CHARLES UNIVERSITY IN PRAGUE

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DEPARTMENT OF ANALYTICAL CHEMISTRY

SETUP AND CHARACTERIZATION OF AN AUTOMATED METHOD FOR SALT-ASSISTED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION USING A LAB-IN-SYRINGE SYSTEM

DIPLOMA THESIS

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Hradec Králové 2016 Ondřej Bešťák

I hereby declare that this thesis is my own original work. All literature and additional sources used have been duly acknowledged and properly cited in the reference section. This thesis has not been submitted for any degree in any university previously.

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……. …….

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Abstract

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Sequential Injection Analysis (SIA) is a technique derived from the Flow Injection Analysis technique. The system generally consists of a computer-controlled syringe pump, a selection valve, and a detector, all connected by inert plastic tubing. It is used to automate laboratory procedures. The “Lab-In-Syringe” technique is a modification of SIA used to carry out parts of the experiment inside the used syringe pump’s void. Using a PTFE-coated magnetic-propelled stirring bar inside the syringe allows, for example, to mix homogenously the syringe content or to perform liquid extraction protocols such as dispersive liquid-liquid microextraction (DLLME).

In this work, the approach to perform salting-out assisted in-syringe DLLME was explored and evaluated for the first time. Starting with a one-phase system, the analyte was extracted from water into n-propanol. For this, a highly-concentrated solution of magnesium sulfate was used to increase the polarity of the aqueous phase. The high polarity causes the separation of the two normally fully miscible liquids.

Astraphloxin and riboflavin were used as model analytes and various conditions, i.e. salt concentration and water/solvent ratio were tested. Measuring the absorbance in the organic phase was done both in-syringe and at the syringe outlet to evaluate the volume of organic phase, time required for phase separation, and for precise analysis of the extracted analytes. The method performance in dependence of the former parameters was studied, evaluated, and improved to achieve a compromise between a high preconcentration factor and fast phase separation.

The highest achieved preconcentration factor was 6.43. The fastest phase separation took less than 5 s. The reproducibility of 3 repetitive extractions was generally below 1 % RSD.

Using n-propanol, even compounds of moderate polarity can be extracted with high-efficiency. Furthermore, n-propanol is a HPLC compatible solvent, so the extract can be optionally analyzed on-line in modern HPLC systems.

In conclusion, the salting-out assisted DLLME presents an interesting approach to perform a fast, precise, and automated extraction in small scale for the analyte preconcentration using an environment-friendly and HPLC compatible solvent.

Abstrakt

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Sekvenční injekční analýza (SIA) je technika odvozená od Průtokové injekční analýzy. Systém se zpravidla skládá z počítačem řízené stříkačkové (pístové) pumpy, selekčního ventilu a detektoru, kdy je vše spojeno systémem inertních plastových trubiček. Používá se k automatizaci laboratorních procedur. Technika „Laboratoř-ve-stříkačce“ je modifikací SIA, která se používá k vykonání částí experimentu v prostoru ve stříkačkové pumpě. Za použití magneticky poháněné míchací tyčinky potažené vrstvou PTFE ve stříkačce je například možné homogenně smísit obsah stříkačky nebo provést různé extrakční protokoly jako je disperzní mikro-extrakce v systému kapalina-kapalina (DLLME).

V této práci je poprvé zkoumán a hodnocen přístup k vysolovací, ve stříkačce prováděné DLLME. Počínaje v jednofázovém systému, analyt byl extrahován z vody do n-propanolu. Pro toto byl použit vysoce koncentrovaný roztok síranu hořečnatého za účelem zvýšení polarity vodné faze. Vysoká polarita způsobuje fázové rozdělení dvou normálně plně mísitelných kapalin.

Astrafloxin a riboflavin byly použity jako modelové analyty a byly otestovány různé podmínky, např. koncentrace soli nebo poměr mezi vodou a rozpouštědlem. Měření absorbance v organické fázi bylo provedeno jak přímo ve stříkačce, tak na výstupu ze stříkačky, aby bylo možné zjistit objem organické fáze, čas nutný pro fázové rozdělení a pro přesnou analýzu extrahovaných analytů. V závislosti na těchto parametrech byly vlastnosti metody studovány, hodnoceny a zlepšovány za cílem dosažení kompromisu mezi vysokým faktorem prekoncentrace a rychlým fázovým rozdělením.

Nejvyšší dosažená hodnota faktoru prekoncentrace byla 6,43. Nejrychlejší fázové rozdělení zabralo méně než 5 s. Reprodukovatelnost tří opakovaných extrakcí byla obecně pod 1 % RSD.

Za použití n-propanolu mohou být efektivně extrahovány i sloučeniny se střední polaritou. N-propanol je navíc rozpouštědlo kompatibilní s HPLC, takže výsledný extrakt může být případně analyzován on-line moderními systémy HPLC.

V závěru, vysolovací ve stříkačce prováděná DLLME představuje zajímavý přístup k rychlé, přesné a automatizované extrakci v malém měřítku pro prekoncentraci analytu za použití k životnímu prostředí šetrného a s HPLC kompatibilního rozpouštědla.

The objectives and assignment of the thesis

The objective in this thesis was to design, optimize and evaluate a method for salt-assisted homogenous liquid-liquid microextraction using a Lab-in-Syringe system. Optimal solvent and salt solution were to be selected, two detection methods were to be tested (in-syringe detection and flow-cell detection) and the method was to be optimized and evaluated using two model analytes. System characteristics, the method parameters and performance were to be explored and evaluated to deliver a proof-of-concept work report.

Cíle a zadání práce

Úkolem této práce bylo navrhnout, optimalizovat a vyhodnotit metodu pro vysolovací homogenní mikro-extrakci v systému kapalina-kapalina za použití systému Lab-in-Syringe. Bylo záměrem zvolit vhodné rozpouštědlo a roztok soli, otestovat dvě detekční metody (detekce ve stříkačce a detekce v průtokové cele) a metoda měla být optimalizována a vyhodnocena za použití dvou modelových analytů. Systémové vlastnosti, parametry a výkon metody měly být prozkoumány a vyhodnoceny, aby bylo možné vypracovat zprávu dokládající funkci tohoto konceptu.

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1. Introduction

Since its invention in 1975 [1], flow techniques have undergone a great deal of progress. Although many commercially available analyzers are still based on flow injection analysis (FIA) or sequential injection analysis (SIA) [2] techniques, various modifications are being employed on the existing systems. During the past five years, the Lab-In-Syringe technique has been developed based on the SIA setup [3,4] and a number of methods has been designed and examined. Some of the recent setups [5,6] feature an in-syringe magnetic stirring system, which makes them suitable to automate different liquid phase microextraction approaches. .

Liquid-liquid extraction (LLE) protocols usually bring the sample into contact with an immiscible solvent. However, by the addition of a highly concentrated salt solution to an initial homogenous mixture of sample and solvent, phase separation can be induced, allowing for a technique known as homogenous LLE. It enables the use of solvents normally miscible with water such as n-propanol or acetonitrile.

In this thesis, a method, which combines dispersive and homogenous extraction techniques in the syringe void of a Lab-In-Syringe system, is being designed, tested and optimized to achieve optimal parameters for the sample pretreatment. Two different detection systems were employed, tested and evaluated. Astraphloxin (a potent cationic colorant) and riboflavin (vitamin B_2) were used as model analytes.

A focus of the research was to find a compromise between a high preconcentration factor and fast sampling rate, while achieving a high reliability, precision and repeatability.

2. Theory

2.1 Description of analytical flow techniques

2.1.1 Flow techniques in general

When performing analytical chemistry laboratory tasks, there are two approaches to be considered. The traditional way is to work "in batch". The idea is to perform e.g. a reaction between analyte and reagents, mixing the compounds homogenously in a flask or chamber and waiting for the reaction to reach its equilibrium. This is what we can call "beaker chemistry". Most tasks are done manually and all processes are finite. This approach often requires a long time and a significant manual work.

The other way is represented by the flow techniques. This way the sample and reagent do not interact in a beaker but rather in a laminar flow of a carrier within a narrow tubing. A typical flow system also includes a pump and a detector, which are connected by the tubing system (manifold) and valves. The analyzer instrument is controlled by a computer and software.

After being injected into the flow, the sample solution is dispersed in a precisely reproducible matter. The process is defined by specific parameters such as time, flow speed and tubing width and length. That way, the reaction conditions are reproducible. The reaction can be observed in a dynamic manner and quantified before it reaches its equilibrium.

Flow techniques present a versatile automation approach for various common wet chemistry tasks. Compared to conventional "beaker chemistry", they also enable faster sampling rate and require smaller amounts of sample and reagents.

Since the invention of Flow Injection Analysis technique (FIA) in 1975 [1], a number of further setups and techniques has been developed, including Sequential Injection Analysis (SIA) [2], Multisyringe Flow Injection Analysis (MSFIA) [7], Lab-On-Valve (LOV) [8], and latest Lab-In-Syringe (LIS) [3,4], which was used in this work.

2.1.2 Flow Injection Analysis

Flow Injection Analysis (FIA) is the original and most basic flow technique. It was developed in 1975 by Růžička and Hansen [1]. A FIA setup can be readily assembled from fairly common labware being a peristaltic pump, an injection valve, an appropriate detection cell, and polymer

tubing, as shown in **Figure 1**. It is easy to understand and it provides a means to automate wet chemistry laboratory procedures.

The pump is generating a flow of a carrier solution. Typically, the sample is injected into the carrier stream and, if required, more reagents are added by confluences or are present in the carrier. The sample moves through the tubing in a zone, which is dispersed over time. At the zone borders, the analyte can react with a reagent present in the carrier. Higher dispersion increases the penetration of the zones and therefore leads to better solution mixing and potentially higher product yield but also sample dilution.

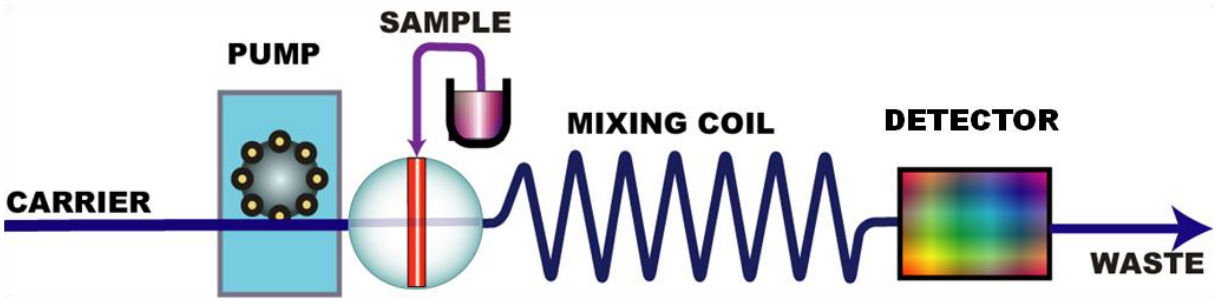

Figure 1: A diagram of a basic FIA system. Picture taken from www.flowinjectiontutorial.com [9]

In laminar flow in a round tube, the sample disperses in a specific fashion. Dispersion in axial directions is promoted by the flow because the central part of the sample zone moves faster than the parts near the tube wall. This results in a forward-facing parabolic shape with a long trailing. Diffusion and various irregularities in the tubing, such as coils, curves or confluence points promote dispersion in radial direction. This is why FIA systems use a coiled mixing tubes. With the axial and radial dispersion together, we finally achieve a non-symmetric shape with distinct tailing.

The resulting reaction is then observed in a detector. For detection, optical methods, i.e. spectrophotometry, are typically used.

Since the dispersion always occurs in the same way (assuming reproducible flow and timing), the whole process is reproducible, including the reaction kinetics. With that in mind, the reaction product can be detected before the reaction finishes, which speeds up the whole process significantly.

2.1.3 Sequential Injection Analysis

Sequential Injection Analysis (SIA) was developed in the 1990s, the first paper being published in 1990 by Růžička and Marshall [2]. In contrast to FIA, a SIA system features a syringe pump and a selection valve connected by tubing (holding coil) as depicted in **Figure 2**. The syringe pump allows to pump a small and precise volume of analyte in both directions – to and from the syringe. The selection valve can be switched between multiple channels, which can be used for the aspiration or dispense of different samples and reagents in a given sequence – hence the sequential injection. It should be pointed out that it is intended that the solutions never reach the syringe but remain between aspiration and renewed dispense in the holding coil, which is in contrast to the technique explained in the following.

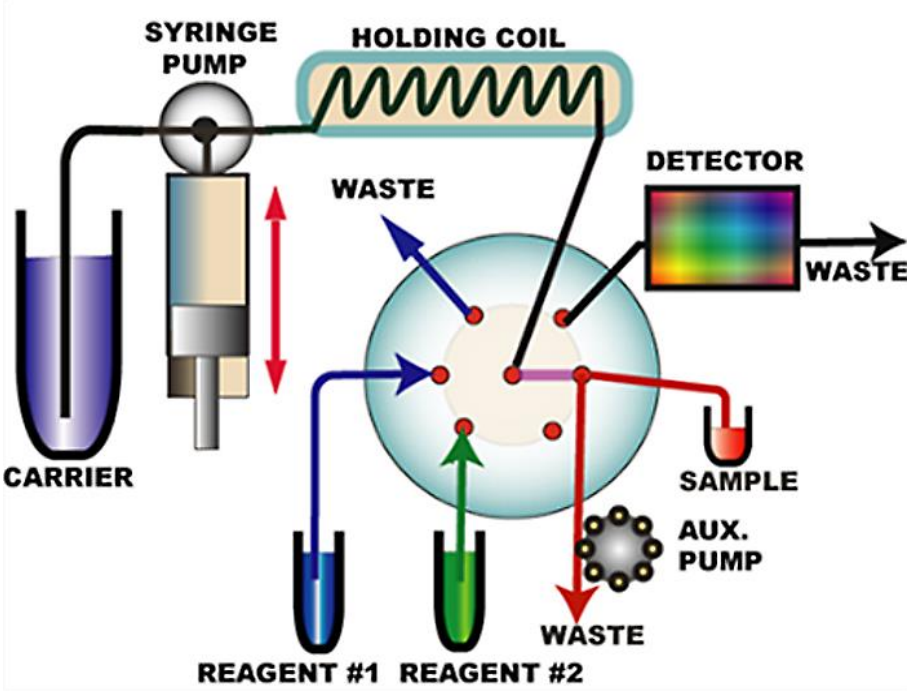

Figure 2: A diagram of a basic SIA system. Picture taken from www.flowinjectiontutorial.com [10]

Employment of a computer controlled syringe pump also enables stopping or reversing the flow direction. This is useful for more thorough radial dispersion and better mixing of the sample and reagents. Stopping the flow with the sample zone inside the detection cell also allows us to observe the reaction kinetics in the process.

While FIA analyzers are generally simpler and faster, SIA instruments are much more versatile, allowing for more complex reactions to be performed.

2.1.4 Lab-In-Syringe

The Lab-In-Syringe (LIS) is a relatively new flow technique, in which the syringe void is used as reaction and mixing chamber rather than a tubing system, as shown in **Figure 3**.

The first work of such automation was reported by Maya et al. (2012) [3], the use of later added in-syringe stirring in different modes was invented by Horstkotte et al. (2013, 2014) [5,6].

