This habilitation thesis has overviewed the development and applications of one of the youngest offspring of FTs, nowadays known as *Lab-In-Syringe* – LIS, as a new automation of sample preparation and analytical procedures. To this end, the thesis describes the two main concepts of wet chemistry automation – *in-batch* and *in-flow* – detailing their strengths, weaknesses, modes, and main features: parallel operation of sample handling in individual containers yielding steady state conditions by homogeneous mixing in batch automation and flow transport of a sample segment in a tubing network undergoing mixing processes with reagents by confluent additions or via penetration of solution segments by dispersion in flow automation.

The introduction and the main part of the theoretical chapter, focus on the description of the main earlier approaches to FTs in terms of their development, features, and their individual advantages and drawbacks. Their differentiation is based on operational and instrumental characteristics, such as sample introduction modes, mixing schemes, configurations of the tubing manifold, or other operation individualities. Their common denominator is straightforward miniaturization of analytical and preparative procedures, speed of processing, high reproducibility achieved with a minimum of instrumentation required, and consequent cost-efficiency.

In the second, the development of the LIS technique and the different operation modes and resulting characteristics are explained. LIS uses for the first time the void of an automatic syringe pump as a mixing, reaction, and extraction chamber and optionally also serves as a detection cell. In most cases, a magnetic stir bar is used inside the syringe void for homogeneous and instantaneous solution mixing that is driven by a rotating magnetic field generated outside the syringe. The instrumentation of a LIS system resembles that of SIA with the peculiarity of omitting the HC while operations follow those applied in batch automation: liquid mixing in a chamber with the possibility of stepwise addition or expulsion of solutions. The developed analyzer modalities and the resulting features are consequently explained. LIS combines the advantages of both automation concepts, above all, high operational flexibility, and overcomes a substantial limitation of FTs: difficulty to automate multistep procedures and LPME approaches in a compact analyzer. Moreover, it circumvents the need of a tubing manifold. The LIS technique has been mostly used for the automation of sample preparation methodologies. Therefore, the theoretic section briefly delineates the respective methodologies for analyte preconcentration and matrix removal used in FT and LIS: DLLME, HLLE, SDME, and SPE.

The thesis continues with detailed comments on publications that are divided according to individual steps of the LIS development, applications, and evaluation of the technique into four main categories. The first one describes the use of LIS for preparative procedures connected to optical detection. This chapter is further divided into four sections: the first one corresponds to early works of automated DLLME, aided by a dispersion solvent, in combination with chromogenic assays before the introduction of the concept of *in-syringe* stirring. The second one deals with automated *in-syringe* stirring-assisted DLLME in combination with chromogenic assays that also includes two standard procedures for total indices. The third one describes the automation of SDME in combination with spectrophotometry, and the fourth one handles automated *in-syringe* stirring-assisted DLLME in combination with ICP-AES. DLLME assisted by a dispersion solvent proved to be efficient with preconcentration factors of up to 30.

However, the usage of the stir bar inside the syringe void rendered higher performance when automating the same assay, and a simpler method on a more compact system was feasible. For coupling LIS to ICP-AES, two automated procedures with *in-syringe* DLLME were developed that enabled the determination of heavy metal cations in salt-laden matrices either by analyte back-extraction into an aqueous ligand-oxidizing solution or by injecting the organic extract directly using a high-temperature spray chamber to achieve extract compatibility. The development of a new system element, a syringe piston with flow-through channel facilitated the automation of HS-SDME and on-drop detection. A new mode for DI-SDME – in drop stirring – was developed that achieved considerably increased extraction kinetics for this typically slow methodology.

Two chapters report on using LIS-automated LPME and SPE coupled online to gas or liquid chromatography, respectively. This included the automation of double-stage analyte preconcentration by coupled DLLME to online-SPE following loosely the QuEChERS principle as well as centrifugation-less sample deproteination and analyte extraction. Moreover, a new modality for the automation of headspace extraction was developed that benefited from the intrinsic feature of size-adaptability of the syringe void. In terms of SPE automation, the LIS proved to be efficient for the automation of sorbent dispersion as well as able to hyphenate with the methodology of bead injection.

The last chapter deals with four reviews and puts the LIS technique into perspective with other works reporting on the automation of LPME, highlighting the versatility of the LIS automation approach. Moreover, a trend article discusses the current role and potential future of the LIS and FTs as valuable tools in research and for niche applications such as process monitoring or automation of on-site sample preparation. Finally, a tutorial on the LIS techniques aims to help newcomers in using the technique.

