CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF ANALYTICAL CHEMISTRY

DIPLOMA THESIS

Study of deproteinization of milk samples via salting-out induced phase separation and automation by Lab-In-Syringe

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Co-supervisor: PharmDr. Kateřina Fikarová, Ph.D.

Hradec Králové, 2021

Daniel Machián

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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13. 5. 2021

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Date/Datum Daniel Machián

I would like to thank and express my most genuine gratitude and respect to my supervisors, M.Sc. Burkhard Horstkotte, Ph.D. and PharmDr. Kateřina Fikarová, Ph.D., for their assistance, guidance, patience, and professionalism at all times. I consider them great scientists and people as they help make science more accessible.

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Abstract

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Diploma thesis title: Study of deproteinization of milk samples via salting-out induced phase separation and automation by Lab-In-Syringe

A novel approach to sample deproteinization based on homogeneous liquid-liquid extraction with salting-out induced phase separation was explored. A methodology was developed, experimentally tested, and optimized that allows automated centrifuge-less deproteinization of milk samples using the flow-batch technique Lab-In-Syringe.

As in other sample preparation methods, the aim was to eliminate the sample matrix and obtain an organic phase that could be submitted to analysis. The thesis includes a range of preliminary experiments performed offline, i.e. without the intended instrumentation, as well as optimization experiments performed online using a Lab-In-Syringe system. Salting out agents and three solvents were tested as well as phase ratios and operational parameters on the Lab-In-Syringe system, including system configuration. In the method optimized by the work reported in this thesis, milk proteins were found effectively denatured and precipitated by the water-miscible solvent acetonitrile, which also serves as possible extraction solvent for analytes of interest in the milk sample. Phase separation was induced by the addition of a mixed solution of $MgSO_4$ and NaCl as salting-out agents.

The automation was based on the Lab-In-Syringe technique with in-syringe magnetic stirring, providing thorough and homogenous mixing inside the syringe void upon the aspiration of the sample and individual liquid reagents. For automated centrifuge-less deproteinization and phase separation, the following procedure was found optimal: diluted milk sample treated by acetonitrile and subsequently by the previously mentioned salting-out solution. Moreover, cleaning protocols were critically examined. In the extracts obtained from the optimized protocol, no traces of proteins were found. All experiments were documented by photographs.

The developed system and method will be coupled online to HPLC in a future work to which this thesis was aimed to contribute the first part, system setup and method development.

Abstrakt

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Název diplomové práce: Studie deproteinizace vzorků mléka pomocí fázové separace indukované vysolováním a automatizace v Lab-In-Syringe

V této práci byl zkoumán nový přístup k deproteinizaci vzorku založený na homogenní kapalinové extrakci s vysolováním indukovanou fázovou separací. Byl vyvinut, experimentálně otestován a optimalizován postup, který umožňuje automatizovanou deproteinizaci vzorků mléka pomocí techniky Lab-In-Syringe, bez potřeby centrifugace.

Stejně jako v jiných metodách přípravy vzorků bylo cílem eliminovat matrici vzorku a získat organickou fázi, která by mohla být podrobena analýze. Práce zahrnuje řadu předběžných experimentů prováděných offline, tj. bez zamýšleného instrumentace, jakož i optimalizační experimenty prováděné online pomocí systému Lab-In-Syringe. Byly testovány vysolovací činidla a tři rozpouštědla, stejně jako fázové poměry a provozní parametry systému Lab-In-Syringe, včetně jeho konfigurace. V metodě optimalizované postupy popsanými v této práci, bylo dosaženo účinné denaturace a vysrážení mléčných proteinů vodou mísitelným rozpouštědlem, acetonitrilem, který také slouží jako možné extrakční rozpouštědlo pro analyty, které jsou předmětem zájmu ve vzorcích mléka. Separace fází byla indukována přidáním směsného vysolovacího roztoku $MgS0_4$ a NaCl, jakožto vysolovacích činidel.

Automatizace byla založena na technice Lab-In-Syringe s magnetickým mícháním přímo v injekční pumpě, které zajišťuje důkladné a homogenní promíchání vzorku a jednotlivých kapalných činidel při jejich nasátí. Pro automatizovanou deproteinizaci bez užití centrifugy a fázovou separaci byly shledán optimálním následující postup: ke vzorku zředěného mléka byl přidán acetonitril a následně výše uvedený vysolovací roztok. Kromě toho byly kriticky prozkoumány postupy čištění systému. V extraktech získaných z optimalizovaného protokolu nebyly nalezeny žádné stopy proteinů. Všechny experimenty byly dokumentovány fotografiemi.

Vyvinutý systém a metoda budou přímo napojeny na HPLC v budoucí práci, ke které bylo touto prací zamýšleno přispět první částí, tedy nastavením systému a vývojem metody.