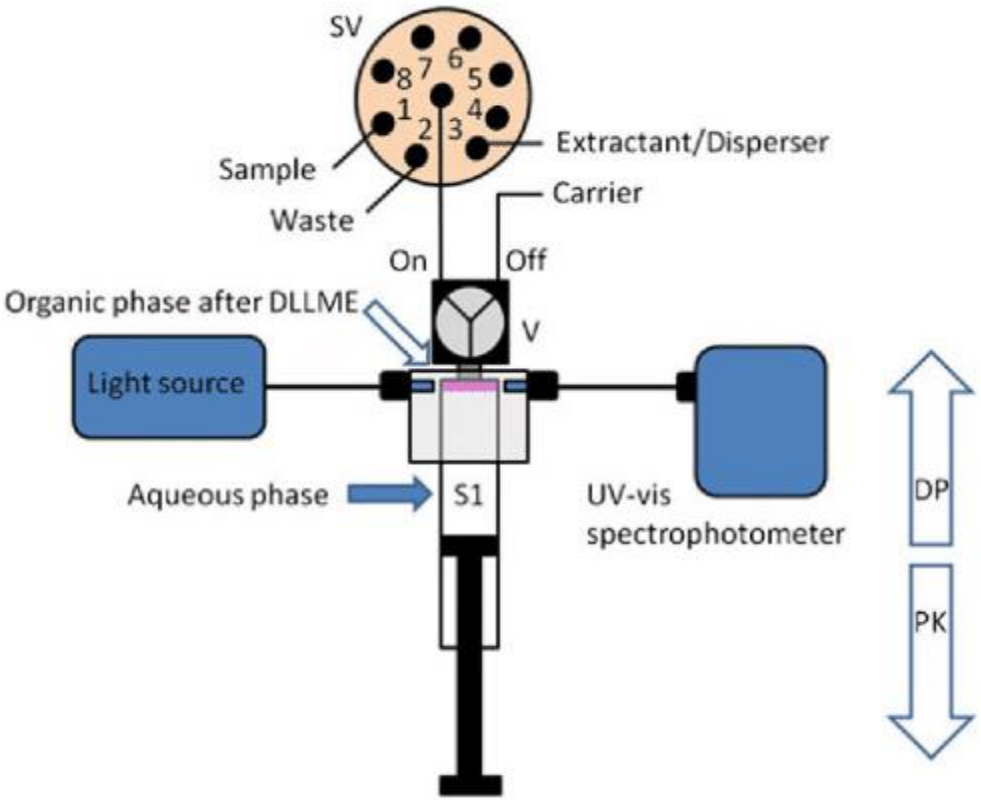

Figure 3: A diagram of a basic LIS system, as depicted by Maya et al. [4]. SV = selection valve, V = syringe valve, S1 = syringe, DP = dispense, PK = pickup.

The LIS system is usually a slightly modified/upgraded SIA or MSFIA system. A key benefit of the LIS is the ability to perform procedures, which benefit from large sample volumes and thorough mixing of solutions such as liquid-liquid extraction (LLE). This can be achieved for example by the aspiration of a small volume of extraction solvent into the syringe. By the placement of a magnetic stirring bar inside the syringe, the aqueous phase and a small volume of extracting solvent can be brought into thorough contact.

The detection can also be carried out in-syringe as reported by Maya et al. (2012) [4]. Because the syringe is made from a transparent material, i.e. glass, and of precise width, it allows to be used as a photometric cuvette.

The LIS can be considered as both a flow technique and a batch technique. For some uses, such as LLE, the flow injection system is only employed for transportation of sample and reagents in the syringe void, where the reaction takes place, often homogenously and up to the point of reaching an equilibrium. On the other hand, reactions can be performed in a flow matter as well. The capabilities of a SIA analyzer can be further expanded in a LIS system by adding new batch functionalities.

2.2 Extraction techniques

2.2.1 Liquid-liquid extraction

A liquid-liquid extraction (LLE) is a well-known separation method used in most of the chemistry fields. Importantly, LLE is employed in bioanalysis for an extraction of an analyte from its biological matrix.

In the basic setup of LLE, the sample solution (generally aqueous) is brought into direct contact with another immiscible liquid (generally an organic solvent). The analyte molecules, present in the original solution or "sample" will distribute between both phases according to the Nernst's distribution law [11], as shown in the following equation:

$$\mathbf{P = c_1/c_2}$$

P = distribution constant, c_1 = analyte concentration in phase 1, c_2 = analyte concentration in phase 2

As the equation states, the ratio between concentrations of the analyte in both phases is a constant. This is valid for a constant temperature and as long as the analyte does not form any associates with the solvent molecules.

An ideal extraction solvent shows a much higher dissolving capacity for the analyte than the original solvent, generally water, but none for other, potentially interfering components of the sample, denoted "sample matrix".

Since the two liquids are immiscible, a boundary layer is formed between them. The extractable molecules pass through the interface. The larger the contact area surface is, the higher the rate of the extraction. Therefore, it is beneficial to expand the interfacial area as much as possible to allow faster extraction. This can be achieved by emulsification of the two liquids (sample and extraction solvent) as reviewed by Moradi et al. in 2014 [12].

The LLE is often scaled down to a smaller form factor. Miniaturized extraction is called microextraction. Miniaturized LLE is often referred to as liquid-liquid microextraction (LLME). Based on the emulsification process, we distinguish between two types of LLME:

Dispersive liquid-liquid microextraction (DLLME)

In DLLE, the emulsion is formed by external forces. The dispersion can be caused e.g. by kinetic energy, such as a rapid injection of the solvent, stirring, shaking or vortexing, or by radiation factors, such as ultrasound or microwave energy. This process results in an interdispersion of the two phases with a large contact area. After the external factors causing the dispersion are ceased, the emulsion quickly disintegrates into two liquid layers by droplet floatation or sedimentation or phase separation is forced by centrifugation. The DLLME was first described by Rezaee et al. in 2006 [13].

Homogenous liquid-liquid extraction (HLLME)

In HLLE, the two liquids initially form a homogenous solution, which is then separated into two phases by a change of physical parameters. This can be done by chemistry-based methods, such as change of pH or addition another reagent (e.g. a salt), or by change of the solution temperature. At application of at least one of these measures, a very fine emulsion is formed, which then disintegrates into two separate phases. The extraction has the fastest rate at the moment when the emulsion is being formed, i.e. when the contact area surface is the largest.

Homogenous and dispersive modes of LLE can be combined to improve the extraction rate. That can be achieved by applying a homogenous extraction technique and further mix the two emerging phases.

2.2.2 Salting-out assisted LLE

Salting-out assisted LLE is one mode of HLLE. Normally, the LLE would require using two phases with a notable difference in polarity. When dealing with an aqueous sample, a lipophilic solvent, by this immiscible with water, is required. This in turn prevents an extraction of substances with low lipophilicity.

Some organic solvents, such as 1-propanol, 2-propanol, or acetonitrile are miscible with water thus cannot be used for classic LLE. However, addition of salt or a highly saturated salt solution to the mixture strongly increases the polarity of the aqueous phase, resulting in a decrease of

the solvent miscibility. A phase separation is then forced between the two otherwise miscible liquids. This way, an extraction can be carried out between aqueous sample and the aforementioned solvent.

By adding the highly polar salt solution, the equilibrium between phases is shifted. While a more hydrophilic/polar solvent can be used, the aqueous phase also becomes more polar. Substances that would normally favor the aqueous phase are extracted into the organic phase.

The same principle is also employed in bioanalysis, when proteins are precipitated from solutions by the change in polarity due to some protein insolubility in highly polar solution. This process does not require the use of another phase, as the proteins from the sample form a solid phase instead.

The Hofmeister series classifies common cations and anions by their ability to salt-out or salt-in proteins. The series are shown below. It is noteworthy that anions have a significantly larger effect than cations [14].

$$\mathbf{F^- \approx SO_4^{2-} > HPO_4^{2-} > acetate > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^- > SCN^-}$$

$$\mathbf{NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > guanidinium}$$

These ionic substances increase the surface tension of the solvent and decrease the solubility of non-polar compounds. Equal principles are applied in salting-out assisted LLE. Hitherto, the Hofmeister series can be taken into account when choosing a salt for salting-out-assisted HLLE.

QuEChERS is an established set of methods for sample pretreatment and analyte extraction from organic matrixes [15]. The word QuEChERS is the abbreviation of the words “Quick, Easy, Cheap, Effective, Rugged and Safe”. Among other techniques, QuEChERS includes the salting-out assisted homogenous-dispersive LLME based on magnesium sulfate plus sodium chloride mixture as a salt.

2.3 Liquid phase microextraction performed by Lab-In-Syringe

DLLME can be performed in syringe of a flow system. DLLME in a LIS system was first described by Maya et al. in 2012 [4]. Initially, the dispersion was caused by a rapid aspiration of a solvent mixture into a syringe. By this, the same principle was used as in the first work describing DLLME by Rezaee et al. (2006), albeit not automated [13].

In 2013, DLLME in a LIS system with magnetic in-syringe stirring was firstly described by Horstkotte et al. [5]. In this setup, an in-syringe magnetic stirring system is developed for more controlled dispersion. Since then, this approach has been used for different analytes and tasks including chromate determination by Henríquez et al. [16], automation of methylene blue active substances assay for determination of anionic surfactants by Suárez et al. [17] or estrogens determination by González et al. [18].

A similar system can be used in a different fashion as well, as demonstrated by head-space single-drop microextraction by Šrámková et al. [19]. In that work, a significant portion of air was aspirated into the syringe, and a single drop of solvent was used to absorb volatile ethanol fumes from the sample solution below. The single drop was then dispensed through a flow cell and the extracted ethanol contents were measured.

In the present work, magnetic-stirring assisted salting-out DLLME in a LIS system was explored for the first time.

2.4 Model analytes

Astraphloxin (also known under the names Astra Phloxine FF, Astra Phloxine G, Astra Phloxine and many others) is a quaternary ammonium cation, structure depicted in **Figure 4**. Its MW = 392.96 g/mol. Thanks to its cationic nature it can also be used for the determination of anionic substances (extraction as ion-pair) [20,21]. It is soluble in water or lower aliphatic alcohols. Its absorption maximum is at 539 nm in water and 548 nm in n-propanol. It has a relatively low toxicity rated at 18 mg/kg (LD50 in rat) [22].

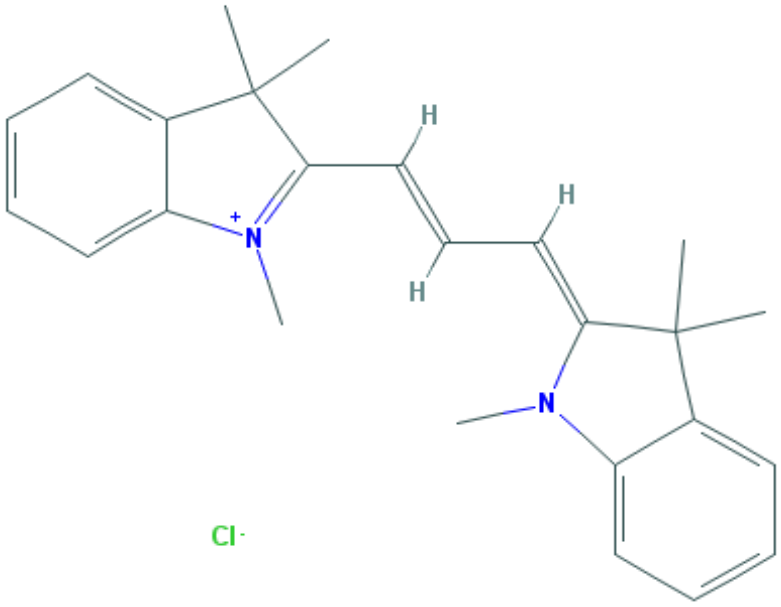

Figure 4: Astraphloxine structure. [22]

Astraphloxine is a potent colorant, even in concentration levels around 1 µmol/l it produces a distinctive red/magenta color. Therefore, the procedure in the syringe could be observed by spectrophotometer as well as by the naked eye.

Riboflavin (also known as vitamin B_2) is a flavin derivate, structure shown in **Figure 5**, MW = 376.36 g/mol. As a precursor to flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD), it is an essential human nutrient. It is found mainly in yeast, milk, eggs or liver. When isolated, it forms orange crystals, it is soluble in water (85 mg/l in 25°C) and slightly soluble in lower aliphatic alcohols. In aqueous solutions it produces a distinctive yellow color in concentrations around 10 μmol/l. It is non-toxic. [23]

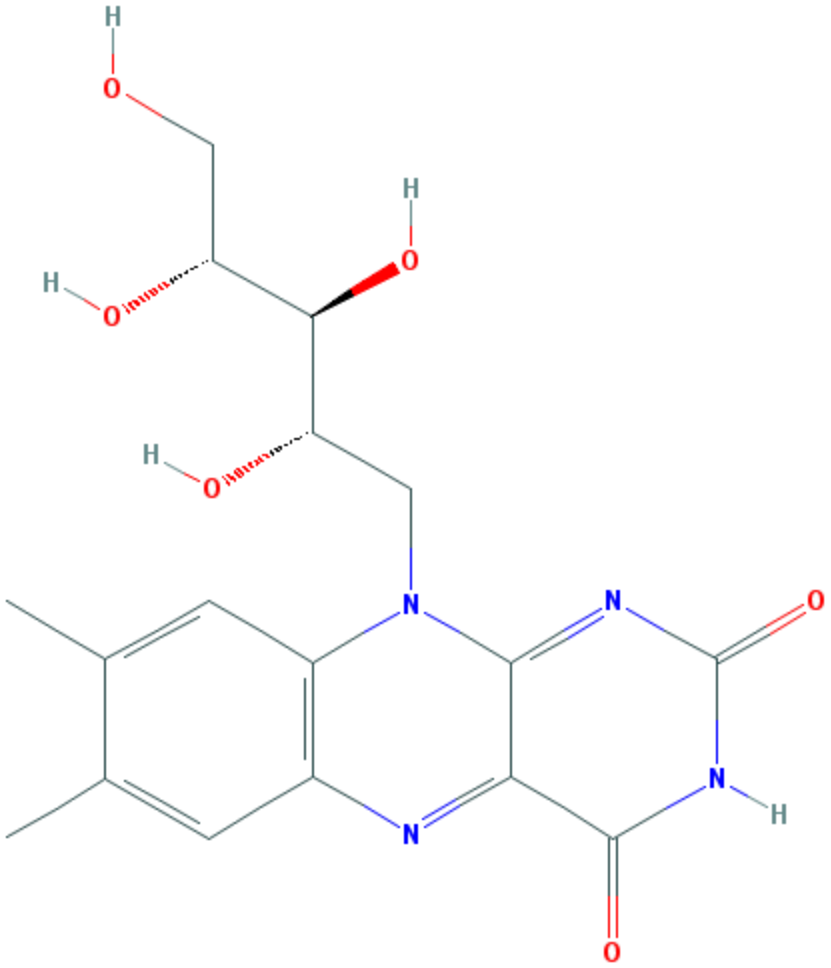

Figure 5: Riboflavin structure. [23]

Riboflavin was selected as a continuance to the experiment to test the method with an analyte of much lower lipophilicity. It also promised some real-life usability for the method, since it is found in many drinks and food supplements.

3. Objectives

The following objectives were set in this thesis:

1. Determination of an optimal solvent and salt component to use for a salt-assisted in-syringe homogenous liquid-liquid extraction (HLLE).
2. Setup of an analyzer instrumentation and evaluation of its characteristics such as the dead volume inside the syringe.
3. Preparation of an operation method for automated homogenous liquid-liquid extraction.
4. Evaluation of the procedure performance characteristics phase separation time, preconcentration factor, extraction efficiency, repeatability, and sampling rate using astraphloxine and riboflavin as two model analytes by variation of final salt concentration and the ratio of organic solvent to aqueous phase using both in-syringe and at-syringe detection.
5. Comparison and discussion of both detection modes and outcomes of the performed optimization
6. Choice of an optimal working conditions under consideration of maximal repeatability, sampling rate, and preconcentration factor.
7. Testing and evaluating the method using astraphloxine and riboflavin as a second model analyte of higher hydrophilicity.

4. Materials and method

4.1 Reagents and analytes

Water of bidistilled quality provided by a Milli-Q purification system from Merck Millipore (Darmstadt, Germany) and reagents of analytical grade were used throughout the entire research.

As extraction system, n-propanol, isopropanol, and acetonitrile, all of analytical reagent grade and 100 % V/V purchased from Sigma Aldrich (Darmstadt, Germany) were tested.

As salt component, a highly concentrated aqueous solution of magnesium sulfate was used. The solution was prepared by adding 550 g of $MgSO_4 \cdot 7\ H_2O$ into 900 ml of water and the mixture was stirred and heated up in a water bath to a point when there were no observable particles of the solid salt. After the solution was let to cool down to ambient temperature, it was filled up to 1000 ml.

For initial tests, a solution of ammonium acetate was used. The solution was prepared in the exact same fashion as for the magnesium sulfate, with the concentration being the same as well – 550 g of NH_4CH_3COO per 1000 ml of solution.

To prevent precipitation, both salt solutions were stocked at the lab temperature.

The first model analyte used was astraphloxine, IUPAC name (2Z)-1,3,3-trimethyl-2-[(E)-3-(1,3,3-trimethylindol-1-ium-2-yl)prop-2-enylidene]indole;chloride, MW = 392.96 g/mol.