The thesis ends with a critical discussion of five major questions related to the impact, role of in-syringe stirring, potential, limitations, and future development of the LIS technique with conclusions given in the next chapter. The presented habilitation thesis gives a comprehensive overview of flow-automated sample preparation and one of the youngest FTs. Moreover, via the included publications, it reports on various inventions and problems solutions that can be applied to other FTs or to improve the automated procedures in manual performance as hereafter concluded.

7. Conclusion

In the frame of this thesis, the possibilities of flow automation of sample preparation methodologies were discussed with a focus on the features and potential of the automation technique Lab-In-Syringe. For this purpose, background to this technique was provided in the theoretical introduction including details on operation modes, delimitation towards other FTs, and a summary on related publications. Furthermore, the role and importance of sample preparation and its automation for analytical chemistry were addressed with selected sample preparation approaches briefly described.

Moreover, instrumental, methodological, and application developments related to 22 experimental works and publications were summarized and debated as well as four reviews, a trend article, and a tutorial, which have surveyed and discussed milestones, applications, and future expectation, as well as system setup and method development of the LIS technique. This comprised also the invention, study, and proposal of new methodological approaches to the confronted analytical problems and sample preparation procedures that are not primarily related to their automation.

Finally, the prospects, advantages, and limitations of the technique were critically discussed together with a debate on the use of FTs in general. In this sense, this thesis is hereinafter concluded.

- LIS is a novel and efficient approach to flow-batch automation using the instrumentation of a sequential injection analyzer and the void of the automatic syringe pump as mixing chamber. Operational possibilities were augmented by enabling homogeneous mixing of the liquid content by a magnetic stir bar placed inside the syringe void and use of the syringe pump either in upright or upside-down orientation depending on the aimed procedure, e.g., the density of the extraction solvent for DLLME. An instrumental progress was the development of a piston with a flow channel, fiber-optic spectrophotometry inside the syringe void, and different modes to induce stirring.

- The LIS has proven to be a highly versatile technique for the automation of sample preparation procedures with reported applications based on in-syringe DLLME, HLLE, DI-SDME, HS-SDME, HS-E, DSPE, as well as CPE and protein precipitation. Advantages towards prior FTs are the possibility of software-instructed homogeneous instantaneous mixing, independently from volume ratios. In respect to other flow-batch approaches, in particular, very high stirring rates that enable efficient solvent dispersion, the possibility to confine gas, and straightforward change of pressure inside the syringe, more efficient system cleaning, and compact and commercially available instrumentation are to be highlighted as the syringe void acts as both liquid container and pump.

- The effectiveness of the LIS techniques for the automation of chromogenic assays in combination with analyte preconcentration, standard procedures for total indices relying on LLE, and development of sample preparation coupled online to advanced analytical instrumentation HPLC, GC, and ICP-AES was demonstrated. Benefits included analyte preconcentration as well as matrix removal and increased analyte selectivity with adequate reproducibility and analyte recoveries achieved to be applied to real sample analysis.

- The LIS tool presents a valuable tool for analytical chemistry education: due to homogeneous mixing, operations are intuitive, and the extraction processes can be visually followed as shown by

commented videos on the Youtube channel of the thesis' author that were intended to contribute to a better understanding of the LIS technique and operation.

- Furthermore, LIS has been, like other FTs, a tool of inventive science: Inventions for problems solving included the proposal of Ba^{2+} for interference masking of SDS and extract washing for the determination of total cationic detergents, background modeling for multivariant spectrum analysis, oxidate back-extraction for matrix clean-up before ICP-AES, modification of a polymeric HLB sorbent with magnetite nanoparticles, adiabatic headspace gas compression for head-space extraction of BTEX, in-drop stirring as novel and time-efficient approach to DI-SDME, in-syringe HS-SDME aided by negative pressure application, continuous DLLME by counter-direction of sample flow and droplet floatation, and centrifugation-less deproteination via HLLE.

- The LIS technique met current trends in FT development, i.e., integration of batch operations, computer-control of all parameters, and simplification of operation and analyzer as well as in analytical chemistry, being the development of sample preparation methodologies for biological, food, and environmental samples directed to advanced instrumental analysis. It further presents a contribution to greener sample preparation by enabling procedural miniaturization resulting in lower solvent consumption and waste production.