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List of used abbreviations

Abbreviation	Definition
ACE	acetone, propan-2-one
anh.	anhydrous
approx.	approximately
Cit.	citrate
DC	direct current
DLLME	dispersive liquid-liquid microextraction
d-SPE	dispersive solid-phase extraction
e.g.	exempli gratia, for example
EtAc	ethyl acetate
et al.	et alii; collective
FIA	Flow Injection Analysis
GC	gas chromatography
HLLE	homogenous liquid-liquid extraction
HPLC	high-performance liquid chromatography
i.d.	inner diameter
i.e.	id est; that is
iPrOH	isopropanol, propan-2-ol
LOV	Lab-On-Valve
LIS	Lab-In-Syringe
LLE	liquid-liquid extraction
LLME	liquid-liquid microextraction
MeCN	acetonitrile
MeOH	methanol
MS	mass spectrometry
NH_4Ac	ammonium acetate
PLA	polylactic acid
PrOH	propan-1-ol
PTFE	polytetrafluoroethylene, Teflon™
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe techniques
RP-HPLC	reversed-phase high-performance liquid chromatography
SALLE	salting-out assisted liquid-liquid extraction
SIA	Sequential Injection Analysis
SDS	sodium dodecyl sulphate
Tar.	tartrate
UV/VIS	ultra-violet/visible electromagnetic spectrum

1. Introduction

Chemical analysis of biological, food, and environmental samples is almost as old as the use of the substances analysed in them [1]. The initial methods consisted of the analyte extraction with appropriate solvents and subsequent qualitative and quantitative determination by chemical reactions. Today, this role is taken over by separation techniques such as high-performance liquid chromatography or gas chromatography combined with qualitatively quantitative detection techniques such as mass spectrometry, photometry of various spectra or electrochemical methods [2,3]. For them, samples must be prepared and treated to get a concentrated extract compatible with the given instrument. Techniques such as liquid-phase extraction or solid-phase extraction took over this category [4].

The automation of these methods is of interest to many analysts as it represents a way to achieve results in a faster, cheaper, more accurate, or more environmentally friendly way [5,6].

In this work, we focus on combining efficient and proven procedure for sample preparation, salting-out assisted liquid-liquid extraction, and flow technique for automation in laboratories, the Lab-In-Syringe.

2. Theoretical part

2.1. Background on performed extraction technique

Qualitative and quantitative analysis of biological samples has been developing extensively in recent years [1]. The need for improvements in analytical methods is driven by demands on easier to perform, faster, and more environmentally friendly (greener) procedures [7]. A brief evolution of biological sample preparations, including the extraction of analytes, is presented in this chapter. It helps to understand the inspiration for the treatment of milk samples objected in this work.

2.1.1. Need for sample preparation in food and biological analysis

With a rapidly growing population over the last century, the need and consumption of food are equally growing [8], resulting in mass crop production, animal husbandry, and aquaculture. To maintain the production volume and the low food prices, active chemical compounds are used [8], namely antibiotics, hormones, and other medicines for animals as well as pesticides and herbicides in plant cultivation. Residues of these substances in animal and plant products may pose a health risk to humans and a hazard to the environment. In addition to those, residues of pollutants such as persistent organic compounds or heavy metals can be present [9-12]. Methods for the analysis of environmental, food, and biological samples are used to keep control over these residues. Among others, wastewater, soil, crops, fruit and vegetable products, honey, milk, meat, or eggs are of interest [9,10,13-15].

The characteristic of these samples is the complexity of the matrices. For example, proteins, polysaccharides, lipids, and other metabolic products are typical components of biological matrices, making direct analysis impossible as they would cause interferences in qualitative analysis and even cause damage to used instrumentation, e.g. by clogging or absorption on active surfaces [10,13,16-18]. For this reason, the samples are chemically and physically treated, and the evaluated compounds (analytes) are subsequently extracted. In other words, a sample preparation consists of removing the matrix and obtaining an extract with analytes, corresponding to the needs of the used separation and detection techniques [19].

With higher needs for this branch of analysis, for the reasons mentioned above, new extraction methodologies or new modifications to older approaches are developed constantly [4,7,14].Selected examples of which are described in the following chapters.

2.1.2. Liquid-liquid extraction

One of the essential and oldest extraction techniques is liquid-liquid extraction (LLE), sometimes also denoted as solvent extraction [20,21]. In principle, the compound of interest is distributed between two immiscible liquid phases based on its solubility in each of them. In practice, one of the phases is hydrophilic, primarily aqueous, and the second is hydrophobic, an organic solvent or combinations of multiple solvents [22]. The extraction itself is determined by several factors. The first being the partition coefficient P which describes a ratio of the compound concentrations in octanol and water at the point of equilibrium. A more common representation is the decimal logarithm of P (logP) [23]. Positive values of logP indicate an affinity of a compound to the hydrophobic phase, while negative values an affinity to water. The value depends on temperature and in the case of an analyte with ionizable groups, on the pH value.