A stock solution I was prepared by diluting 78.6 mg of astraphloxine into 10 ml of water corresponding to a concentration of 20 mmol/l. This stock solution I was kept in a dark vial at 4°C. A second stock solution (II) in the concentration of 100 µmol/l was prepared from stock solution I and used typically for not more than a week. It was stored in a volumetric flask likewise at 4°C. Working solutions showed concentrations in the range of 0.1–5 µmol/l. These were prepared ad hoc by appropriate dilution of stock solution II.

The second model analyte was riboflavin, also known as vitamin B_2, IUPAC name 7,8-dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4-dione, MW = 376.36 g/mol.

A stock solution was prepared by diluting 18.8 mg of riboflavin into 250 ml of water corresponding to a concentration of 200 µmol/l. This stock solution was stored in a volumetric

flask at 4°C. Working solutions showed concentrations in the range of 1–30 µmol/l. These were prepared ad hoc by appropriate dilution of the stock solution.

A solution of potassium chromate, MW = 194.19 g/mol, was used to determine the syringe dead volume. The solution was prepared by dissolving 485.5 mg of K_2CrO_4 in 100 ml of water resulting in a concentration of 25 mmol/l.

All substances used were of analytical grade quality and used and stored in concordance with their stability requirements. Aside from potassium chromate, all substances were non-toxic and ecologically safe.

4.2 Instrumentation

The main instrument used was a FIAlab-3500 Sequential and Flow Injection Analyzer from FIAlab Inc. (Seattle, WA, USA). It is equipped with a rotatory multiposition valve of 8 positions, a high resolution syringe pump equipped with a 5 ml glass syringe of 6 cm stroke, and a 4 channel peristaltic pump. The peristaltic pump was not used in this work. The syringe pump featured a rotary head valve with two positions, namely IN and OUT for connection with the multiposition valve and waste, respectively.

The instrument's software is compatible with various detection techniques such as UV/VIS spectrophotometry, fluorescence, chemiluminescence, and electrochemical detection. The FIAlab-3500 was connected to a PC with Windows 7 operating system and controlled using the FIAlab for Windows control software, version 5.11.10 (FIAlab Inc.), supporting all of the above-mentioned detection techniques.

The schematics of the entire system with both detection systems is depicted in **Figure 6A**.The setup featured an in-syringe detection system (**Figure 6** – 1) as well as an outer detection cell connected at the syringe output (**Figure 6** – 2). The in-syringe detection system consisted of a polymer adaptor ring (**Figure 6B**) with mounting connectors on the opposite sides for attaching a bright white LED as light source on one side and an optical fiber leading to a spectrophotometric detector to the other side as shown in **Figure 7**. The ring itself was placed onto the syringe barrel and held in place with rubber rings as it can be seen in **Figure 8**. The second detection cell was placed at the syringe outlet to waste (syringe head valve to position OUT) via a 10 cm long PTFE tubing. It consisted of a flow-cell made from ULTEM resin for spectrophotometric measurement with a light path channel of 10 mm length and 1 mm width.

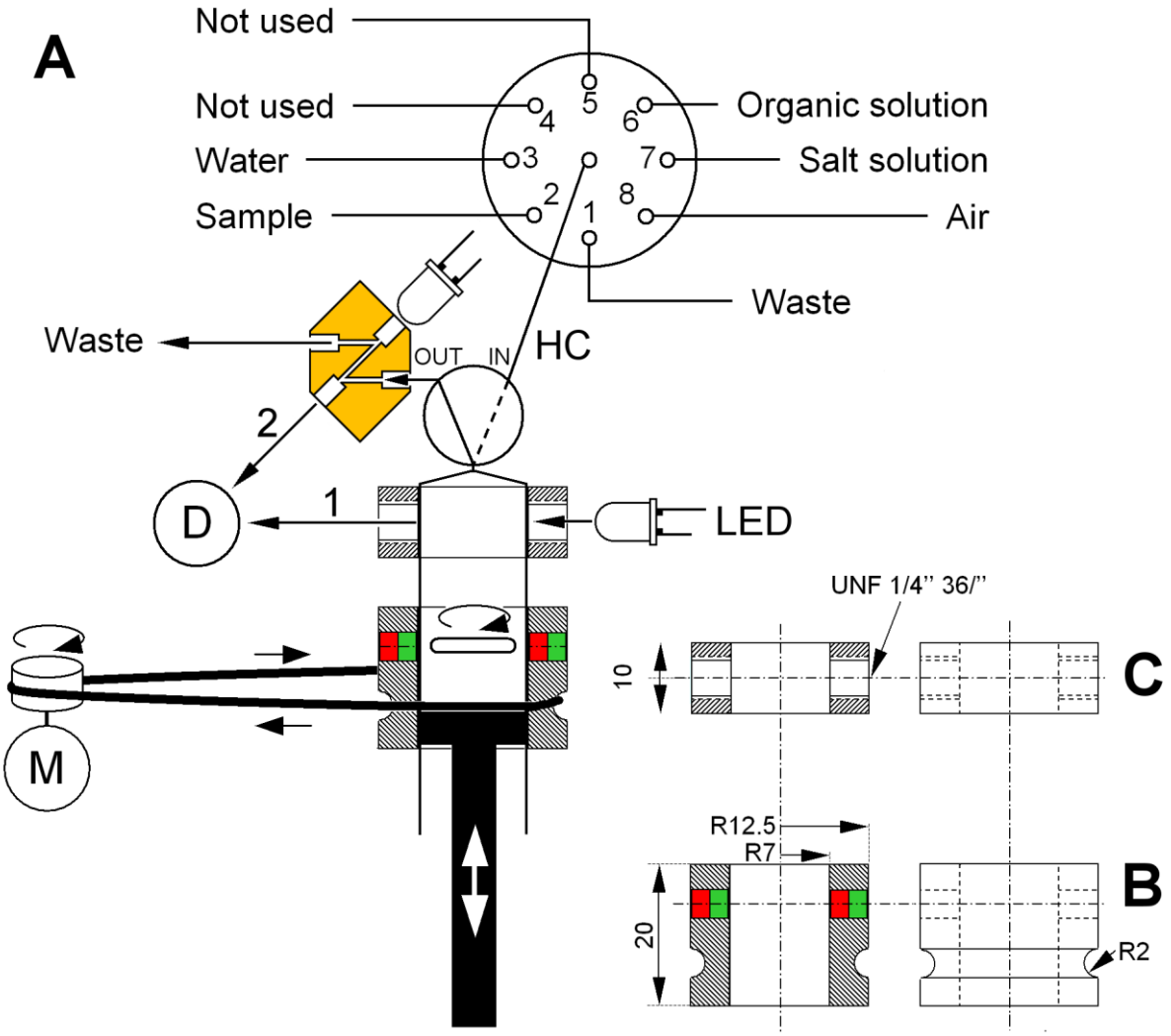

Figure 6: The schematics of the used system. A: The connection layout of the selection valve; B: the dimensions of the stirring system ring; C: the dimensions of the in-syringe detection adaptor ring.

The setup integrated a miniature fiber optic diode array spectrophotometer from Ocean Optics Inc. (Dunedin, FL, USA), type USB2000. The spectrometer was connected to the computer by USB 2.0 and to either the at-syringe detection flow cell (for measurement on the syringe outlet, configuration as in **Figure 6** – 2) or to the adaptor ring (for in-syringe measurement, configuration as in **Figure 6** – 1, the adaptor ring shown in **Figure 7**) by an optical fiber (1 mm quartz core diameter) purchased from Ocean Optics Inc.

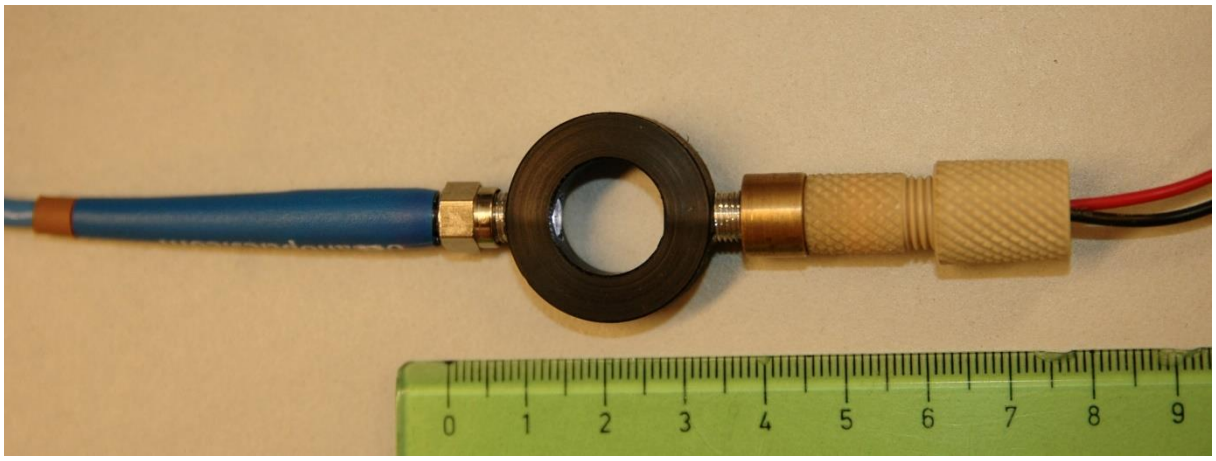

Figure 7: The polymer adaptor ring used for in-syringe detection with an LED attached on the right and an optical fiber leading to the spectrophotometer on the left.

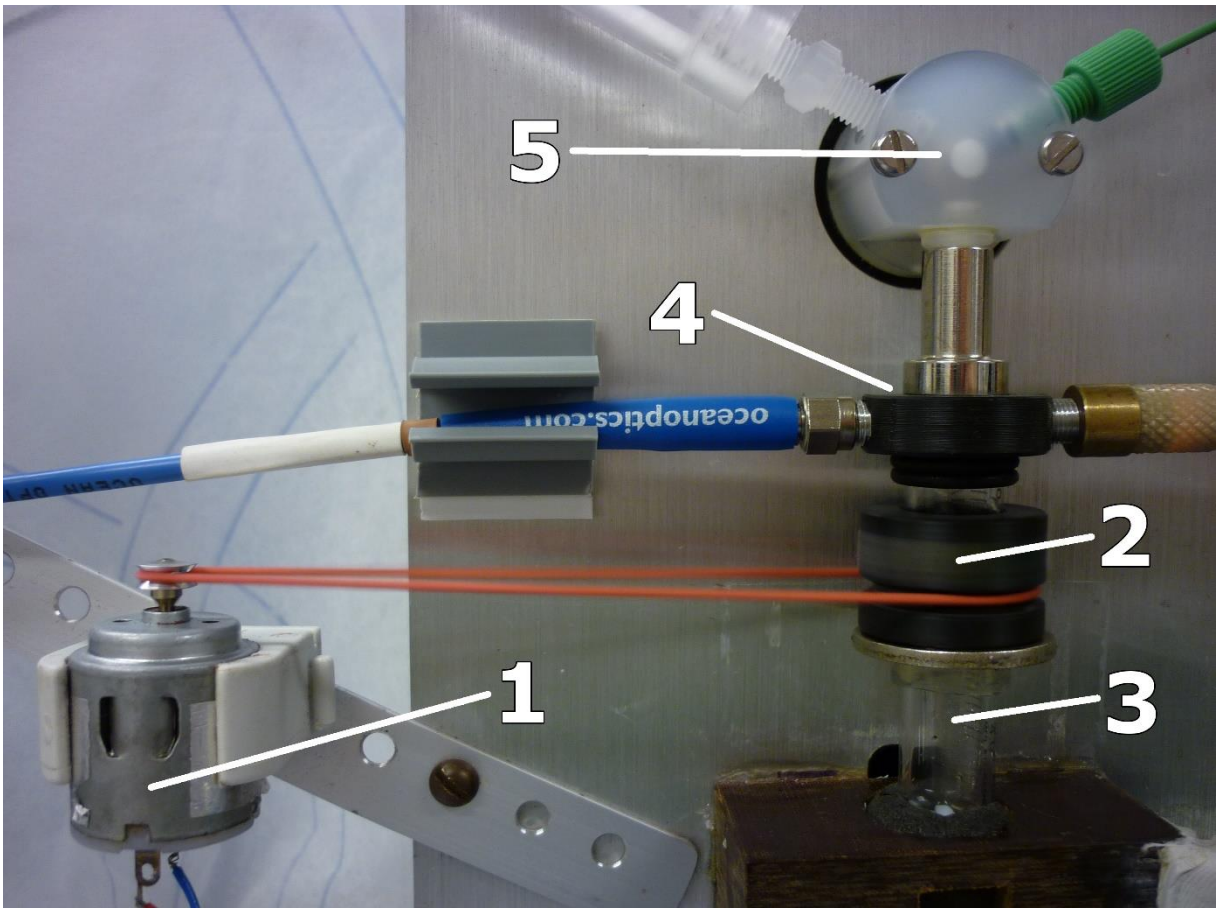

Figure 8: The stirring system in motion. 1 – Motor; 2 – Stirring ring with magnets; 3 – Syringe; 4 – Optical fiber adaptor ring for in-syringe detection; 5 – Syringe head valve.

An in-syringe stirring system was implemented, consisting of a PTFE-coated magnetic stirring bar inside the syringe and a plastic ring holding two neodymium magnets, placed onto the

syringe barrel and rotating around the syringe axis (**Figure 6C**). It was driven by a rubber band and connected electric motor as shown in **Figure 8**.

The stirring system was activated by the control software. At switching the motor on, its rotor propelled the rubber band used as an adaptor belt for the plastic ring. With the ring rotating, the neodymium magnets inside it forced a corresponding movement in the magnetic stirring bar inside the syringe.

In addition, a spectrophotometer model 8453 from Hewlett-Packard (Palo Alto, CA, USA) was used for the acquisition of spectra of the model analytes.

4.3 Operation methods

The instruments and the software have been started and properly initialized. All solutions have been attached by a plastic tubing to their respective positions at the selection valve in the following order (also seen in **Figure 6**):

Table 1: The selection valve connection order.

channel	*connection*
1	waste
2	sample
3	water
4	not used
5	not used
6	organic solvent
7	salt solution
8	air

A 1 l waste bottle was connected to the position 1 as well to the position “OUT” of the syringe head valve.

At the beginning of each day, the entire system was cleaned with water. The syringe was then filled with water and a dark scan and a blank scan were performed with the LED switched off

and on, respectively. After these steps have been carried out, the system was ready for the measurement procedure.

The pre-extraction procedure consisted of the following steps:

Table 2: The pre-extraction procedure in steps.

step	*action*
1.	The spectrometer is set to proper wavelengths.
2.	The syringe is emptied into the waste.
3.	The syringe is cleaned twice with solvent, twice with water and finally filled with water.
4.	The reference scan is performed.
5.	The syringe is emptied into the waste.
6.	The syringe is cleaned three times with sample and filled with sample.
7.	The sample scan is performed.
8.	The syringe is emptied into the waste.
9.	The syringe is cleaned once with solvent.

The pre-extraction procedure precedes the extraction procedure. The actual extraction steps are depicted in **Figure 9**. The extraction procedure consisted of the steps as shown in

Table 3. Usually in one procedure batch, the pre-extraction procedure was performed once and the extraction procedure was performed multiple times with various settings.