- LIS has enlarged the field of application where FTs can be used with high efficiency beyond former constraints and can be combined advantageously with autosamplers. Moreover, interest in the LIS technique is growing among FT practitioners. The interest in the concept is indicated also by finding similar approaches to LIS both from companies as well as in recent scientific literature based on other means to mix the syringe content.

- The required instrumentation is simple and cost-efficient and the few elements needed can be easily produced by 3D printing as shown in the example of various elements in our tutorial. Discussed limitations of the LIS technique included commercial support yet with one collaboration already started, improved software for method development, instrumental control, and data acquisition, and competition with existing FT concepts and particularly with autosampler-based automation based on disposable consumables. The future will show if merging both concepts, e.g., for automation of matrix precipitation will be accepted by users outside academia.

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9. Contributions of the author to the contemplated works

The following chronological list of publications have been contemplated in this habilitation thesis. It indicates for each publication the impact factor corresponding to the year of publication or, where this was not possible, the most recent published impact factor of the journal. Furthermore, the quartile (Q1-Q4) of the journal based on given impact factor and year of publication as well as an electronic link based on the DOI number of the respective publication to facilitate online access of the respective article. Finally, the types of contributions of the author [20] of this habilitation thesis are given for which the terminology proposed by the editorial Elsevier was adapted [21].

The corresponding author is underlined, the candidate for the associate professorship and author of this habilitation thesis is printed in bold. The citation scores according to the database SCOPUS including and excluding self-citations are given at the date of 9th March 2023.

1. FERNANDO MAYA, **BURKHARD HORSTKOTTE**, JOSÉ MANUEL ESTELA, <u>VÍCTOR CERDÀ</u>, Analytical and Bioanalytical Chemistry 404 (2012) 909-917. *Lab in a syringe - Fully automated dispersive liquid-liquid microextraction with integrated spectrophotometric detection.*
   IF_{2012} = 3.659 $Q(IF_{2012})$ = 88.67% (**Q1**)   Cited: 83 / 65
   http://doi.org/10.1007/s00216-012-6159-4

   Contributions: Assistance in planning, design and fabrication of system components, participation in writing: review and editing, discussion of results

2. **BURKHARD HORSTKOTTE**, MICHAL ALEXOVIČ, FERNANDO MAYA, CARLOS M. DUARTE, VASILhttp://www.scopus.com/authid/detail.url?origin=resultslist&authorId=8647785900&zone= ANDRUCH, <u>VÍCTOR CERDÀ</u>, Talanta 99 (2012) 349-356. *Automatic determination of copper by in-syringe dispersive liquid-liquid microextraction of its bathocuproine-complex using long path-length spectrophotometric detection.*
   IF_{2012} = 3.498 $Q(IF_{2012})$ = 84.67% (**Q1**)   Cited: 71 / 62
   http://doi.org/10.1016/j.talanta.2012.05.063

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[20] The individual contributions are to be considered as moderate ("assistance in"), balanced against other authors ("participation in"), or considerably (no indication). This is particularly valid for technical items (system setup, method development, component preparation, formal analysis) or when being the first author of the respective publication.

[21] Conception and planning (main conceptual ideas, theory development, setting goals and aims of study), literature research, design and fabrication of system components (not commercially available elements produced e.g. by 3D printing or subtractive machining, e.g. optical fiber adapters, stirring systems, detection cells, etc.), setup of flow system and optimization of operation method (preparing the instrumental setup, computer control, and software instruction protocol), experimental work (experimental planning, optimization of method parameters including data evaluation, method characterization, validation, and application to samples), formal analysis (development of mathematical models, experimental design, statistics), visualization (preparation of figures from data, photographies, etc.), and writing of original draft, review and editing (of manuscript draft), discussion of results, supervision (involving critical feedback, teaching, overall direction and planning), first author, and corresponding author (article submission and communication with editorial)

Contributions: Conception and planning, literature research, setup of flow system and optimization of operation method, participation in experimental work, visualization, and writing of original draft, review and editing, supervision of second author, first author

3. **BURKHARD HORSTKOTTE**, FERNANDO MAYA, CARLOS M. DUARTE, <u>VÍCTOR CERDÀ</u>, Microchimica Acta 179 (2012) 91-98. *Determination of ppb-level phenol index using in-syringe dispersive liquid-liquid micro-extraction and liquid waveguide capillary cell spectrophotometry.*
    IF$_{2012}$ = 3.434   Q(IF$_{2012}$) = 83.33 (**Q1**)   Cited: 27 / 22
    http://doi.org/10.1007/s00604-012-0866-6