The second factor is the surface area of the two phases since the transition of the compound is only possible there. Thus, with the larger area, the extraction is more effective, i.e., it reaches the equilibrium in a shorter time. Therefore, an essential step of LLE is thorough mixing, creating a dispersion with a large surface area. Phase separation occurs spontaneously or is forced, typically by centrifugation. The overall effectiveness of LLE can be increased with multiple repetitions of the procedure as the transition is always fractional, based on the logP value. A compound can be extracted from the aqueous phase into the organic or vice-versa. When it undergoes a second extraction back to a solvent with similar characteristics, e.g., into clear water or appropriate buffer, the process is referred to as back-extraction.

LLE can be performed with large volumes in the separatory funnel, or it can be down-scaled to microlitres by various approaches, often comprised as liquid-liquid microextraction (LLME). Since biological samples are often limited to small volumes of a few hundred microlitres or less, in particular LLME technique is frequently the method of choice [24].

To lower the volume of used extraction solvent to a minimum, a dispersive solvent can be added to the extraction solvent. Together, the solvents are rapidly injected into the sample upon which cloudy dispersion of small solvent droplets is formed, which increases the surface area immensely. This technique, named dispersive liquid-liquid microextraction (DLLME), for its advantages, became popular for the sample preparation throughout all fields of analysis [25,26]. In many common protocols, described for various uses [14,15,27,28], mixing and solvent dispersion are done by vortexing and phase separation is forced by centrifugation.

As immiscible extraction solvents are not ideal for injection to some detection instruments, above all, HPLC, the collected organic phase is often evaporated to dryness under a flow of inert gas. The analytes are then redissolved into an appropriate solvent according to the needs of the subsequent separation and detection technique.

2.1.3. Homogeneous liquid-liquid extraction and QuEChERS

With the need for even faster, easier, cheaper, and greener methods for sample preparation, a novel form of LLE was developed, called homogenous liquid-liquid extraction (HLLE).

HLLE is taking advantage of an initially homogeneous solvent system, i.e., one liquid phase and no boundary layer separating the aqueous sample and the organic solvent. Therefore, the extraction of a solute is possible in the entire volume, increasing the effectivity, sometimes described as if the surface area for LLE was infinite [29]. The homogeneity is achieved with a consolute (mutually soluble in all proportions) solvent with amphiphilic properties, e.g., acetonitrile (MeCN) or methanol (MeOH), dissolving a small volume of an otherwise immiscible extraction solvent, e.g., chloroform or tetrachloroethan, in the aqueous sample [30,31]. No vigorous stirring is required, and the volume of toxic organic solvent is lowered. Then, phase separation is induced, e.g., by change of temperature or pH or addition of salt, disrupting the dissolution equilibrium of the ternary solvent system. The earliest reports on HLLE [32], described the use of acetone (ACE) and the addition of salt to induce phase separation.

There are many adaptions to this setup, but the principle of using an initial homogenous system is the same. For example, if an analyte can be extracted directly into a water-miscible solvent, the immiscible solvent is omitted. Alternatively, the extraction is taking place in the initial homogenous solution of two miscible organic solvents, subsequently separated by the addition of water. Systems based on ionic-pairing interactions, surfactants, or pH balance have also been described [29,33].

One particular adaptation, presented in 2003 as the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method, has experienced a boom in biological sample preparation and was presented in wide variations [7]. It is based on HLLE performed with an aqueous sample and using a water-miscible organic extraction solvent and a single salt or multiple salts to induce phase separation. The extract is then cleaned up by dispersive solid-phase extraction (d-SPE). Generally, not the analyte but the interferents are adsorbed to the sorbent. The original method was developed for the analysis of pesticides in fruit and vegetables. With the increasing popularity of QuEChERS, different improvements and adaptations have been reported [7]. New applications include monitoring of drugs in pharmacy and medicine as well as contaminants in the food industry or environment.

New combinations of reagents and experimental conditions were successfully tested, resulting in a wide range of options. Here, few examples of used agents are presented. MeCN, MeOH, or ACE as extraction solvents. Salts such as $MgSO_4$, NaCl, Na_2SO_4, $ZnSO_4$, or sodium acetate, often in various combinations for induction of phase separation [7,34]. Modified silica gel, polyvinylpolypyrrolidone, or salts for d-SPE clean up.