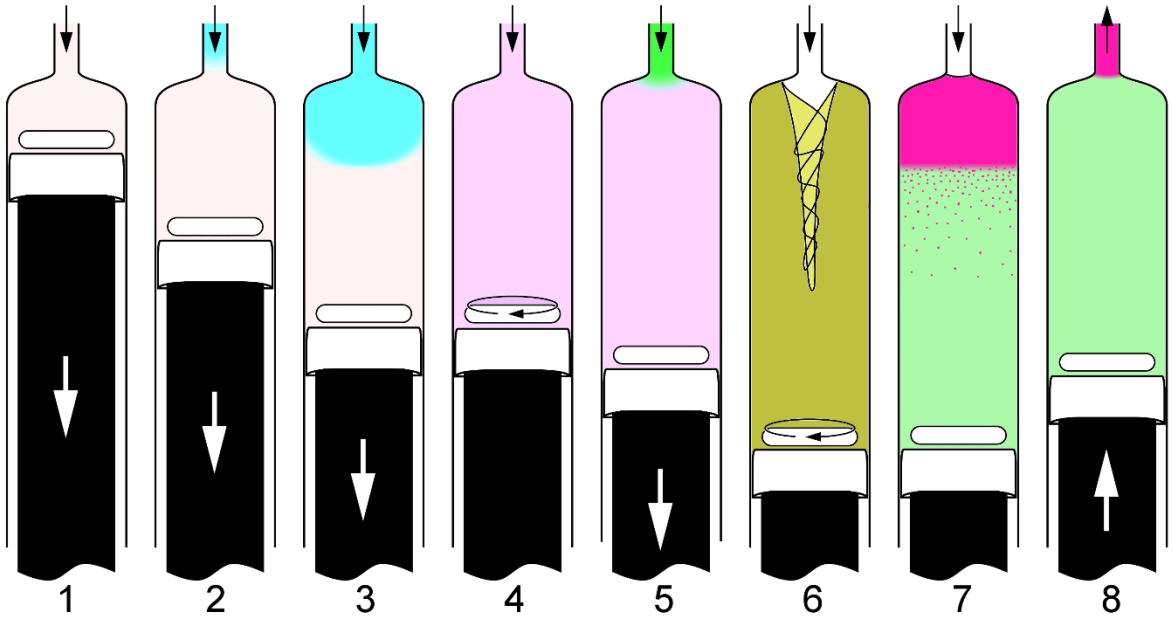

Figure 9: Schematic representation of in-syringe HLLE. 1 – a sample is aspirated, 2–3 – the solvent is aspirated, 4 – the sample is mixed with the solvent into a homogenous solution, 5 – the salt solution is aspirated, 6 – the syringe content is mixed, 7 –phase separation occurs, 8 – the resulting extract is dispensed through the detection flow-cell to waste. Note: the numbers of the steps are not equivalent with the numbers used in **Table 3**.

Table 3: The extraction procedure in steps.

step	*action*
1.	The syringe is cleaned three times with sample.
2.	The sample is been aspirated into the syringe.
3.	The solvent is been aspirated into the syringe.
4.	The sample and solvent are mixed by the in-syringe stirring system.
5.	While the salt solution is being aspirated, the syringe contents are being mixed by the in-syringe stirring system.
6.	While the syringe content is being mixed, 200 µl of air is aspirated into the syringe.
7.	After 10 s wait-time the in-syringe stirring is stopped.
8.1	If the in-syringe detection is employed, the absorbance scanning is being performed for 120 s. Afterwards, the syringe is emptied to waste.
8.2	If the detection at the syringe output is employed, the mixture in the syringe is let to stabilize for 120 s. Afterwards, the syringe is being slowly (50 µl/s) emptied into the waste, while the absorbance scanning was being performed.

5. Results and discussion

5.1 Identifying problems

In the beginning of the work, there was a number of problems to be addressed. The general idea about how to perform the homogenous-dispersive LLME has been outlined by previous works. Performing a DLLME in a LIS system is a known method developed by Maya et al. [3,4], various method of emulsion-based LLE have been reviewed by Moradi et al. [12]. The task at hand was to combine a salting-out assisted HLLME with DLLME and to develop a fully functional method in a LIS system based on the aforementioned protocols.

When performing procedures in a LIS system, there are certain volume limitations. The sample, the organic solvent and the salt solution have to fit in the volume of the syringe.

Considering the spectrophotometric detection, proper wavelengths had to be determined. Finally, the system also needs to be thoroughly and rapidly cleaned between the extractions. Some of the issues during the early experiments were system malfunctions, mainly of the stirring system.

Selecting proper salt solution and solvent, determining their volumes, analyte absorption wavelengths and other procedure parameters are input variables that had to be outlined in order to achieve favorable output variables. These include a fast sampling rate, a high preconcentration factor, quantitative analyte extraction, and high precision and accuracy.

5.2 Dead volume problematic

Due to the use of the stirring bar inside the syringe, the syringe cannot be emptied completely. The resulting dead volume, i.e. the remaining liquid at syringe emptying is ideally given a cylinder of the height of the stirrer and diameter as the inner diameter of the syringe minus the stirring bar volume. Moreover, the holding coil and the syringe head valve also contribute to the dead volume.

The only mode to achieve complete syringe emptying would be to turn the syringe upside down, so that air, accumulating in the syringe or actively aspirated in the beginning of the procedure, would press out the solutions at emptying. However, such configuration was used rather for solvent of higher density than water [6].

Because these dead volumes would contribute to the volume of aqueous phase in the syringe, which was of interest to be known in this work to calculate the final phase ratio, prior to any measurements, experiments with potassium dichromate were performed to determine the combined dead volume of the syringe, syringe valve, and holding coil. Dichromate was chosen as it shows an intense color but does not stick to any surfaces such as organic indicators.

A volume of 0.4 ml of a 2.5 mmol/l solution of $K_2Cr_2O_7$ was diluted with 3.6 ml in-syringe as well as offline. The experiments in-syringe were performed in triplicate. For in-syringe dilution, the syringe was washed with water three times before each test so that the dead volume would be filled only of water. Both resulting solutions were analyzed by in-syringe spectrophotometric detection. For measuring the off-line prepared solution, the syringe was cleaned with the same solution. From the difference between the absorbance values it was calculated that the dead volume is approximately 0.57 ml as deduced below:

For the evaluation of the dead volume inside the syringe ($V_{Syringe}$), first, it was cleaned with water (three-times aspiration of water through the holding coil and discharge through the secondary head valve port). Then, 400 µL of 25 mmol/l chromate ($V_{Chromate}$) and 3.6 ml of water (V_{Water}) were aspirated through the holding coil into the syringe and mixed. The measured absorbance A_1 was compared to the absorbance obtained, when the syringe was cleaned and filled with an equal mixture, but which was prepared manually, A_0. Preparing the solution inside the syringe resulted in a 7.7 % lower absorbance, i.e. 0.823 AU for A_1 and 0.892 AU for A_0. Using formula shown in **Equation 1**, the dead volume inside the syringe, evaluated from threefold repetition, was then 336 µL. The volume of water, which was aspirated into the syringe (3.6 ml) did not include the volume of water remaining in the holding coil and head valve, so that these dead volumes did not contribute to the calculation.

$$V_{Syringe} = \left(\frac{A_0}{A_1} - 1 \right) \cdot \left(V_{Chromate} + V_{Water} \right)$$

Equation 1: The dead volume calculation. A_0 = the absorbance of offline mixture, A_1 = the absorbance of in-syringe mixture, $V_{Chromate}$ = potassium dichromate solution volume, V_{Water} = water volume.

To evaluate the dead volume including the holding coil and head valve, the difference was to aspirate the remaining water in these compartments by aspiration of air, so that all water would go into the syringe. The difference in the calculated dead volume from the first and second

experiment can then be related to the ex-syringe dead volumes. The resulting absorbance value was detected at 0.782 for A_1.

Dead volume of syringe……...336 µl
Dead volume of syringe, holding coil and syringe head valve……...567 µl
Dead volume of holding coil and syringe head valve…….231 µl

All methods feature an aspiration of 250 µl of air following all liquids before the extraction. This was done to push the entire dead volume of holding coil and syringe head-valve into the syringe. All calculations are therefore adjusted for the combined dead volume of the syringe, holding coil and syringe head-valve.

It was decided that rather than correcting all future results based on this error, it is both simpler and more accurate to wash the syringe before each extraction cycle three times with the sample solution. This ensured that the dead volume consisted of the sample, and as sample was the first liquid aspirated in the syringe before the actual extraction, it would not affect the results in any way. This triple-washing was implemented in each tested method, both at the beginning of each extraction and before all sample-standard scans. Before reference scan, the syringe was washed twice with solvent and twice with water. The drawback was a higher consumption of the sample.

The dead volume however decreased the salt concentration and affected the phase ratio. This effect was taken into account in the following experiments and calculations.

5.3 Finding a suitable salt solution

In a manually performed salting-out extraction, salt can be added to the sample in form of a powder to achieve oversaturation with salt. This mode is done also e.g. in the QuEChERS sample preparation technique [15].

In this work however, a solution, i.e. a reagent form that could be handled by the syringe pump, had to be used. To be able to use a minimum amount of solvent, the final salt concentration had to be maximized, i.e. the salt solution added to the sample-solvent mixture should be as concentrated as possible, ruling out salts of low solubility.

Looking for the most potent salt solution, there are two things to be taken into account. The salting out potency increases with the salt concentration in the solution, which means that a salt

with very high solubility in water has to be chosen. The other thing is that ions have different salt out potential described by Hofmeister [14]. When selecting a suitable salt, we were inspired by QuEChERS as well as by Hofmeister series

Magnesium sulfate was one candidate to test for salting out since it is successfully employed as main salting out component in QuEChERS protocols. In this work, magnesium sulfate was tested as simple solution, not in combination with a second salt component. Another considered candidate was ammonium acetate not only because it is well soluble but also it presents a volatile salt so rests of salt present in the final extract would be less problematic e.g. if analyzed by mass spectrometry.

In manually performed preliminary experiments, equal volumes of model analyte solution, n-propanol, and salt solution were mixed. The concentrations of the salt solutions were 550 g of $MgSO_4 \cdot 7\ H_2O$ per liter and 550 g of NH_4CH_3COO per liter, being close to the saturation point and high concentrations would have been impractical to use facing problems like salt precipitation. A fast phase separation occurred using the magnesium sulfate solution, whereas using the ammonium acetate the solution remained homogenous. Therefore, we selected magnesium sulfate as salt for all experiments.

Another salt that was considered was the ammonium sulfate. However, the $(NH_4)_2SO_4$ is more soluble in organic solutions than $MgSO_4$. Moreover, $MgSO_4$ is the standard salt used in the QuEChERS protocols, which is why it was deemed to be a better choice.

5.4 Finding a suitable solvent

Usually in DLLME, a water immiscible solvent such as n-hexanol or chloroform is employed. To perform HLLE, a solvent is needed, which is fully miscible with water or highly soluble in water, but which also easily reaches oversaturation if salt is added. In order to use the syringe in the usual manner, the solvent also needs to have a lower density than water, so it can float at the top part of the syringe, where the in-syringe detection takes place. Last but not least, solvents which would be compatible with HPLC were favored as this work was considered a proof of concept of automation of an HPLC friendly sample pretreatment procedure.

Based on their partition coefficient log(P-octanol/water) values, density, and solubility in water, several solvents were considered for this work, given in **Table 4**.

Table 4: Physical properties of the considered solvents.

substance	*solubility in water [g/l]*	*log(P)*	*density [g/cm3]*
n-butanol	73	0.84	0.810
n-propanol	unlimited	0.25	0.803
iso-propanol	unlimited	0.05	0.786
acetone	unlimited	-0.24	0.785
acetonitrile	unlimited	-0.34	0.786

Since the solvent should be miscible with water, i.e. at least in ratios of 1:2, the use of n-butanol was ruled out from the beginning. The higher the log(P) value, the less salt would be theoretically needed to divide a homogenous phase into the two phases. The problem in flow techniques is that the salt has to be added as a concentrated solution which becomes diluted by the sample solution. In consequence, the ion concentration required for phase separation with acetonitrile, used typically in QuEChERS, can hardly be reached. Also, other solvents might not be as easily available in laboratories as n-propanol, e.g. iso-butanol or methylacetate.

The initial tests showed that phase separation did neither occur with acetonitrile nor acetone using a concentrated solution of $MgSO_4$.

Further manual tests were then conducted with n-propanol and iso-propanol. For this, equal volumes of sample solution, solvent and salt solution were mixed. A 1:1 mixture with n-propanol showed to separate into two phases in less than 5 s at the addition of the salt solution, whereas the same mixture but with iso-propanol remained homogenous for almost 1 min before slowly dividing into two phases.

Another test was done with the sample solution, solvent and salt solution in the ratio of 3:2:3. This time, the mixture with n-propanol produced two phases in 40 s, while the mixture with iso-propanol still remained homogenous.

Since the highest possible volume of sample and the lowest possible volume of solvent is needed in order to achieve a maximal preconcentration factor, and the speed of the extraction is also an important feature, n-propanol was chosen as optimal solvent. No further tests were conducted with other solvents since they show a lower partition coefficient, which would lead to even less favorable extraction parameters than with iso-propanol.

5.5 Determination of analytical wavelengths

Quantification of the analyte in both sample and extract was performed with UV/VIS absorption spectrophotometry. To determine the most suitable wavelengths, absorbance spectra were taken for solutions of both analytes for both solvents. The astraphloxine solutions showed concentration of 29 µmol/l, the riboflavin solutions showed concentration of 20 µmol/l. The spectra were analyzed at the Hewlett-Packard spectrometer model 8453.

Astraphloxine showed an absorption maximum at 539 nm in aqueous solution as shown in **Figure 10**. For the organic solvent, a spectral shift towards longer wavelengths was observed – the determined maximum was 548 nm – as shown in **Figure 11**. Therefore, we decided to measure synchronously at both wavelengths and to determine the resulting absorbance at its higher value. A reference wavelength to compensate absorbance effects which are not specific to the analyte was set to 600 nm. In addition, a vertical spectral shift was observed. To compensate for this, all readouts at 548 nm have been decreased by 3.7 %.

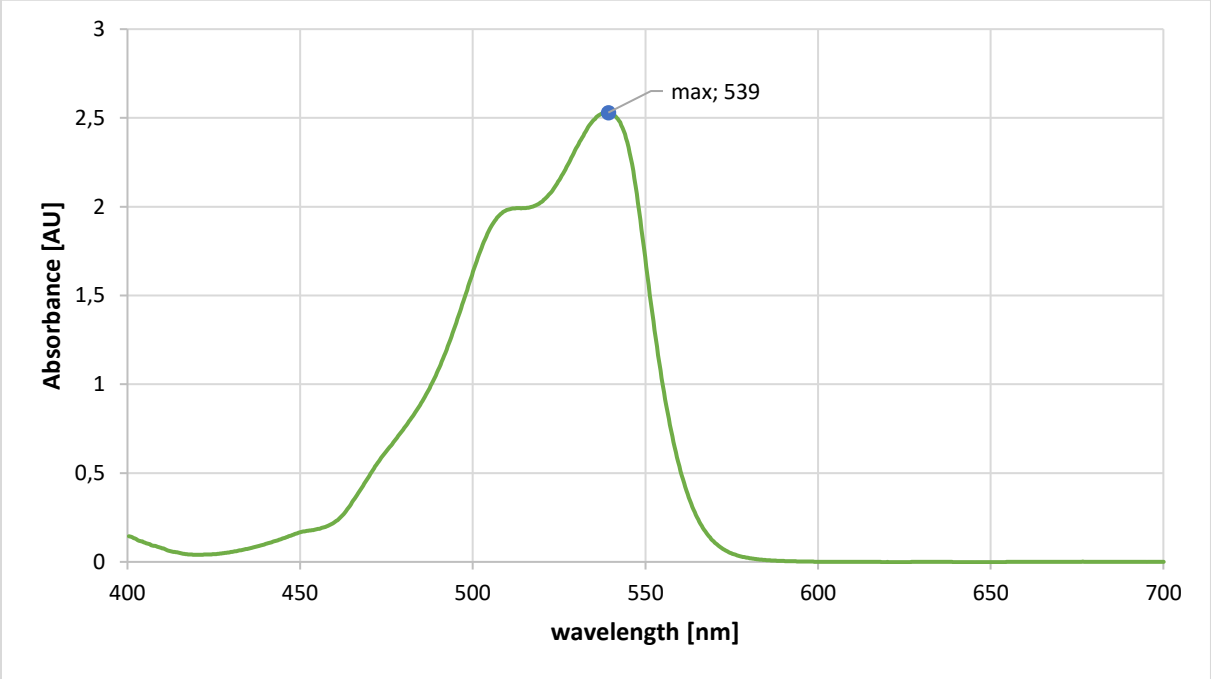

Figure 10: The absorption spectrum of astraphloxine in water at 29 µmol/l.