    Contributions: Conception and planning, literature research, setup of flow system and optimization of operation method, experimental work, visualization, writing of original draft, first author

4. RUTH SUÁREZ, **BURKHARD HORSTKOTTE**, CARLOS M. DUARTE, <u>VÍCTOR CERDÀ</u>, Analytical Chemistry 84 (2012) 9462-9469. *Fully-automated fluorimetric determination of aluminium in seawater by in-syringe dispersive liquid-liquid microextraction using lumogallion*.
    IF$_{2012}$ = 5.695   Q(IF$_{2012}$) = 96,67% (**Q1**)   Cited: 50 / 43
    http://doi.org/10.1021/ac302083d

    Contributions: Conception and planning, design and fabrication of system components, setup of flow system and optimization of operation method, participation in experimental work, participation in visualization, assistance in writing of original draft, review and editing, supervision of first author

5. **BURKHARD HORSTKOTTE**, RUTH SUÁREZ, PETR SOLICH, <u>VÍCTOR CERDÀ</u>, Analytica Chimica Acta 788 (2013) 52-60. *In-syringe-stirring: A novel approach for magnetic stirring-assisted dispersive liquid-liquid microextraction*.
    IF$_{2013}$ = 4.517   Q(IF$_{2013}$) = 94.08% (**Q1**)   Cited: 74 / 57
    http://doi.org/10.1016/j.aca.2013.05.049

    Contributions: Conception and planning, design and fabrication of system components, setup of flow system and optimization of operation method, experimental work, visualization, writing of original draft, first author

6. CAMELIA HENRÍQUEZ, **BURKHARD HORSTKOTTE**, PETR SOLICH, <u>VÍCTOR CERDÀ</u>, Analytical and Bioanalytical Chemistry 405 (2013) 6761-6769. *In-syringe magnetic-stirring assisted liquid-liquid microextraction for the spectrophotometric determination of Cr(VI) in waters.*
    IF$_{2013}$ = 3.578   Q(IF$_{2013}$) = 86.18% (**Q1**)   Cited: 37 / 28
    http://doi.org/10.1007/s00216-013-7111-y

    Contributions: Conception and planning, design and fabrication of system components, setup of flow system and optimization of operation method, participation in experimental work, assistance in formal analysis, visualization, participation in writing of original draft, review and editing, supervision of first author

7. **BURKHARD HORSTKOTTE**, RUTH SUÁREZ, PETR SOLICH, <u>VÍCTOR CERDÀ</u>, Analytical Methods 6 (2014) 9601-9609. *In-syringe magnetic stirring assisted dispersive liquid-liquid micro-extraction with solvent washing for fully automated determination of cationic surfactants*.
IF_{2014} = 1.821 $Q(IF_{2014})$ = 43.92% (**Q3**)   Cited: 29 / 19
http://doi.org/10.1039/C4AY01695E

   Contributions: Conception and planning, design and fabrication of system components, literature research, setup of flow system and optimization of operation method, experimental work, formal analysis, visualization, writing of original draft, first author

8. IVANA ŠRÁMKOVÁ, **BURKHARD HORSTKOTTE**, PETR SOLICH, HANA SKLENÁŘOVÁ, Analytica Chimica Acta 828 (2014) 53-60. *Automated in-syringe single-drop head-space micro-extraction applied to the determination of ethanol in wine samples*.
IF_{2014} = 4.513 $Q(IF_{2014})$ = 93.92% (**Q1**)   Cited: 43 / 36
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   Contributions: Conception and planning, setup of flow system and optimization of operation method, participation in experimental work and formal analysis, visualization, participation in writing of original draft, review and editing, supervision of first author, corresponding author

9. FERNANDO MAYA, **BURKHARD HORSTKOTTE**, JOSÉ MANUEL ESTELA, <u>VÍCTOR CERDÀ</u>, Trends in Analytical Chemistry 59 (2014) 1-8. *Automated in-syringe dispersive liquid-liquid microextraction*.
IF_{2014} = 6.472 $Q(IF_{2014})$ = 97.97% (**Q1**)   Cited: 73 / 63
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   Contributions: Assistance in writing original draft, review and editing, critical discussion and feedback to help in shaping the manuscript