A particular HLLE approach, called salting-out induced liquid-liquid extraction (SALLE) is commonly used for sample preparation as well [1,35-37]. As the name suggests, it is HLLE where phases are separated upon the addition of a salting-out agent (Figure 1B). Since there is no d-SPE clean up, compared to QuEChERS, the extract must be either processed to suit the analysing technique or in a directly applicable form.

In both SALLE and QuEChERS techniques, the addition of salts is used to disrupt the equilibrium of a homogenous system of solvents, resulting in phase separation. A decrease in the solubility of an organic solvent and an analyte in an aqueous phase, dependent on an increase in the salt concentration, is called the salting-out effect, as depicted in Figure 1 [34].

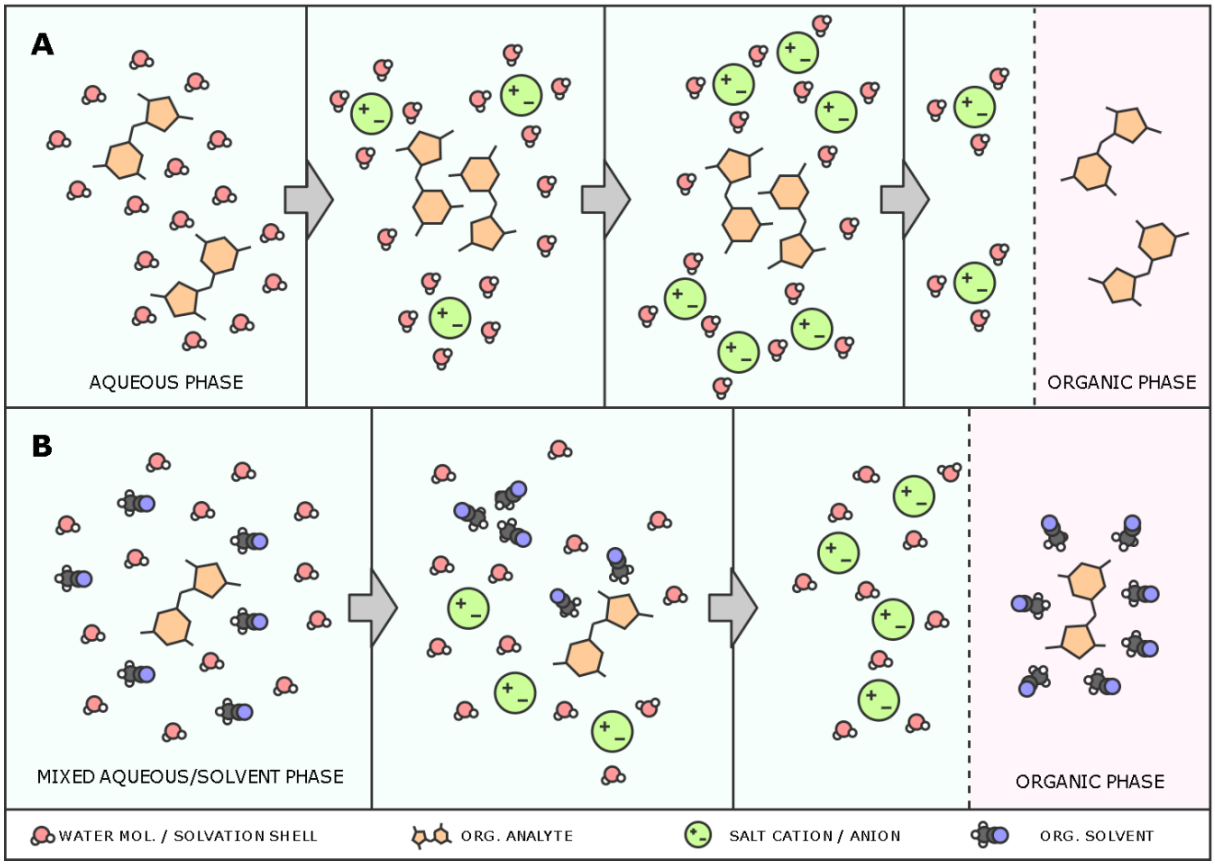

Figure 1. Representation of the salting-out effect in extraction methods. **A:** Salting-out induced extraction of polar analyte into water-immiscible organic phase. **B:** Salting-out assisted liquid-liquid extraction. The salting-out effect induces phase separation of initial homogenous mixture of aqueous sample containing an analyte and water-miscible solvent. [34]

As described over a century ago [34,38], different cations and anions show an unequal salting-out ability, which is based on the physical background of the salting-out effect. In brief, charged salt ions show higher solvation potential than a water-miscible organic solvent, i.e., the solvation capacity of water molecules is more likely to be used up by the salt ions. Solvation of a solute by water (hydration) is provided by hydrogen bonding as well as ionic and van der Waals interactions [38,39]. It was discovered that the salting-out abilities of different ions correspond with the Hofmeister series, which is series of anions and cations ordered by their ability to change protein solubility [40], as shown in Figure 2. Based on the behaviour in an aqueous solution, the ions are classified as the kosmotropes (order-making) and chaotropes (chaos-making), as shown in Figure 2. The kosmotropes have the ability to rearrange the water molecules in effective way to increase the hydration potential. In addition, the salting-out effect of anions is superior to the effect of cations [34].