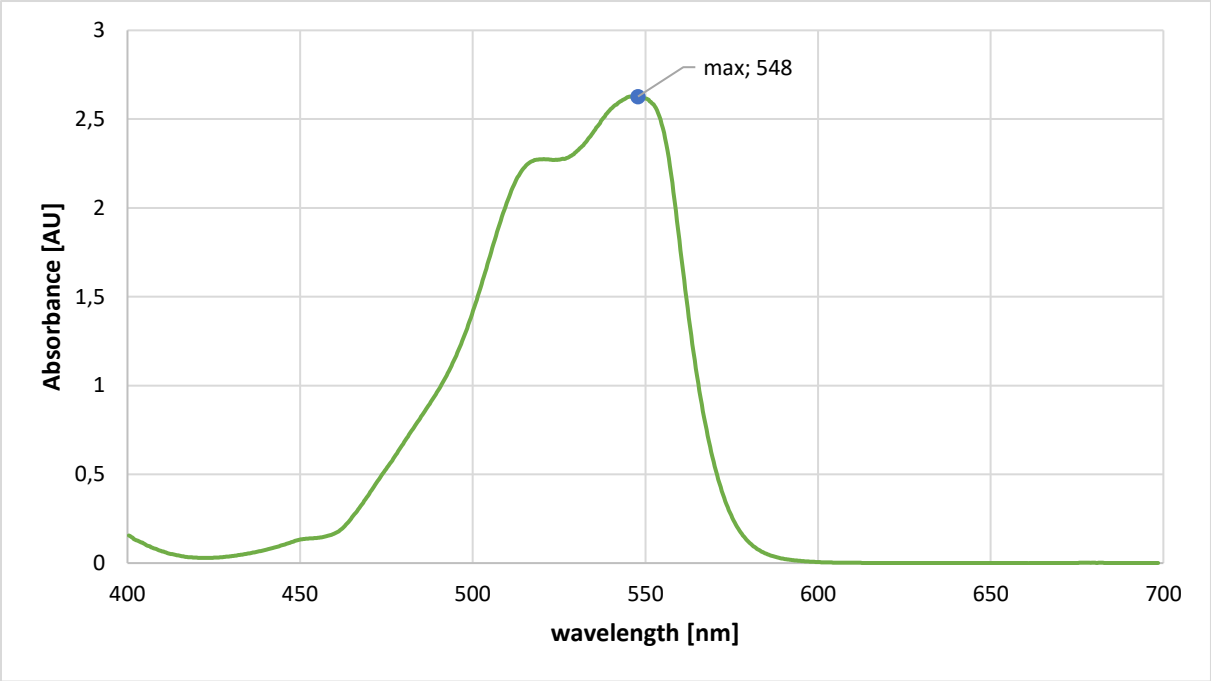

Figure 11: The absorption spectrum of astraphloxine in n-propanol at 29 µmol/l.

Riboflavin showed two absorption maxima at the ultraviolet part of the spectrum at 222 nm and 270 nm, and one maximum in the visible part of the spectrum at 447 nm. The spectral shift due to the different solvents was negligible. The wavelength of 447 nm was selected to perform detection scans, the reference wavelength was set to 600 nm.

5.6 Initial estimates

Occurrence of phase separation, final ratio of organic and aqueous phase, and extraction performance would depend strongly on the initial phase ratio and salt concentration in the aqueous phase. It can be estimated that:

1. Phase separation will not occur for very low salt concentration and low amount of solvent, as it would completely dissolve in the aqueous phase.
2. Phase separation time, and correspondingly the procedure sampling rate will be slower for low salt concentrations and low solvent volume.
3. The preconcentration factor will be higher for higher sample volume and lower solvent volume. As there will be more analyte present and smaller organic phase to extract it to, the extract concentration will increase.

4. The extraction efficiency will be higher for higher salt concentration in the aqueous phase and for the higher volume of the organic phase.
5. Repeatability should not correlate with the given parameters, only with the proper method design and thorough in-process system cleaning.

Some of the observed variables are in conflict to each other. For example, a high preconcentration factor can correlate with a long extraction and phase separation time and therefore slow down the overall sample throughput. With this fact in mind, it was determined to outline a method with multiple parameter-settings, each one optimized for an individual parameter.

In **Figure 12**, a three-dimensional chart shows an estimation of the dependency of the preconcentration factor on the salt concentration and the phase ratio. This has been outlined after some manual initial tests, but before any actual measurement. Generally speaking, the lower the solvent amount and the salt content, the higher preconcentration factor is possible to achieve, although not enough solvent or salt results in failed phase separation.

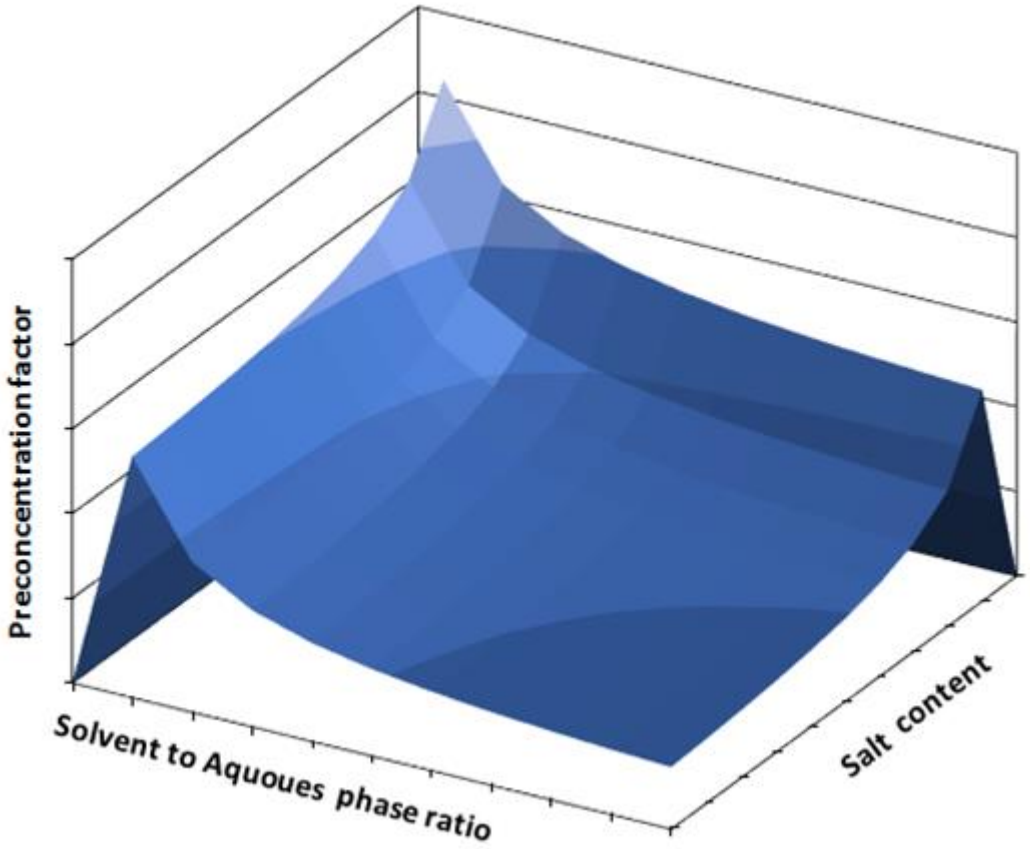

Figure 12: General dependency of the preconcentration factor on the salt concentration and phase ratio. Zero preconcentration factor indicates that the phases would not separate.

5.7 The systematic approach to the experiments

After some initial manual tests and considerations, the necessary solutions and solvents were selected and prepared. All experiments were carried out using 0.5 µmol/l astraphloxine as a model sample unless explicitly said otherwise.

Preliminary experiments were done with 1.0 ml of solvent and 1.5 ml of sample solutions varying the volume of the salt solution. After these tests it was decided to test systematically series of given solvent volume from 0.7 ml to 1.3 ml varying simultaneously the volumes of sample and salt solutions to yield a total volume of 3.0 ml.

After studying solvent volumes of 0.7, 0.9, 1.1 and 1.3 ml, another series using 0.8 ml of solvent was added. This was done because the results for solvent volumes of less than 1.1 ml showed

higher levels of preconcentration. Also, while the results using 1.1 ml and 1.3 ml of solvent were similar, there was a significantly larger difference in results using 0.7 and 0.9 ml of solvent.

It was decided to measure first the phase separation kinetics as described in section **5.11.1** for which in-syringe detection had to be used. After the phase separation time was known, it would be possible to advance with evaluating the preconcentration factor, extraction efficiency and sampling rate. This second task was first attempted using the flow-cell detection measurements as described in section **5.12** to allow determination of the total volume of obtained solvent after phase separation. Later, another method was tested using the in-syringe detection, as described in section **5.11.2**. This was to update the cleaning procedures and improve the format of the resulting data, and subsequently to determine the preconcentration factor, sapling rate and repeatability.

Upon finalizing the study with astraphloxine, the second model analyte riboflavin was tested to evaluate the applicability to an analyte of high hydrophilicity, described in in section **5.13**.

5.8 Phase separation characteristics

One of the important parameters of the method was the time required for phase separation because it presents a limitation for the total time of analysis. Phase separation was indicated by the completed division of the extraction emulsion into two homogenous phases, the colored organic phase with the extracted analyte being positioned at the top and the aqueous phase with salt positioned below (shown in **Figure 9-7**). To evaluate this time, detection inside the syringe was used to record an absorption signal.

When the phase separation occurs, it indicates that the extraction procedure was carried out successfully. The time of the phase separation limits the overall speed of the procedure. The shorter the time of phase separation, the faster sampling rate can be achieved.

The phase separation time was determined from the in-syringe measurements. Once all solutions have been aspirated into the syringe and the mixing process has been finished, the measurement was started. The signal of a successful extraction followed a sigmoid-like shape as shown exemplarily in **Figure 13**.

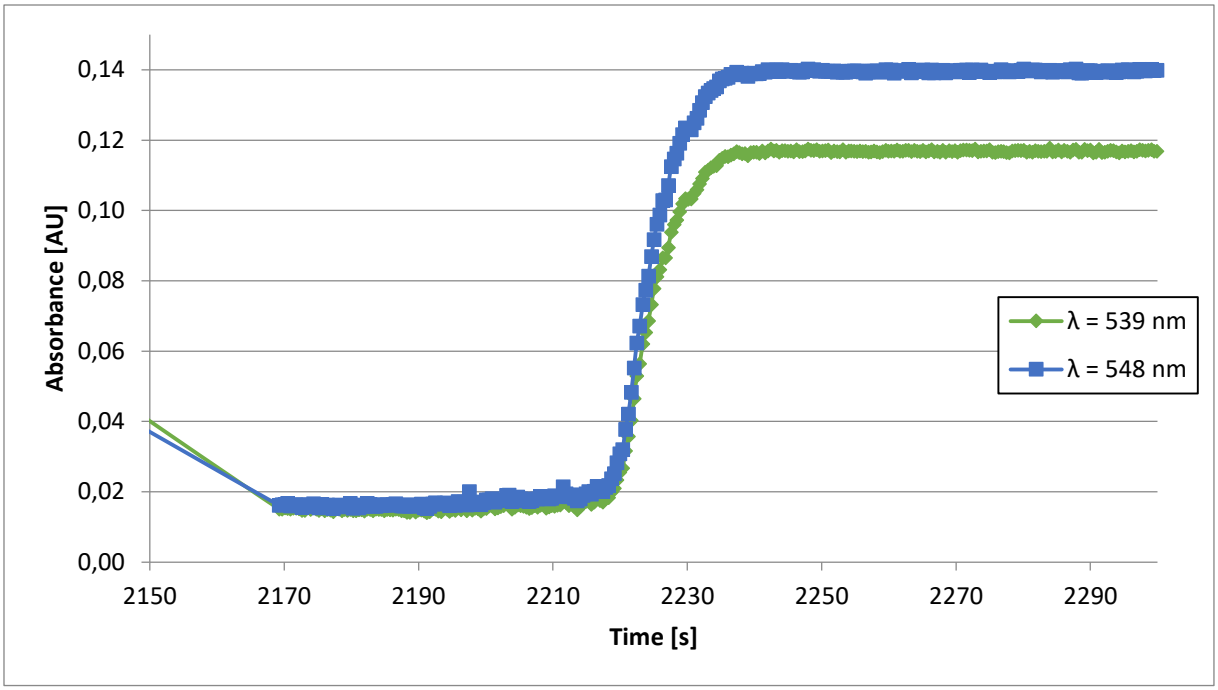

Figure 13: A typical segment of the chart from the in-syringe detection measurements with astraphloxine, capturing the phase separation phenomenon. The procedure was using was using 0.7 ml of solvent, 1.8 ml of salt solution and 1.2 ml of sample.

As it can be seen, the signal course started from a stable low absorbance value being the absorbance of the homogenous mixture minus the absorbance at a reference wavelength to compensate the scattering effect due to the dispersed droplets (2170-2190 s). After some time the values gradually increase because the solvent droplets with the extracted analyte float (2190-2230 s) and accumulate at the top of the syringe and then even in front of the optical fiber entrance (2230-2300 s) leading to the spectrophotometer. Finally, a stable level is reached indicating that the phase separation is finished.

It is important to note that the evaluated time is a good estimation but not identical with the time required for phase separation. This is because detection in-syringe was done on a fixed height. So a phase separation resulting with a very small final volumes of solvent would not be detected because the solvent would float above the fiber optics unregistered. On the other side, for very large volumes of solvent after phase separation, the process might proceed still while the signal is already stable because the boundary layer is located below the optical fiber. The final volume of solvent and with that a correction of the estimated phase separation time could only be done by measuring the amount of organic solvent in the outflow of the syringe (at-syringe detection cell, configuration as in **Figure 6-2**), which was tested in section **5.12**.

As it can be seen, the solvent phase with the extracted analyte is homogenous as the signal does not change once it reaches the upper level. The phase separation time is then specified by the difference between the time of the first recorded point and the first recorded point of constant values (+/- 5 %).

In some cases a phase separation did not occur. The resulting signal course is shown exemplarily in **Figure 14**. This can be caused by a too low ratio of the volumes of solvent and sample or by a too low concentration of salt in the aqueous phase. Obviously, the HLLME protocol cannot work without achieving phase separation so that these parameter settings were omitted and settings with more solvent and/or more salt were studied.

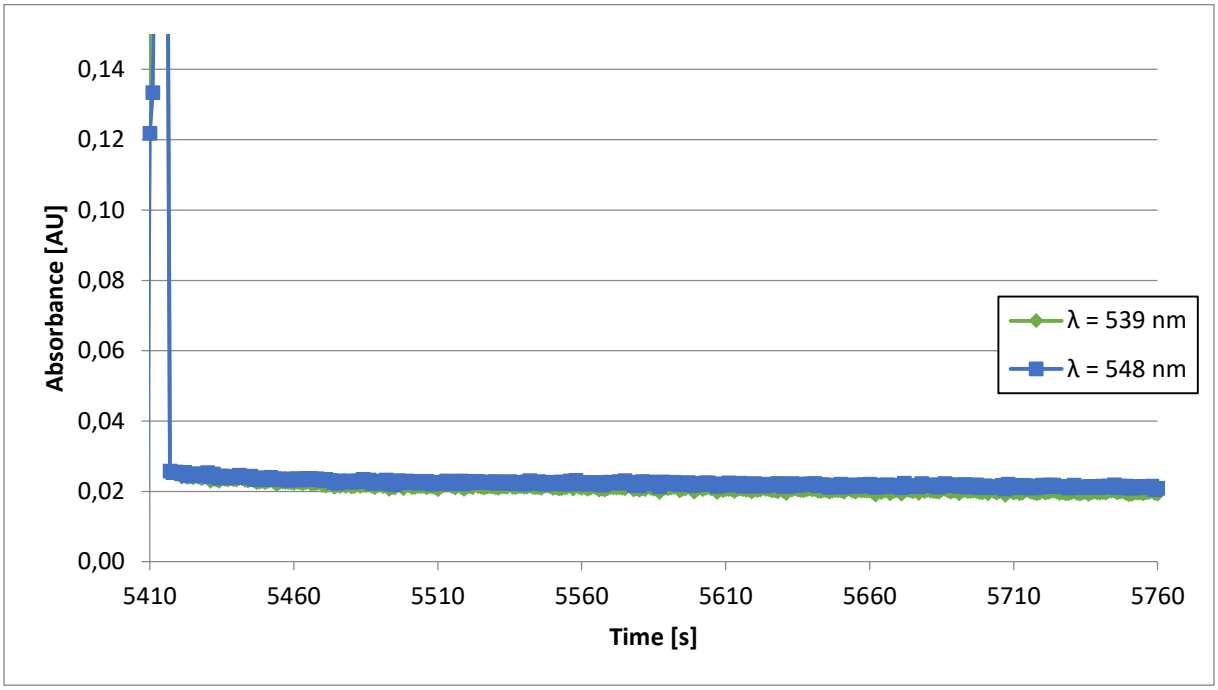

Figure 14: A typical segment of a chart from the in-syringe detection measurements with astraphloxine when phase separation did not occur. The procedure was using 0.9 ml of solvent, 1.2 ml of salt solution and 1.8 ml of sample.