10. RUTH SUÁREZ, **BURKHARD HORSTKOTTE**, <u>VICTOR CERDÀ</u>, Talanta 130 (2014) 555-560. *In-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction for automation and downscaling of methylene blue active substances assay*.
IF_{2014} = 3.545, $Q(IF_{2014})$ = 85.81% (**Q1**)   Cited: 27 / 24
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   Contributions: Conception and planning, setup of flow system and optimization of operation method, assistance in experimental work, participation in visualization, assistance in writing of original draft, review and editing, supervision of first author

11. <u>MICHAL ALEXOVIČ</u>, **BURKHARD HORSTKOTTE**, PETR SOLICH, JÁN SABO, Analytica Chimica Acta 906 (2016) 22-40. *Automation of static and dynamic non-dispersive liquid phase microextraction. Part 1: Approaches based on extractant drop-, plug-, film- and microflow-formation.*
IF_{2016} = 4.950, Q(IF_{2016}) = 91.45% (**Q1**)   Cited: 77 / 68
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 Contributions: Equal contribution as first author (Statement also in publication): Conception, literature research, visualization, writing of original draft, review and editing

12. <u>MICHAL ALEXOVIČ</u>, **BURKHARD HORSTKOTTE**, PETR SOLICH, JÁN SABO, Analytica Chimica Acta 907 (2016) 18-30. *Automation of static and dynamic non-dispersive liquid phase microextraction. Part 2: Approaches based on impregnated membranes and porous supports.*
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 Contributions: Equal contribution as first author (Statement also in publication): Conception, literature research, visualization, writing of original draft, review and editing

13. IVANA ŠRÁMKOVÁ, <u>**BURKHARD HORSTKOTTE**</u>, HANA SKLENÁŘOVÁ, PETR SOLICH, SPAS D. KOLEV, Analytica Chimica Acta 934 (2016) 132-144. *A novel approach to Lab-In-Syringe Head-Space Single Drop Microextraction and on-drop sensing of ammonia.*
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http://doi.org/10.1016/j.aca.2016.06.039

 Contributions: Conception and planning, setup of flow system and optimization of operation method, participation in experimental work and formal analysis, visualization, participation in writing of original draft, review and editing, supervision of first author, corresponding author

14. <u>MICHAL ALEXOVIČ</u>, **BURKHARD HORSTKOTTE**, IVANA ŠRÁMKOVÁ, PETR SOLICH, JÁN SABO, Trends in Analytical Chemistry 86 (2017) 39-55. *Automation of dispersive liquid–liquid microextraction and related techniques. Approaches based on flow, batch, flow-batch and in-syringe modes.*
IF_{2017} = 7.030   Q(IF_{2017}) = 96.91% (**Q1**)   Cited: 80 / 71
http://doi.org/10.1016/j.trac.2016.10.003

 Contributions: Equal contribution as first author (Statement also in publication): Conception, literature research, visualization, writing of original draft, review and editing

15. RAQUEL SÁNCHEZ, **BURKHARD HORSTKOTTE**, KATEŘINA FIKAROVÁ, HANA SKLENÁŘOVÁ, SALVADOR MAESTRE, MANUEL MIRÓ, JOSÉ-LUIS TODOLÍ, Analytical Chemistry 89 (2017) 3787-3794. *Fully automatic in-syringe magnetic stirring-assisted dispersive liquid–liquid microextraction hyphenated to high-temperature torch integrated sample introduction system-inductively coupled plasma spectrometer with direct injection of the organic phase.*
    IF$_{2017}$ = 6.042   Q(IF$_{2017}$) = 95.68% (**Q1**)   Cited: 28 / 25
    http://doi.org/10.1016/j.chroma.2018.04.055

    > **Contributions:**   Setup and optimization of flow system and operation method, participation in experimental work, assistance in writing of original draft, review and editing

16. **BURKHARD HORSTKOTTE**, KATEŘINA FIKAROVÁ, DAVID J. COCOVÍ-SOLBERG, HANA SKLENÁŘOVÁ, PETR SOLICH, MANUEL MIRÓ, Talanta 173 (2017) 79-87.
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    IF$_{2017}$ = 4.244   Q(IF$_{2017}$) = 88.27% (**Q1**)   Cited: 20 / 18
    http://doi.org/10.1016/j.talanta.2017.05.063

    > **Contributions:**   Conception and planning, setup of flow system and optimization of operation method, experimental work, visualization, writing of original draft, supervision of second author, corresponding and first author