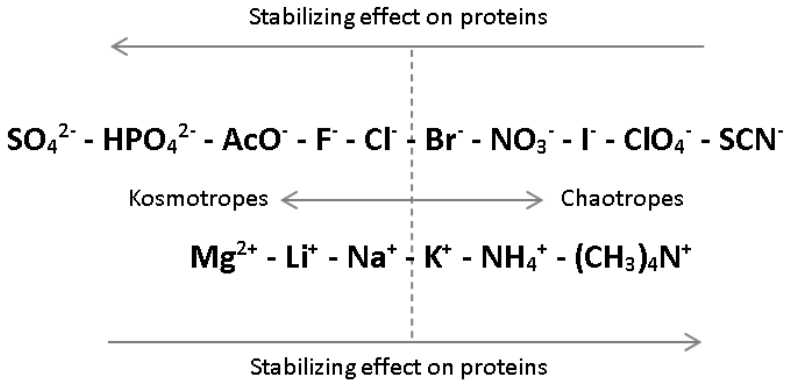

Figure 2. The Hofmeister series of anions and cations. Diagram shows the effect of ions on protein stability and division into kosmotropes or chaotropes. [38]

With the help of the Hofmeister series, the efficiency of salts, available for use in SALLE can be estimated. Nevertheless, the exact salting-out properties of different ions always depend on conditions and the nature of the initial mixture. With respect to the Hofmeister series and from established procedures of SALLE and QuEChERS, anions as SO_4^{2-}, CO_3^{2-}, Cl^- or acetate, and cations as Na^+, K^+, Li^+, Mg^{2+} or NH_4^+ work as salting-out agents [1,7,35,36]. Moreover, other highly polar molecules such as saccharose or glucose were also used to induce phase separation in sample preparation technique, denoted as sugaring-out liquid-liquid extraction [41].

The salting-out effect can also force the transition of a polar analyte from an aqueous sample into a separate organic phase. Similar to a water-miscible solvent, the analyte solubility is decreased by the salting-out effect of salt ions, disrupting the solvation shell around the analyte (Figure 1A). This principle is used for extracting highly polar analytes by LLE, as described in section 2.1.2.

2.1.4. Solvent-aided deproteinization

As mentioned in section 2.1.1, matrices of biological samples often include proteins. Thus, an important part of the preparation of these samples is deproteinization, i.e., elimination of proteins [19]. In practice, it is a conversion of in-sample soluble proteins to insoluble precipitate. Protein precipitation is induced by chemical and physical stimuli, e.g., change of pH, the addition of a water-miscible organic solvent, salting-out, or as a result of protein denaturation [42]. The efficiency of the deproteinization depends on the concentration of the precipitating agent and subsequent separation of proteins, typically achieved by centrifugation or filtration [19].

The use of an organic solvent for deproteinization is common in protocols for biological sample preparation. Either the proteins or other molecules, e.g., a pharmaceutical substance present in the sample can be the target, i.e., the analyte, of the subsequential analytical method. When the analytes do not require extraction from the sample, e.g., quantitative determination by spectrophotometry or thin-layer chromatography, solvent-aided deproteinization is done separately before analysis [43]. Contrariwise, in extraction techniques, like LLE, HLLE, SALLE, and QuEChERS, deproteinization is naturally achieved with the extraction solvent. Furthermore, protein precipitation is enhanced by the salting-out effect in SALLE and QuEChERS.

The basic design of solvent-aided deproteinization protocol consists of the addition of an organic solvent, e.g., MeOH, MeCN or ethanol, and precipitating the proteins [19,43]. The sample is then centrifuged, and the proteins are formed into a compact pellet that can be easily disposed of or used for protein analysis [44].

2.2. Background on used automation technique

Automation in general, has been a point of interest in the field of chemical analysis for many decades. It provides the possibility to process a large number of samples and widely independently from human labour [5]. Thus, it often leads to faster, more reliable, and, also by the typically involved downscaling, cheaper analysis in a long-term perspective. It is also considered that automation and miniaturization are a way to greener chemical analysis [5]. The automation technique used in this experimental work, as well as antecedent and related techniques, are described in this chapter.