It must be said that for a resulting large volume of organic phase after phase separation, the real time could be still ongoing while already a stable signal level is registered. This is because the part of the liquid in which droplets are still floating is below the optical fiber. This only has a negligible effect though, because an accurate measurement is possible as soon as the phase stabilizes at the top level, since there were never any changes in the signal recorded past that point. Also, large volumes of resulting organic phase logically correlate with very fast phase separation times.

5.9 Preconcentration factor evaluation

The preconcentration factor is the main important parameter of the extraction. It characterizes the resulting increase of the analyte concentration. Thus, it is calculated as the ratio between the concentration of the analyte in the organic phase after the extraction and the concentration of the analyte in the original sample solution. According to Lambert-Beer Law, the concentration is commensurable to the absorbance, so the factor can be calculated from the absorption values as shown in the following equation in case that the molar absorbances for both organic phase and aqueous phase are equal:

$$F_c = c_{org}/c_{sample} \approx A_{org}/A_{sample}$$

F_c = preconcentration factor, c_{org}, A_{org} = concentration of the analyte in the organic extract, resp. absorbance of the extract at the wavelength suitable for organic solutions, c_{sample}, A_{sample}, concentration of the analyte in the sample, resp. absorbance of the sample at the wavelength suitable for aqueous solutions.

Both absorbance values can be determined using either in-syringe detection and detection at the flow-cell at the syringe output. It was therefore necessary to measure the sample absorbance before the extraction at some point of measurement. This was usually done at the beginning of the experiment. In this work, the preconcentration factor was calculated from the values recorded during in-syringe detection measurements. This allowed us to read out the preconcentration factor from the same set of results as the phase separation time.

5.10 Extraction efficiency and resulting phase volumes

Besides the phase separation time and the preconcentration factor, there were two other parameters of interest, the extraction efficiency and the phase volumes.

The extraction efficiency can be described as a percentage of the solute extracted from the sample into the organic phase – the extract. For two immiscible solvents it can be calculated be the following formula:

$$\mathbf{EE = 1-(c_{aq}/c_{sample}) \approx 1-(A_{aq}/A_{sample})}$$

EE = extraction efficiency, c_{sample}=concentration of analyte in original sample, A_{sample} = absorbance of analyte in original sample, c_{aq} = concentration of analyte in resulting aqueous phase, A_{aq} absorbance of analyte in resulting aqueous phase.

This is because the analyte concentration is commensurable to molar amount, but only if the volume of the phase does not change significantly. However, using a miscible solvent as in this work a significant part of the solvent will be dissolved into the aqueous phase. In addition, a dead volume is present, which needs to be considered.

With both the in-syringe detection and flow-cell detection methods, it is not possible to measure the volume of the resulting aqueous phase. However, it is possible to measure the volume of the extract i.e. the organic phase because it is expulsed from the syringe first and it passes first through the flow-cell. That means that both the start and the end of the organic phase are observable in the flow-cell measurements and the volume is determined by the flow rate and the time difference from start to end point of the observance of the organic phase. Only the start of the aqueous phase is however visible using this type of detection. Another way to calculate the extraction efficiency is then by calculating the amount of analyte in the extract from its concentration and the organic phase volume. Then, this molar amount can be compared with the original amount in the original sample present in the syringe in the form of aspired sample and the dead volume (which consists of sample):

$$\mathbf{EE_{HLLE} = (c_{org} \times V_{org}) / [(c_{sample} \times (V_{sample} + V_{DV})] \approx}$$
$$\mathbf{\approx (A_{org} \times V_{org}) / [(A_{sample} \times (V_{sample} + V_{DV})]}$$

EE_{HLLE} = extraction efficiency, c_{sample}, A_{sample} = sample concentration, absorbance, c_{org}, A_{org} = concentration of organic phase after extraction, absorbance, V_{DV} = dead volume.

It is noteworthy that both the absorbance values and the organic phase volumes have to be read-out from the syringe out-flow detection in the flow-cell. The flow-cell measurements were however considered less reliable than the ones using the in-syringe detection. This was due to the fact that the resulting data were less accurate as the organic phase was pushed through the flow-cell, as shown and described in the chapter **5.12**. This means that the extraction efficiency and phase volumes could not be calculated precisely.

5.11 Astraphloxine measurements using the in-syringe detection

Based on the preliminary experiments, a decision was made to progress systematically and test multiple series of a given solvent volume and varying sample and salt solution volumes. The parameters were limited by the syringe volume, so the sample and salt solution volumes were limited at 3.7–4.3 ml in total. It was decided to use 3.0 ml of sample and salt volume combined to allow for a reserve for the syringe dead volume and the aspirated air. Using less salt enabled using a larger volume of sample. The solvent volume was different for each series of the measurement, selected to be 0.7 ml, 0.8 ml, 0.9 ml, 1.1 ml and 1.3 ml respectively.

5.11.1 The phase separation time determination

Our objective was to determine the phase separation time at first. Using in-syringe detection the entire table of settings was tested, as shown in **Table 5**. Each measurement with a successful phase separation was shown as a string of values in a sigmoid-shaped chart as described in **Figure 13**. The phase separation time was read out as the difference between the time of the first point in that chart and the time of the first point of the stabilized upper "plateau" part. Readouts from three repeated measurements were averaged.

The difference in the charts with a fast or slow phase separation can be seen in the following examples. **Figure 15** shows one of the shortest times, **Figure 16** shows one of the longest.

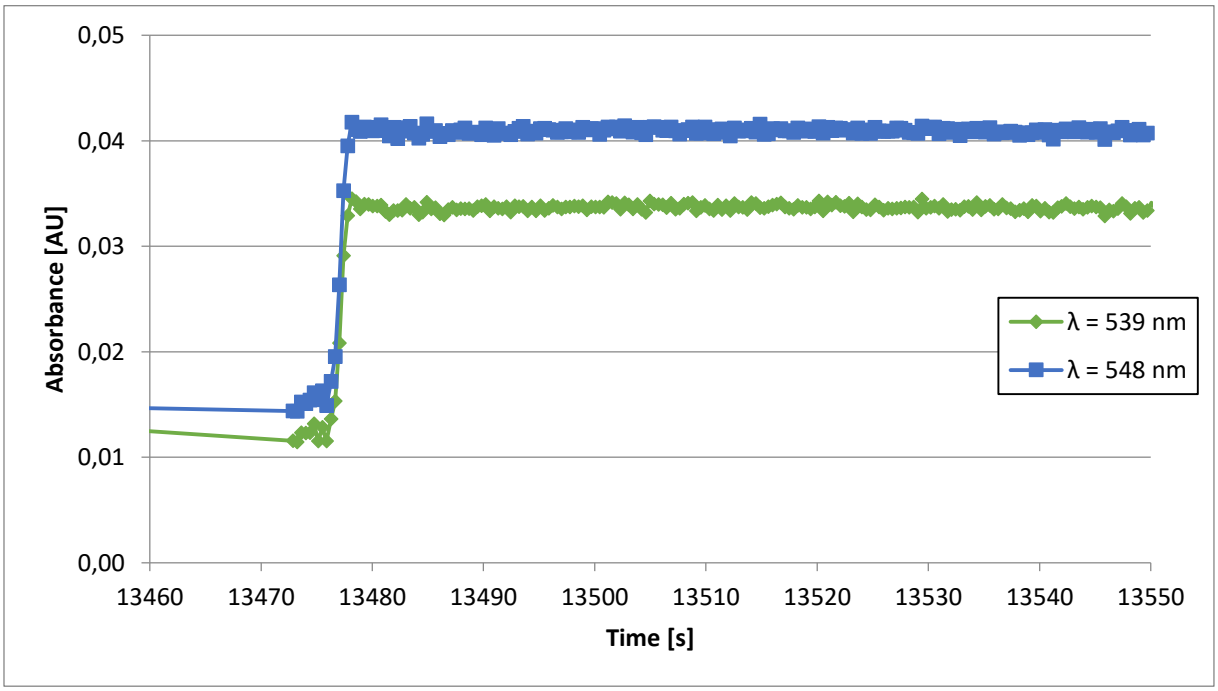

Figure 15: A segment of a chart from a test with 1.3 ml of solvent, 1.0 ml of sample and 2.0 ml of salt. Average phase separation time is 5 s.

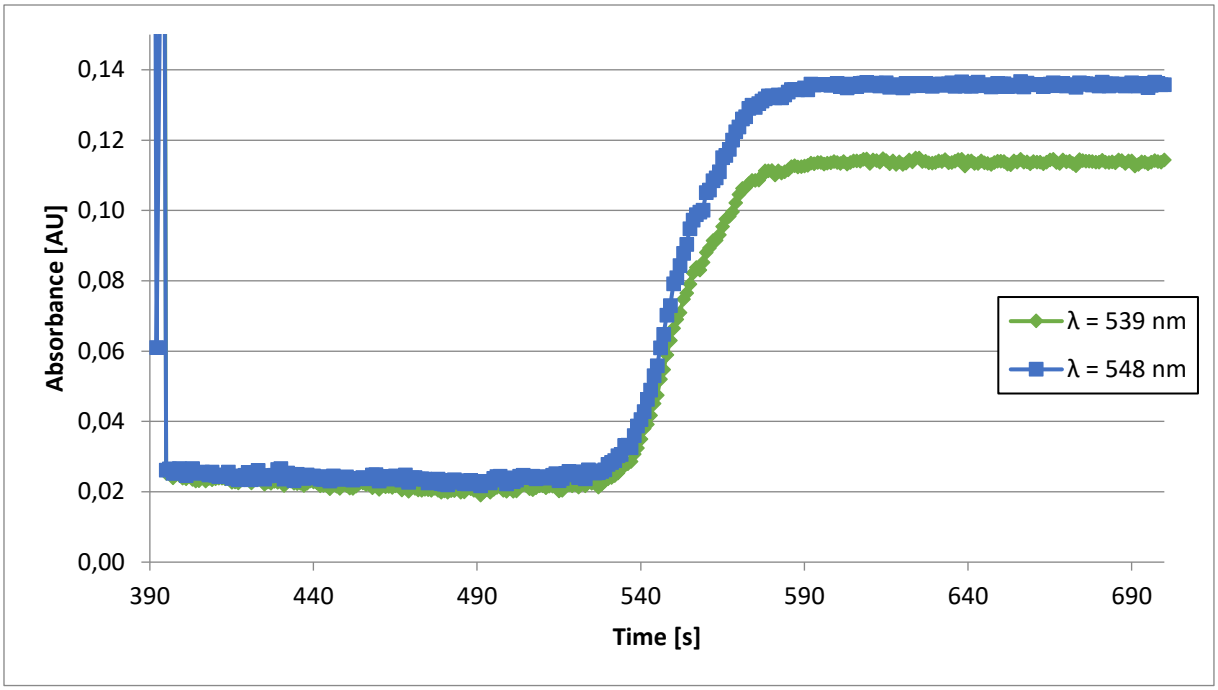

Figure 16: A segment of a chart with 0.8 ml of solvent, 1.4 ml of sample and 1.6 ml of salt. Average phase separation time is 78 s.

Table 5: Phase separation time of individual settings. If not listed, phase separation did was not observed. Using n-propanol as solvent, 0.5 µmol/l astraphloxine solution as sample and 550 g/l MgSO4 solution as salt solution .

solvent volume [ml]	*salt volume [ml]*	*sample volume [ml]*	*phase ratio*	*salt concentration in water phase [g/l]*	*coefficient Cps [g/l]*	*phase separation time [s]*
0.7	2.0	1.0	0.20	308.38	60.52	**46**
0.7	1.8	1.2	0.20	277.54	54.47	**67**
0.7	1.6	1.4	0.20	246.71	48.41	**400-450***
0.7	1.4	1.6	0.20	215.87	42.36	–
0.7	1.2	1.8	0.20	185.03	36.31	–
0.7	1.0	2.0	0.20	154.19	30.26	–
0.8	2.0	1.0	0.22	308.38	69.16	**34**
0.8	1.8	1.2	0.22	277.54	62.25	**40**
0.8	1.6	1.4	0.22	246.71	55.33	**78**
0.8	1.4	1.6	0.22	215.87	48.41	–
0.8	1.2	1.8	0.22	185.03	41.50	–
0.8	1.0	2.0	0.22	154.19	34.58	–
0.9	2.0	1.0	0.25	308.38	77.81	**29**
0.9	1.8	1.2	0.25	277.54	70.03	**33**
0.9	1.6	1.4	0.25	246.71	62.25	**41**
0.9	1.4	1.6	0.25	215.87	54.47	**68**
0.9	1.2	1.8	0.25	185.03	46.69	–
0.9	1.0	2.0	0.25	154.19	38.90	–
1.1	2.0	1.0	0.31	308.38	95.10	**6**
1.1	1.8	1.2	0.31	277.54	85.59	**8**
1.1	1.6	1.4	0.31	246.71	76.08	**29**
1.1	1.4	1.6	0.31	215.87	66.57	**36**
1.1	1.2	1.8	0.31	185.03	57.06	**56**
1.1	1.0	2.0	0.31	154.19	47.55	–
1.3	2.0	1.0	0.36	308.38	112.39	**5**
1.3	1.8	1.2	0.36	277.54	101.15	**7**
1.3	1.6	1.4	0.36	246.71	89.91	**8**
1.3	1.4	1.6	0.36	215.87	78.67	**19**
1.3	1.2	1.8	0.36	185.03	67.43	**35**
1.3	1.0	2.0	0.36	154.19	56.20	**67****

*Note: * – the phase separation time was extremely long and unreliable. ** – the phase separation was unreliable.*

The phase separation time and occurrence were strongly depending on the salt concentration and the phase ratio. Higher salt concentration in the aqueous phase and larger volume of solvent caused quicker phase separation. Upon multiplying these two numbers, a new value was created, which for future references is called coefficient C_{ps} [g/l], as shown below:

$$\mathbf{C_{ps} = V_{solvent}/(V_{sample} + V_{salt} + V_{DV}) \times (V_{salt} \times c_{salt})/(V_{sample} + V_{salt} + V_{DV})}$$

C_{ps} = coefficient C_{ps}, $V_{solvent}$ = solvent volume, V_{sample} = sample volume, V_{salt} = salt solution volume, V_{DV} = dead volume, c_{salt} = concentration of the salt solution in g/l.

These calculations were adjusted by the dead volume. The coefficient C_{ps} was compared with a phase separation time and a convincing polynomic correlation of $R^2 = 0.941$ was found as shown in **Figure 17**.

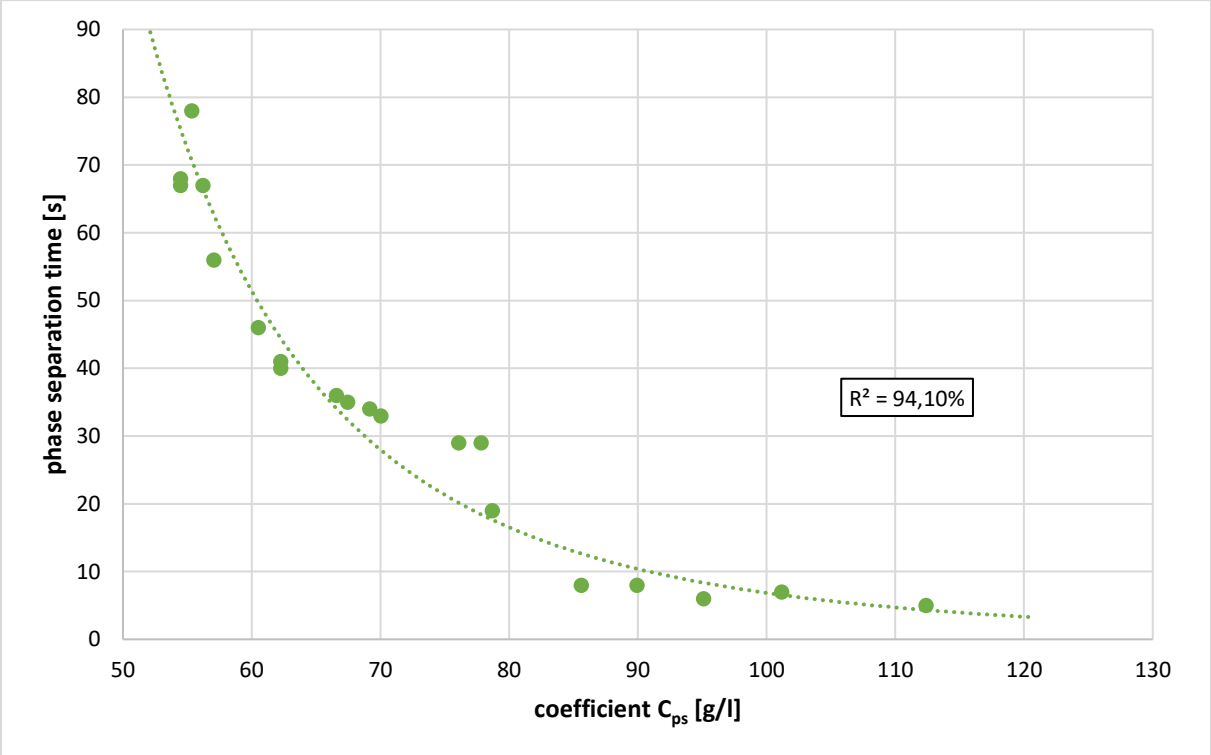

Figure 17: A general estimate of the dependence of the phase separation time on a C_{ps} coefficient.