17. **BURKHARD HORSTKOTTE**, NATALIA LOPEZ DE LOS MOZOS ATOCHERO, PETR SOLICH, Journal of Chromatography A 1555 (2018) 1-9. *Lab-In-Syringe automation of stirring-assisted room-temperature headspace extraction coupled online to gas chromatography with flame ionization detection for determination of benzene, toluene, ethylbenzene, and xylenes in surface waters.*
    IF$_{2018}$ = 3.858   Q(IF$_{2018}$) = 82.74% (**Q1**)   Cited: 13 / 16
    http://doi.org/10.1016/j.chroma.2018.04.055

    > **Contributions:**   Conception and planning, design and fabrication of system components, setup of flow system and optimization of operation method, experimental work, visualization, writing of original draft, supervision of second author, corresponding and first author

18. IVANA ŠRÁMKOVÁ, **BURKHARD HORSTKOTTE**, KATEŘINA FIKAROVÁ, HANA SKLENÁŘOVÁ, PETR SOLICH, Talanta 184 (2018) 162-172. *Direct-immersion single-drop microextraction and in-drop stirring microextraction for the determination of nanomolar concentrations of lead using automated Lab-In-Syringe technique.*
    IF$_{2018}$ = 4.916   Q(IF$_{2018}$) = 87.50% (**Q1**)   Cited: 37 / 35
    http://doi.org/10.1016/j.talanta.2018.02.101

Contributions: Conception and planning, setup of flow system and optimization of operation method, literature research, participation in experimental work, visualization, participation in writing of original draft, review and editing, supervision of first author, corresponding author

19. **BURKHARD HORSTKOTTE**, MANUEL MIRÓ, PETR SOLICH, Analytical and Bioanalytical Chemistry 410 (2018) 6361-6370. *Where are modern flow techniques heading to?*
IF_{2018} = 3.286 $Q(IF_{2018})$ = 79.65% (**Q1**)   Cited: 23 / 18
http://doi.org/10.1007/s00216-018-1285-2

    Contributions: Conception and planning, literature research, writing of manuscript, corresponding and first author

20. KATEŘINA FIKAROVÁ, **BURKHARD HORSTKOTTE**, HANA SKLENÁŘOVÁ, FRANTIŠEK ŠVEC, PETR SOLICH, Talanta 202 (2019) 11-20. *Automated continuous-flow in-syringe dispersive liquid-liquid microextraction of mono-nitrophenols from large sample volumes using a novel approach to multivariate spectral analysis*.
IF_{2019} = 4.916 $Q(IF_{2019})$ = 87.79% (**Q1**)   Cited 10 / 9
http://doi.org/10.1016/j.talanta.2019.04.044

    Contributions: Conception and planning, design and fabrication of system components, setup of flow system and optimization of operation method, literature research, assistance in experimental work, formal analysis, participation in visualization and writing of original draft, review and editing, supervision of first author, corresponding author

21. **BURKHARD HORSTKOTTE**, PETR SOLICH. Molecules 25 (2020) 1612. *The Automation Technique Lab-In-Syringe: A Practical Guide.*
IF_{2020} = 4.401 $Q(IF_{2020})$ = 64.89% (**Q2**)   Cited: 18 / 11
http://doi.org/10.3390/molecules25071612

    Contributions: Conception and planning, literature research, writing of manuscript, corresponding and first author

22. KATEŘINA FIKAROVÁ, **BURKHARD HORSTKOTTE**, DANIEL MACHIÁN, HANA SKLENÁŘOVÁ, PETR SOLICH. Talanta 221 (2021) 12142. *Lab-In-Syringe for automated double-stage sample preparation by coupling salting out liquid-liquid extraction with online solid-phase extraction and liquid chromatographic separation for sulfonamide antibiotics from urine*.
IF_{2021} = 6.556 $Q(IF_{2021})$ = 87.93% (**Q1**)   Cited: 28 / 25
http://doi.org/10.1016/j.talanta.2020.121427

    Contributions: Conception and planning, design and fabrication of system components, setup of flow system and optimization of operation method, literature research, assistance in experimental work, participation in visualization, participation in formal analysis, participation in writing of original draft, review and editing, supervision of first and third author, corresponding author