2.2.1. Flow techniques and available approaches

For automation in analytical chemistry, transfer of chemical reactions into a self-operated and closed system is convenient [5]. The advantages are, for example no contamination from outside or possibility of continuous performance, i.e., 24 hours a day. Flow techniques are based on systems of a narrow tubing, denoted manifold, inside which mixing steps and reactions are taking place as well as a required detection. Such system is controlled by a computer, therefore, can be easily automated.

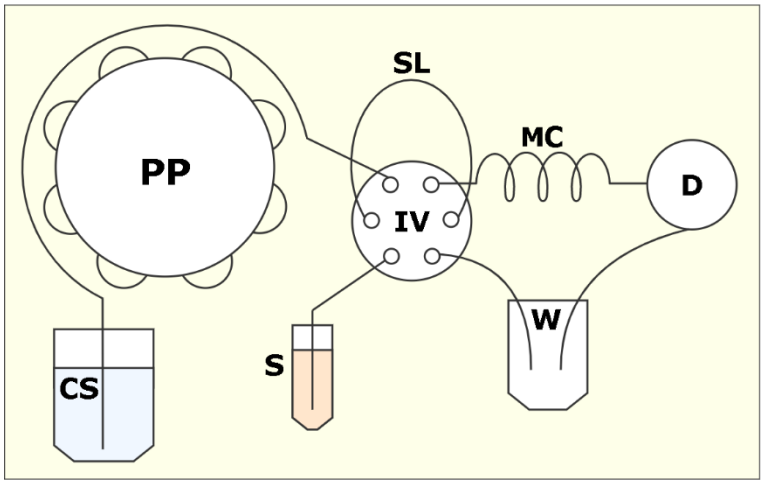

Figure 3. Diagram of a possible setup of instrumentation for FIA. CS: carrier solution, D: detector, IV: injection valve, MC: mixing coil, PP: peristaltic pump, S: sample, SL: sample loop, W: waste.

One of the earliest flow techniques, the so-called Flow Injection Analysis (FIA) [45], is composed of a peristaltic pump providing a continuous laminar flow of a carrier solution through the tubing manifold, as depicted in Figure 3. A sample is injected into the carrier solution via an injection valve. Reagents used as the carrier solution and the sample are mixed directly inside the manifold at the each-other penetrating zones, denoted dispersion. The reaction time is controlled by the length of the tubing section between the injection valve and a detection cell called reaction or mixing coil. The dimensions of this tube are optimized, and serpentine winding or knotting can be used to enhance radial dispersion in addition to the axial component typical for laminar flow, thus, providing improved mixing with minimal zone broadening.

The detected signal is characterized by tailing peaks due to wall friction. Most often, spectroscopic detection methods are used. The main advantage of FIA is its reproducibility since the experimental conditions, e.g., flowrate of the carrier solution or volumes of reagents, are fixed by the tubing dimensions and the set flow rate of the carrier. In consequence, the dispersion and mixing pattern, as well as the reaction kinetics, are constant in FIA and detection can take place before the reaction equilibrium is reached, thus providing the possibility for very fast analysis.

A new concept of flow automation was set by the invention of Sequential Injection Analysis (SIA), featuring the use of a computer-controlled bidirectional pump, generally a syringe pump, and a multiposition selection valve [46], as depicted in Figure 4. The central port of the selection valve is connected via tubing, denoted holding coil, to the syringe pump. The syringe pump is filled and refilled as required by the carrier solution. The continuous carrier flow inherit to FIA is replaced with a combination of aspiration and dispensation steps from and towards the valve performed by the syringe pump in a programmed sequence. Typically, solution zones of sample and reagents are aspirated into the holding coil where mixing via dispersion occurs. Upon flow reversal through one of the lateral ports of the selection valve towards a detector, the mixing is enhanced as the axial dispersion acts in both ways. The reacted sample is passed through the detection cell and towards waste while acquiring signal data. In contrast to FIA, the volumes and flow rates are easily adapted not by physical exchange of tubing but by adaptation of the operation program on the controlling computer.