It has to be said that this is merely a correlation. It can only be used for *estimation* whether the extraction procedure can be carried out or how fast the phase separation will be. For $C_{ps} < 54$ g/l, phase separation was not observed.

Since the measurement data follow a sigmoid-shaped trend, it was difficult to read out the time value properly at which phase separation could be considered as terminated. More specifically,

it was required to do this time estimate in a reproducible manner. To capture the entire process of phase separation, the measurement was started directly after the stirring was stopped. However, once the phase separation time was known, it was possible to first wait for that time or longer, and then start measuring. This would return only the desired values from the top flat part of the chart.

It was noticed that the first measurement of each method execution yielded high absorbance values of the extract compared to the following extractions. It was found, that this was caused by astraphloxine getting absorbed at the PTFE-coated stirring bar, piston head, and syringe inlet. Since the syringe was washed in the beginning of the method execution, the astraphloxine concentration was approximately 5–7 % higher in the first run. Washing the syringe with water alone was found as insufficient, so an additional syringe wash with solvent was added after each extraction eliminating this problem.

5.11.2 Final method, the sampling rate, preconcentration factor, and repeatability determination

Another series of experiments was conducted with altered settings. First, single washing of the syringe with solvent at the end of each pre-extraction procedure was implemented (**Table 2**). Second, data acquisition was not started directly after end of stirring but after a waiting time of 90 s over a period of 15 s. This allowed us to average all the detected values without further treatment. From this set of experiment, the preconcentration factors and the measurement repeatability were calculated.

This final method took on average 307 s per extraction. This included 90 s of waiting time, which could be considerably shortened for fast phase separation times. For some settings, e.g. 1.3 ml of solvent/1.0 ml of sample/2.0 ml of salt solution, it was possible to be shortened to 10 s without any impact on accuracy, which will decrease the average procedural time to 227 s. The method using the-in-syringe detection and the finalized program as shown in **Annex** – the working program for astraphloxine can therefore achieve a sampling rate of **15.9 h^{-1}** for the fastest phase separation or **11.7 h^{-1}** for the slowest phase separation.

The pre-extraction procedure took 381 s. It is however worth noting that the pre-extraction procedure was used for system setup including dark and reference measurement. This way one pre-extraction procedure can be used for multiple extraction procedures and multiple actual measurements of one analyte.

Table 6: Preconcentration factor (F_c) values and repeatability of the astraphloxine in-syringe measurements calculated as a relative standard deviation. Using n-propanol as solvent, 0.5 µmol/l astraphloxine solution as sample and 550 g/l MgSO4 solution as salt.

solvent volume [ml]	*salt volume [ml]*	*sample volume [ml]*	*phase separation time [s]*	*standard absorbance at 539 nm*	*extract absorbance at 548 nm**	*preconcentration factor (Fc)*	*relative standard deviation*
0.7	2.0	1.0	**46**	0.024	0.125	**5.20**	**1.38%**
0.7	1.8	1.2	**67**	0.024	0.155	**6.43**	**1.03%**
0.7	1.6	1.4	**400-450**				
0.7	1.4	1.6	–				
0.7	1.2	1.8	–				
0.7	1.0	2.0	–				
0.8	2.0	1.0	**34**	0.026	0.094	**3.66**	**0.89%**
0.8	1.8	1.2	**40**	0.026	0.109	**4.28**	**0.93%**
0.8	1.6	1.4	**78**	0.026	0.131	**5.13**	**0.22%**
0.8	1.4	1.6	–				
0.8	1.2	1.8	–				
0.8	1.0	2.0	–				
0.9	2.0	1.0	**29**	0.027	0.075	**2.79**	**0.33%**
0.9	1.8	1.2	**33**	0.027	0.085	**3.16**	**0.48%**
0.9	1.6	1.4	**41**	0.027	0.101	**3.74**	**2.04%**
0.9	1.4	1.6	**68**	0.027	0.118	**4.40**	**1.27%**
0.9	1.2	1.8	–				
0.9	1.0	2.0	–				
1.1	2.0	1.0	**6**	0.028	0.053	**1.93**	**2.15%****
1.1	1.8	1.2	**8**	0.028	0.059	**2.13**	**0.38%**
1.1	1.6	1.4	**29**	0.028	0.064	**2.33**	**0.93%**
1.1	1.4	1.6	**36**	0.028	0.067	**2.44**	**0.48%**
1.1	1.2	1.8	**56**	0.028	0.073	**2.63**	**0.99%**
1.1	1.0	2.0	–				
1.3	2.0	1.0	**5**	0.028	0.043	**1.55**	**1.45%****
1.3	1.8	1.2	**7**	0.027	0.047	**1.70**	**0.16%**
1.3	1.6	1.4	**8**	0.027	0.050	**1.84**	**0.93%**
1.3	1.4	1.6	**19**	0.027	0.053	**1.93**	**0.49%**
1.3	1.2	1.8	**35**	0.027	0.055	**2.01**	**0.40%**
1.3	1.0	2.0	**67**				

*Note: * = values were adjusted for a vertical spectral shift observed for astraphloxine. ** = an outlayer was excluded from the standard deviation calculation, which was then calculated from two results only.*

It can be seen that the preconcentration factor is highest for the lowest amount of solvent, while the amount of sample is of less importance.

The repeatability was calculated as the relative standard deviation for three separate extraction and measurements performed with the same settings. It was below 1 % for most settings but a value over 3 % was found twice. Upon examining the results it was found that both values were affected by the first measurements, which was attributed to unsatisfactory cleaning of the system from the extraction measurements before. If that is indeed the case, it could be corrected by even more thorough system cleanup or by ignoring the first measurement in a series. To obtain results with high precision confidence, it is therefore advisable to revise the cleaning protocol and test for outlayers. In conclusion, the typical reproducibility was < 1 % of relative standard deviation.

5.12 Astraphloxine measurements using the flow-cell detection

Employing detection in the flow-cell at the syringe out-flow, a different set of results was obtained. This time, the extraction was allowed enough time for phase separation and afterwards the syringe was expulsed from the syringe through the detection cell.

Unlike using in-syringe detection extraction kinetics cannot be observed. However, it is possible to observe the composition of the entire syringe content after the extraction has been finished, as it passes through the flow-cell. While most of the pumping steps were executed at a flow rate of 200 µl/s, the discharge of the syringe content through the flow-cell was done at 50 µl/s. This was done to allow for measurement of the solvent with higher precision and to reduce the turbulent mixing in the tubing and the flow-cell to allow better identification of the start and end point of the organic phase at passing the detector.

A typical output data set acquired during passing the syringe liquid content through the detection cell can be seen in **Figure 18**. The recorded values in the chart begin with some noise from the residues of the procedures carried out before. The absorbance than spikes upward sharply, marking the beginning of the organic phase. Afterwards, the absorbance stabilizes at an elevated value. A sudden drop in the absorbance marks the end of the organic phase and the beginning of the aqueous phase passing the detection cell.

The signal shape can be explained by the parabolic flow profile, typical for laminar flow, while a block-flow would yield an ideal rectangular signal shape. A further problem is that in the

corners of the flow cell, rests of water would remain from the previous syringe cleaning, which would dissolve in the organic phase at passing, by this leading an apparently higher absorbance.

The organic volume has been calculated as the difference in time between the first and last visibly elevated absorbance values, multiplied by the syringe discharge velocity of 50 µl/s. The absorbance of the organic volume has been read-out as the last recorded absorbance value in a consistent string of values corresponding to the organic phase. This was because the initial detected values showed a spike of higher absorbance, after which the values slowly stabilized. Although it is unknown if they have been fully stabilized in each case, the last data point is likely to be the most accurate. In case there was another spike in absorbance at the end of organic phase, the value before was used for averaging.

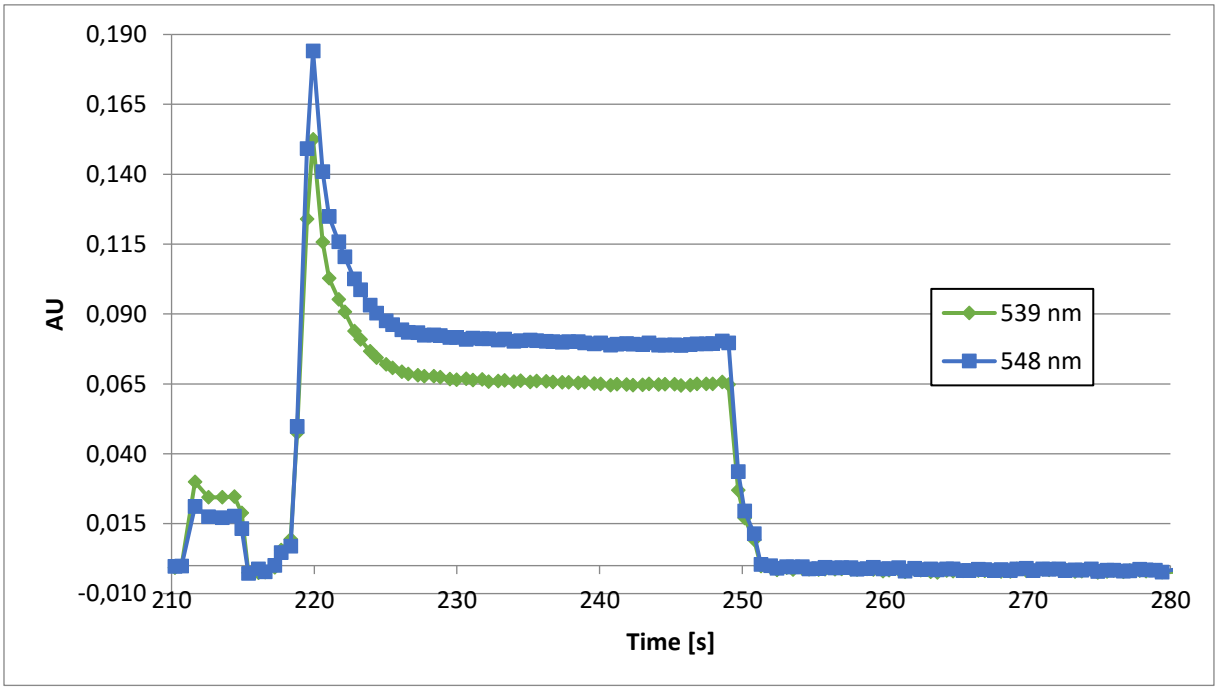

Figure 18: A segment of a chart from a test with 1.3 ml of solvent, 1.0 ml of sample and 2.0 ml of salt.

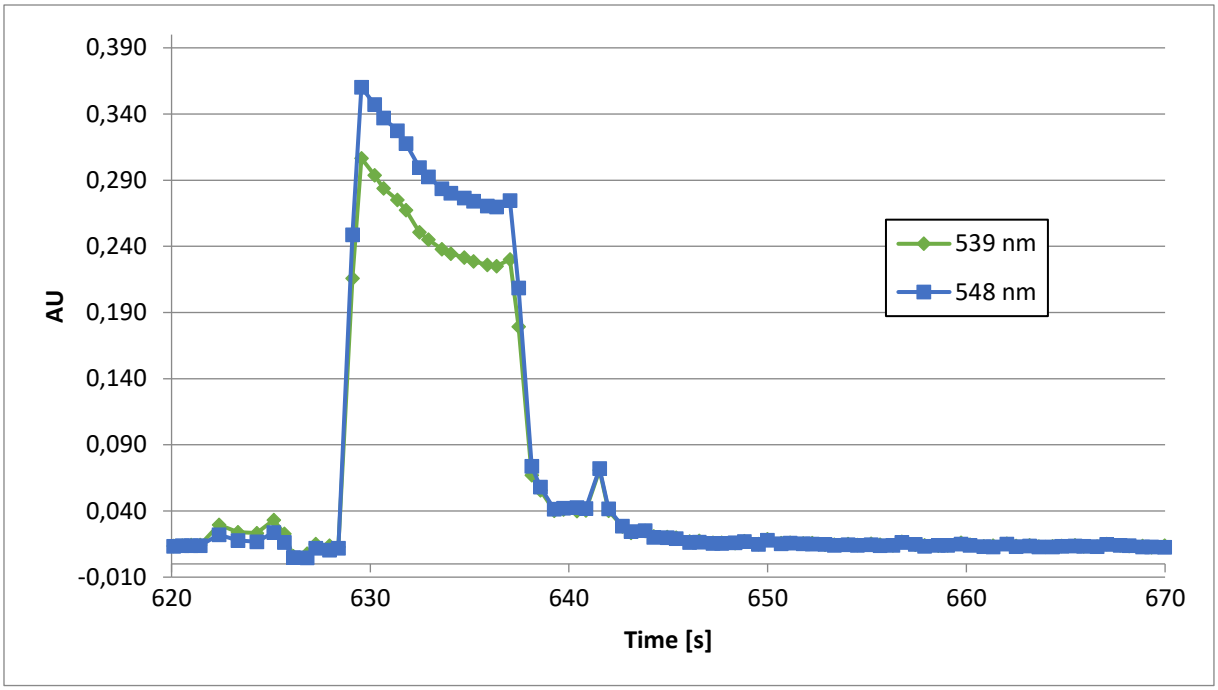

Figure 19: A segment of a chart from a test with 0.8 ml of solvent, 1.4 ml of sample and 1.6 ml of salt.

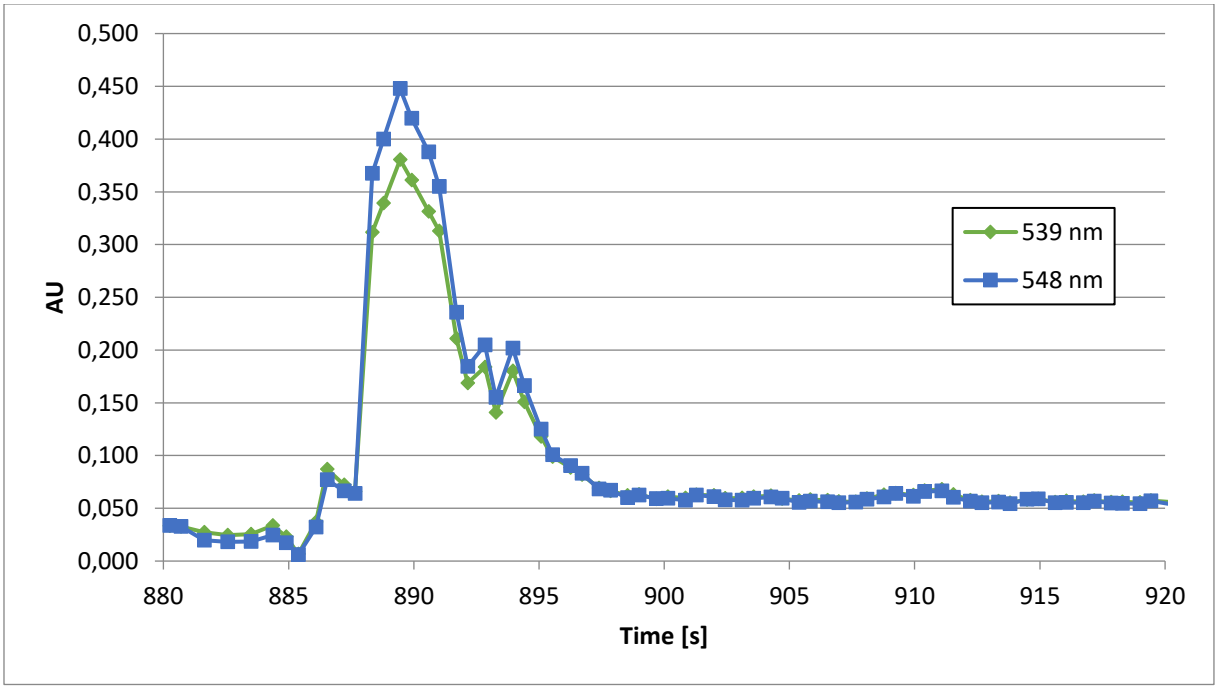

Figure 20: A segment of a chart from a test with 0.8 ml of solvent, 1.6 ml of sample and 1.4 ml of salt.