23. CELESTINE VUBANGSI GEMUH, **<u>BURKHARD HORSTKOTTE</u>**, PETR SOLICH. Molecules 26 (2021) 5358. *Lab-In-Syringe with Bead Injection coupled online to high-performance liquid chromatography as versatile tool for determination of nonsteroidal anti-inflammatory drugs in surface waters.*
    IF$_{2021}$ = 4.927   Q(IF$_{2021}$) = 63.97% (**Q2**)   Cited: 3 / 2
    http://doi.org/10.3390/molecules26175358

    Contributions: Conception and planning, setup of flow system and optimization of operation method, participation in literature research, participation in experimental work, visualization, participation in writing of original draft, review and editing, supervision of first author, corresponding author

24. CELESTINE VUBANGSI GEMUH, MILOSLAV MACHÁČEK, PETR SOLICH, **<u>BURKHARD HORSTKOTTE</u>**. Analytica Chimica Acta 1210 (2022) 339874. *Renewable sorbent dispersive solid phase extraction automated by Lab-In-Syringe using magnetite-functionalized hydrophilic-lipophilic balanced sorbent coupled online to HPLC for determination of surface water contaminants*.
    IF$_{2021}$ = 6.911   Q(IF$_{2021}$) = 89.08% (**Q1**)   Cited: 5 / 1
    http://doi.org/10.1016/j.aca.2022.339874

    Contributions: Conception and planning, setup of flow system and optimization of operation method, participation in literature research, assistance in experimental work, visualization, participation in writing of original draft, review and editing, supervision of first author, corresponding author

25. <u>IVANA H. ŠRÁMKOVÁ</u>, **BURKHARD HORSTKOTTE**, LAURA CARBONELL-ROZAS, JAKUB ERBEN, JIŘÍ CHVOJKA, FRANCISCO J. LARA, ANA MARÍA GARCÍA-CAMPAÑA, DALIBOR ŠATÍNSKY. Membranes 12 (2022) 648. *Nanofibrous online solid-phase extraction coupled with liquid chromatography for the determination of neonicotinoid pesticides in river waters.*
    IF$_{2021}$ = 4.562   Q(IF$_{2021}$) = 61.88% (**Q2**)   Cited: 0
    http://doi.org/10.3390/membranes12070648

    Contributions: Assistance in conception and planning, setup of flow system and operation method, assistance in experimental work, discussion of results, visualization, review and editing

26. SERCAN YILDIRIM, DAVID J. COCOVI-SOLBERG, BENGI USLU, PETR SOLICH, **<u>BURKHARD HORSTKOTTE</u>**. Talanta 246 (2022) 123476. *Lab-In-Syringe automation of deep eutectic solvent-based direct immersion single drop microextraction coupled online to high-performance liquid chromatography for the determination of fluoroquinolones.*
    IF$_{2021}$ = 6.556   Q(IF$_{2021}$) = 87.93% (**Q1**)   Cited: 10 / 9
    http://doi.org/10.1016/j.talanta.2022.123476

    Contributions: Assistance in conception and planning, setup of flow system and operation method, assistance in experimental work, participation in writing of original draft, review and editing, supervision of first author, corresponding author

27. KATEŘINA FIKAROVÁ, DANIEL MACHIÁN, SERCAN YILDIRIM, PETR SOLICH, **BURKHARD HORSTKOTTE**. Analytica Chimica Acta 1233 (2022) 340507. *Automated centrifugation-less milk deproteinization and homogenous liquid-liquid extraction of sulfonamides for online liquid chromatography.*
IF$_{2021}$ = 6.911, Q(IF$_{2021}$) = 89.08% (**Q1**)   Cited: 2 / 1
http://doi.org/10.1016/j.aca.2022.340507

   Contributions: Conception and planning, design and fabrication of system components, setup of flow system and operation method, participation in writing of original draft, review and editing, supervision of first, second, and third author, corresponding author

28. SERCAN YILDIRIM, KATEŘINA FIKAROVÁ, VERONIKA PILAŘOVÁ, LUCIE NOVÁKOVÁ, PETR SOLICH, **BURKHARD HORSTKOTTE.** Analytica Chimica Acta 1251 (2023) 340966.
Lab-In-Syringe automated protein precipitation and salting-out homogenous liquid-liquid extraction coupled online to UHPLC-MS/MS for the determination of beta-blockers in serum.
IF$_{2021}$ = 6.911   Q(IF$_{2021}$) = 89.08% (**Q1**)   Cited: 0

   Contributions: Conception and planning, design and fabrication of system components, setup of flow system and operation method, assistance in experimental work, participation in writing of original draft, review and editing, supervision of first and second author, corresponding author