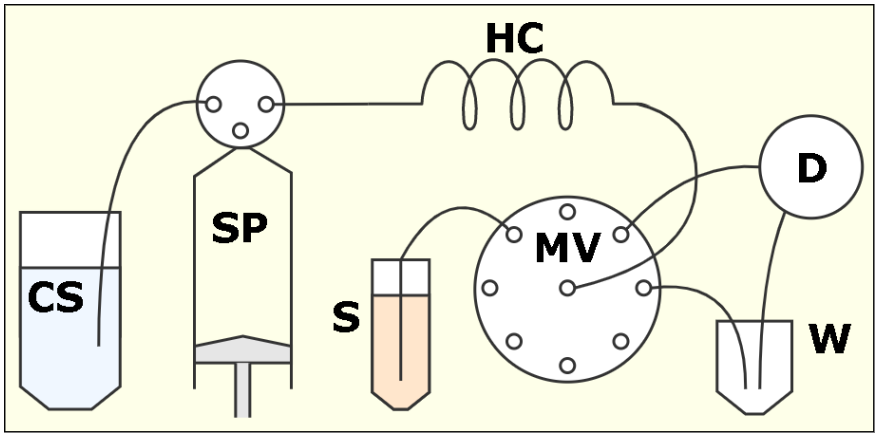

Figure 4. Diagram of a possible setup of instrumentation for SIA. CS: carrier solution, D: detector, HC: holding coil, MV: multiposition valve, S: sample, SP: syringe pump, W: waste.

The versatility of SIA allows performing multistep analytical methods [47,48]. For example, by computer control, the zone of reacting solutions can be positioned precisely into the detection cell so that on-reaction kinetics can be observed in real-time, allowing, for instance, the differentiation of analytes by their different speed of reaction. On the other hand, both FIA and SIA are incapable of separation of similar analytes and are therefore rather used for measuring similar analytes as sum parameters than as separated compounds.

It should be pointed out that FIA and the SIA were modified in multiple ways to be used for various purposes and their capability was enlarged e.g., by incorporating additional valves to re-direct carrier or reagent flows or intelligent use of segmentation air bubbles. Moreover, system components such as mixing chambers, SPE columns, or low-backpressure separation columns were used in many reported methods [47-49]. A noteworthy example of a flow technique and operation concept derived from SIA is the Lab-On-Valve (LOV) technique, where reaction and detection take place directly in a purpose-designed transparent stator of the multiposition valve with great potential to automate SPE protocols [6,50].

2.2.2. Basic principles of Lab-In-Syringe

Similar to the LOV, the Lab-In-Syringe (LIS) technique is a flow technique inspired by SIA that has been developed in 2012 [51]. In LIS, the syringe pump is used for most functions normally integrated in the tubing manifold: e.g., reaction chamber, mixing chamber, and detection cell [52], which is in contrast to SIA where the syringe pump is used only as a pump and is filled only with the carrier. Most importantly, the holding coil is shortened to a minimum as all solutions are aspirated into the syringe void and mixed there, while the typical SIA components, being the multiposition valve and generally a connected detection cell, are kept.

The syringe pumps used for LIS and SIA systems are computer-controlled, thus allowing to operate at a set flow rate. The syringe itself is generally made of a glass tube of few millilitres in volume, the syringe barrel, and the movable piston with a PTFE head. In addition, the syringes comprise a head valve of at least two positions for connection either to the selection valve or a carrier reservoir (SIA), or allowing waste disposal.

Typical for LIS is the aim to minimize the dead volume given by the holding coil [52]. Therefore, in LIS procedures, the air is sometimes used to drive the liquids through and out of the holding coil into the syringe void, or to create a bubble-like segmentation between solutions that should not be mixed in the holding coil but inside the syringe void, as it was the case in this work.

Another feature developed for LIS is the concept and the equipment for in-syringe magnetic stirring [53]. A magnetic stir bar is placed inside the syringe void and is driven by an external rotating magnetic field. For this, a plastic ring holding a pair of neodymium magnets is placed onto the syringe barrel. This stirring ring is then operated by a rotary motor via a rubber belt. Another way allowing slow stirring is to place a motor with a bar magnet fixed on top close to the syringe [52]. In-syringe stirring allows efficient and thorough mixing inside the syringe void and, in consequence, a whole range of possible methodologies to be carried out in LIS.

Among others, LIS has been proven to be a very versatile automation device for extraction techniques such as HLLE [54], DLLME [55,56], SALLE [57], d-SPE [53] or head space extraction [58]. Given the dimensions of the used syringes, those methods were not only automated but also miniaturized, leading to greener alternatives to the traditional concepts [5] while on the other hand, larger volumes (several millilitres) can be treated as well, which is typical for SIA or FIA.

A LIS setup can be set up with two possible orientations of the syringe pump [52]. The first one, called upright, presents the piston at the bottom and the head valve at the top of the syringe. As a result, phases of lower density are always expulsed first as it could be desired for some applications, e.g., liquid-phase extraction with solvent of density lower than water. On the other hand, the stirring bar inside the syringe creates a dead volume that will be always filled by liquid, in other words, the syringe cannot be emptied completely of liquid. In the other orientation, upside-down, the head valve is at the bottom and the piston is at the top. As expected, the emptying order of phases based on the density is reversed and air remains inside the syringe. By aspiration of enough air, it is enabled to push out all liquid content from the syringe void. However, due to the compressibility of air, all liquid movements are delayed, and additional waiting periods must be applied after each dispense or aspiration.