The flow-cell measurements were considered inferior due to the following reasons:

1. The liquid phases underwent mixing in the tubing on the way to the flow-cell and with rests of liquid from former syringe cleaning. Further extraction took place as well, as the tubing was filled by the sample solution, as seen in the usual upward spike in absorbance in the leading part of the organic phase as shown in **Figure 18**.
2. While in-syringe measurements were easily evaluated as the absorbance values stabilized after a given time period, the flow-cell measurements often showed no stable level at all (for small volumes of organic phase). This was probably due to aforementioned further mixing taking place in the tubing. Not only it prevented obtaining reliable results, it also made data analysis less reproducible, as there was no described way of analyzing a precisely defined string of data output values. This is indicated in **Figure 19** and **Figure 20**.
3. The sampling rate of the flow-cell measurements was lower than the one of the in-syringe measurements. The slower expulsion of the syringe contents added 57–66 s to each extraction procedure, when allowing 90 s for phase separation in each scenario. When accounted for the 15 s of detection period in the in-syringe measurements, the actual delay is 42–51 s. This lead to extraction procedure times of 358 s for the slowest settings and 269 s for the fastest. The sampling rate was **10.1–13.4 h^{-1}** using the flow-cell detection procedure, compared to **11.7–15.9 h^{-1}** using the-in-syringe detection procedure. The pre-extraction procedure was prolonged as well, due to the reference scan and sample scan taking place in the flow-cell during an expulsion of water or sample from the syringe at 50 µl/s. This was however considered a negligible issue.

As the flow-cell measurements were conducted right after determining the phase separation time (see chapter **5.11.1**), the final method improvements were not implemented. In particular, the solvent-washing of the syringe was not used, which affected the first measured point in a given series (excluded from calculations). Also, in the earlier stages of the experiments, system malfunctions were more common, in particular failures of the stirring system. These are the reasons to believe the flow-cell measurements results are not fully reliable.

As mentioned in section **5.10**, the extraction efficiency for HLLE can only be accurately calculated knowing the organic volume. This in turn is only possible to find out from the flow-cell measurements. Observations were made and corresponding estimates were outlined in **Table 7**.

Table 7: Results of the flow-cell measurements. Using n-propanol as solvent, 0.5 µmol/l astraphloxine solution as sample and 550 g/l MgSO4 solution as salt.

solvent volume [ml]	*salt volume [ml]*	*sample volume [ml]*	*salt concentration in water phase [g/l]*	*detected organic phase volume [ml]*	*standard absorbance at 539 nm*	*extract absorbance at 548 nm**	*resulting extraction efficiency*
0.7	2.0	1.0	308.38	**0.40**	0.054	0.255	**120.41%**
0.7	1.8	1.2	277.54	**0.30**	0.054	0.307	**96.58%**
0.7	1.6	1.4	246.71	**0.15**	0.054	0.454	**64.05%**
0.8	2.0	1.0	308.38	**0.60**	0.052	0.185	**135.89%**
0.8	1.8	1.2	277.54	**0.55**	0.052	0.215	**128.59%**
0.8	1.6	1.4	246.71	**0.40**	0.052	0.260	**101.49%**
0.8	1.4	1.6	215.87	**~0.1**	0.052	~0.4	**~35%**
0.9	2.0	1.0	308.38	**0.75**	0.051	0.139	**130.59%**
0.9	1.8	1.2	277.54	**0.70**	0.051	0.159	**123.42%**
0.9	1.6	1.4	246.71	**0.65**	0.051	0.182	**117.93%**
0.9	1.4	1.6	215.87	**0.50**	0.051	0.214	**96.72%**
1.1	2.0	1.0	308.38	**1.10**	0.055	0.109	**138.64%**
1.1	1.8	1.2	277.54	**1.10**	0.055	0.118	**133.82%**
1.1	1.6	1.4	246.71	**1.10**	0.055	0.128	**129.99%**
1.1	1.4	1.6	215.87	**1.00**	0.055	0.140	**116.95%**
1.1	1.2	1.8	185.03	**0.80**	0.055	0.156	**95.69%**
1.3	2.0	1.0	308.38	**1.50**	0.049	0.078	**152.69%**
1.3	1.8	1.2	277.54	**1.50**	0.049	0.086	**149.70%**
1.3	1.6	1.4	246.71	**1.50**	0.049	0.091	**142.04%**
1.3	1.4	1.6	215.87	**1.45**	0.049	0.096	**132.59%**
1.3	1.2	1.8	185.03	**1.40**	0.049	0.103	**125.40%**
1.3	1.0	2.0	154.19	**1.05**	0.049	0.109	**91.59%**

*Note: * = values were adjusted for a vertical spectral shift observed for astraphloxine.*

The flow-cell measurements results introduced several facts:

1. The earlier hypothesis about the in-syringe detection not being able to detect too small volumes of organic phase has been confirmed. The detected organic phase volumes of 0.1 ml and 0.15 ml have not been observed by the in-syringe detection.

2. Due to some of the aforementioned process imperfections such as further mixing, further extraction and mostly the contact of the organic phase with more sample in the tubing and the flow-cell, the calculated extraction efficiency exceeded 100 % in most cases. The aqueous phase however showed a very low absorbance values, in most cases at <0.005. That means that in most cases the actual extraction efficiency is at values at >90 %.
3. The extraction efficiency was lower for lower detected organic phase volumes and for higher sample volumes.
4. The calculated volume of some organic phases exceeded the initial solvent volume. That is probably due to some of the sample present in the tubing and the flow-cell dissolving in the organic phase. This could be a major factor causing higher calculated extraction efficiency.
5. Increasing solvent volume decreased the volume of solvent dissolved in the aqueous phase.

Further information about HLLE have been revealed using the flow-cell measurements, as well as design flaws of the flow-cell detection method. Therefore, the method was tested on another analyte using exclusively in-syringe detection.

It is however worth considering using significantly more air aspired into the syringe. The 250 µl of air is enough to push the dead volume of the holding coil and the syringe head-valve into the syringe, but might not be enough to act as a separator between the organic phase and the sample during syringe discharge. This was not tested and could have improved the flow-cell detection method results.

5.13 Riboflavin measurements

In addition to astraphloxine, extraction of riboflavin, being a second model analyte of significant hydrophilicity, was studied. The program was identical to the experiments with astraphloxine, shown in **Annex** – the working program for astraphloxine and the same variables were tested in the same fashion.

The detection wavelength was adapted and set to 447 nm instead of 539/548 nm. Also, only two salt concentrations were tested for each salt concentration, which yielded in the previous experiments with the highest preconcentration factors. This was done because the initial tests with riboflavin indicated that very low preconcentration factor could be expected. The measurements results are shown in **Table 8**.

Table 8: Preconcentration factor (F_c) values and repeatability of the riboflavin in-syringe measurements calculated as a relative standard deviation (n = 3). Using n-propanol as solvent, 10 µmol/l riboflavin solution as sample and 550 g/l $MgSO_4$ solution as salt. Note: the phase separation time is a result of astraphloxine measurements.

solvent volume [ml]	*salt volume [ml]*	*sample volume [ml]*	*phase separation time [s]*	*standard absorbance at 447 nm*	*extract absorbance at 447 nm*	*preconcentration factor (Fc)*	*relative standard deviation*
0.7	2	1	**46**	0.1069	0.1093	**1.02**	**0.18%**
0.7	1.8	1.2	**67**	0.1069	0.1131	**1.06**	**0.49%**
0.8	1.8	1.2	**40**	0.1068	0.1058	**0.99**	**0.45%**
0.8	1.6	1.4	**78**	0.1068	0.1063	**1.00**	**0.67%**
0.9	1.6	1.4	**41**	0.1043	0.0978	**0.94**	**0.70%**
0.9	1.4	1.6	**68**	0.1043	0.097	**0.93**	**0.38%**
1.1	1.6	1.4	**29**	0.1045	0.0891	**0.85**	**0.68%**
1.1	1.4	1.6	**36**	0.1045	0.092	**0.88**	**0.45%**
1.3	1.4	1.6	**19**	0.1011	0.0784	**0.78**	**0.49%**
1.3	1.2	1.8	**35**	0.1099	0.0874	**0.80**	**0.66%**

The highest preconcentration factors were near to 1.0 thus indicating that no preconcentration of riboflavin was achieved. This was probably due to method lower extraction efficiency for riboflavin. Although there is no precise method to determine the extraction efficiency precisely, if we consider astraphloxine 90–100% efficiency and compare the preconcentration factors, it seems that riboflavin extraction efficiency varies between 10–50 %. While the method could

still be used for sample matrix removal, extracting a portion of riboflavin into the organic solvent, it is clear that further modifications of the method would be required such as a different salt component or solvent.

6. Conclusion

A system and method for automated in-syringe salt-assisted homogenous liquid-liquid extraction has been designed, explored and evaluated for the first time. Two detection methods were studied to evaluate both resulting solvent volume and phase separation time in dependency of salt concentration and volumetric ratio of n-propanol employed as solvent.

In-syringe detection was proven to be superior due to imperfections of the flow-cell detection method, such as increased inaccurate absorbance readouts caused by further mixing, dissolving and extraction taking place in the tubing and the flow-cell as well as by turbulent dispersion forces. The flow-cell detection method could perhaps be improved by aspirating higher volume of air before the extraction, as this was not tested. The final method using in-syringe detection has been applied to the extraction of two model analytes of different hydrophobicity being astraphloxine and riboflavin.

From the studies, the following conclusions and characteristics can be summarized:

The higher the salt concentration in the aqueous phase, the more likely the phase separation and the faster phase separation time would be. Also, the organic phase volume would be greater. The preconcentration factor would be lower for the reason of the resulting organic phase volume being larger and because there would be lower volume of sample for a higher volume of salt.

The lower the initial ratio of solvent volume compared to aqueous phase volume, the higher the preconcentration factor, less likely phase separation and slower phase separation time would be.

- The repeatability calculated as the relative standard deviation of three repetition was generally below 1 %.
- The highest preconcentration factor achieved was F_c = 6.43 for the model analyte astraphloxine yielding a sampling rate of 11.7 h^{-1}.
- The fastest sampling rate achieved was 15.9 h^{-1} at a preconcentration factor of F_c = 1.55-2.13 for astraphloxine. This was possible when applying a reduced phase separation time of 10 s instead of 90 s.

For the studies of the two model analytes is was concluded that the method was applicable to the extraction of astraphloxine with a near to 100% extraction efficiency for the majority of settings while for the more hydrophilic riboflavin the extraction efficiency varied between 10-

50 % and can therefore not be applied without further modifications. There was no significant preconcentration achieved for riboflavin, as the preconcentration factor was 0.78–1.06. However, the method would still be useful for both analytes for extracting the analyte from the sample, removing the organic matrix and for desalting.

The studied approach was fully automated and can be considered as environmental friendly due to the solvent employed. It can further be expected, although not tested nor being a task for this thesis, that the extraction method could be coupled to HPLC due the used solvent being HPLC-compatible.

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8. Annex – the working program for astraphloxine

Operation program for salt-assisted HLLE using the in-syringe detection in its final version. This program has been used to determine the preconcentration factor and repeatability of the method using astraphloxine as the model analyte, in-syringe measurement and 1.3 ml of solvent.

```
'Hardware settings
Hardware Settings Wavelength 1 (nm) 539
Hardware Settings Wavelength 2 (nm) 548
Hardware Settings Wavelength 4 (nm) 600
Hardware Settings Use Wavelength 4 as Reference

Hardware Settings Integration Time (msec) 3
Hardware Settings Detectors to Average 10
Hardware Settings Samples to Average 10
Hardware Settings Scan Rate (Hz) 5

SyringePump Command (?) KOR

Contact Closure Off

Variable Define New ValvePos
Variable Define New Repetition

Variable Define New Vol2
Variable Define New Vol7

'Port 1: Waste / 2: Sample / 3: Water / 4: - 5: used by other program / 6: Solvent 1 / 7: Salt / 8: Air


'----------------------------------------------------------------1300----------------------------------------------------------------

'Empty Syringe to Waste
SyringePump Valve In
SyringePump  Flowrate (microliter/sec) 200
SyringePump Empty
SyringePump Delay Until Done

'REFERENCE SCAN
'Clean syringe with solvent
ValvePos = 6
Repetition = 2
Insert File C:\Users\Obsluha\Documents\SIA3500 data\homogeneous DLLME\Procedure
CleanSyringe.fia

'Clean syringe with water
ValvePos = 3
Repetition = 2
Insert File C:\Users\Obsluha\Documents\SIA3500 data\homogeneous DLLME\Procedure
CleanSyringe.fia

'Aspirate and dispense water
Multiposition Valve port 3
SyringePump Valve Out
SyringePump Flowrate (microliter/sec) 200
SyringePump Aspirate (microliter) 4000
SyringePump Delay Until Done
```

```
SyringePump Dispense (microliter) 1000
SyringePump Delay Until Done

Delay (sec) 5
Spectrometer Reference Scan

'Empty Syringe
SyringePump Valve In
SyringePump Flowrate (microliter/sec) 200
SyringePump Empty
SyringePump Delay Until Done

'SAMPLE SCAN
'Clean syringe with sample
ValvePos = 2
Repetition = 3
Insert File C:\Users\Obsluha\Documents\SIA3500 data\homogeneous DLLME\Procedure
CleanSyringe.fia

'Aspirate and dispense sample
Multiposition Valve port 2
SyringePump Valve Out
SyringePump Flowrate (microliter/sec) 200
SyringePump Aspirate (microliter) 4000
SyringePump Delay Until Done
SyringePump Dispense (microliter) 1000
SyringePump Delay Until Done

Delay (sec) 5
Spectrometer Absorbance Scanning
Delay (sec) 15
Spectrometer Stop Scanning

'Empty Syringe
SyringePump Valve In
SyringePump Flowrate (microliter/sec) 200
SyringePump Empty
SyringePump Delay Until Done

'Clean syringe with solvent
ValvePos = 6
Repetition = 1
Insert File C:\Users\Obsluha\Documents\SIA3500 data\homogeneous DLLME\Procedure
CleanSyringe.fia

Vol2 = 1000
Vol7 = 2000

Loop Start (#) 6
Loop Start (#) 3

'Clean syringe with sample
ValvePos = 2
Repetition = 3
Insert File C:\Users\Obsluha\Documents\SIA3500 data\homogeneous DLLME\Procedure
CleanSyringe.fia
'Aspirate sample
Multiposition Valve port 2
SyringePump Valve Out
SyringePump Flowrate (microliter/sec) 200
```

SyringePump Aspirate (microliter) Vol2
SyringePump Delay Until Done

'Aspirate solvent with stirring
Multiposition Valve port 6
SyringePump Valve Out
SyringePump Flowrate (microliter/sec) 100
SyringePump Aspirate (microliter) 1300
Delay (sec) 5
Contact Closure On
SyringePump Delay Until Done
Delay (sec) 5
Contact Closure Off

'Aspirate Salt solution
Multiposition Valve port 7
SyringePump Valve Out
SyringePump Flowrate (microliter/sec) 75
SyringePump Aspirate (microliter) Vol7
Delay (sec) 5
Contact Closure On
SyringePump Delay Until Done

'Aspirate Air with stirring
Multiposition Valve port 8
SyringePump Valve Out
SyringePump Flowrate (microliter/sec) 100
SyringePump Aspirate (microliter) 200
SyringePump Delay Until Done
Delay (sec) 10
Contact Closure Off

Delay (sec) 90
Spectrometer Absorbance Scanning
Delay (sec) 15
Spectrometer Stop Scanning

'Empty Syringe
SyringePump Valve In
SyringePump Flowrate (microliter/sec) 200
SyringePump Empty
SyringePump Delay Until Done

Loop End
Vol2 += 200
Vol7 -= 200
Loop End

Save Data C:\Users\Obsluha\Documents\SIA3500 data\homogeneous DLLME\141027-06 Test w 1300 nPrOH, 1-2 ASPX, 2-1 MgSO4.dat

Delay (sec) 5

Reset Data

Message End