2.3. Background on automated solvent-based sample preparation

In usual biological sample analysis, an extract is gathered from a treated sample, subjected to a technique for analyte separation and detection. The previously mentioned flow techniques are great tools for automation of sample treatment procedures, but the ability to separate similar analytes is limited. Moreover, separation techniques like high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to mass spectrometry (HPLC-MS, GC-MS) or spectrophotometry (HPLC-UV/VIS) are reliable and more popular for these tasks [2]. However, they usually require manual and time-consuming sample preparation. That creates an ideal opportunity to use flow techniques for automated sample preparation and extraction of analytes.

2.3.1. Automation of solvent-aided sample preparation

The automation of analytical methods is a point of interest for many analytical scientists and technicians [59]. At first, busy large laboratories used automated instruments managing dozens of samples at once, usually with programable robotic arms. Those were accessible only for laboratories, dealing with large amounts of samples and with sufficient financial support [60]. Simultaneously, simpler, cheaper, and more accessible techniques were developed based on basic laboratory instrumentation, self-crafted or accessible components. This allows the possibility to widely research automation techniques. In summary, automated sample preparation allows operating multiple samples at once or in sequence without human assistance, leading to faster and more precise results [61,62].

Treatment of multiple samples usually relies on a computer-operated robotic arm with an autosampler syringe, performing simple tasks like sampling, pipetting, or shaking. So prepared samples in multiposition trays are ready for the analysis with the use of auto-sampler. This technique was applied for mass cytometry [63] or HPLC-MS [64].

In the second approach, sequential order of steps is performed by an instrument. The steps are programmed, controlled, and executed by computer software, leading to an automated and precise technique. This is the concept of SIA and LIS automation techniques for sample preparation [47,48]. Besides those, other sequential techniques for liquid-phase sample preparation were reported, based on, e.g., the multi-chamber device where liquids are driven by a stream of pressurized air [65] or the multifunctional autosampler syringe with on/off valve [66].

The preparation of biological samples containing proteins poses a different problem for automation. The use of centrifugation, as mentioned in section 2.2.2., requires work and the intelligence for the task and is hardly automated. On the other hand, the use of filtration, which can be automated, is a viable option. Nevertheless, in a large scale, it is connected to disadvantages such as use of large amounts of filters or the possibility of blockage. Therefore, centrifuge-less techniques for the deproteinization are of interest.

2.3.2. Use of Lab-In-Syringe in liquid-phase sample preparation

Considering the above-mentioned points on the LIS technique, it can be assumed that LIS is ideal for automated liquid-phase sample preparation methods [52]. This statement is supported by three main pillars, the first one being a possibility to precisely aspirate and mix sample and reagents in the syringe in a given sequence, as needed, imitating a manual vial-based protocol. The next being the in-syringe stirring, which provides homogenous mixing comparable to the use of vortexing in manual protocols. The third one is the fact that the content of the syringe void is dispensed in exact order based on the density of a given layer of separated phases. In other words, the extract can be segmented and collected via an assigned port. Other possible features applicable for liquid-phase sample preparation methods are discussed in section 1.2.2.

The first liquid-phase extraction technique automated by LIS was DLLME [53,55,56]. This approach takes advantage of the use of liquid reagents only, which are easily handled inside a tubing manifold and the syringe itself. The final extract can be analysed directly in the manifold using a detection cell or be subjected to advanced separation and detection techniques such as HPLC-UV/VIS.

The merging of HLLE and LIS techniques has also been a focus of interest with the problematic point being the induction of phase separation of the initially homogenous solution inside the syringe. Direct addition of salt, as the most often used approach of salting-out induced phase separation, is unavailable since the salt in solid form cannot be added into the manifold. However, related methods, using a SIA system enlarged by an open-top mixing chamber, have been published [47]. A true HLLE with the LIS approach was developed with the phase separation inducted by addition of an acid or an alkaline solution, i.e., changing the pH value [48,54]. This method can be considered as a theoretical bridge to perform SALLE with the LIS technique. That is because the chemical principle of the salting-out and the pH change is similar. In both techniques, the solvation shells are broken down by ionic or non-covalent interactions between the charged ions, water, and solvent molecules.

The disadvantage to the addition of acid or alkaline solution is a possible affection of the analyte stability as the pH changes. On the other hand, the use of a liquid reagent for the induction of the phase separation is convenient.

Based on this, a novel method presenting the use of the LIS and SALLE was published recently [57]. It promotes the use of a highly concentrated solution of a salting-out agent to induce the phase separation. As this work deals with a similar matter, the development of deproteinization and salting-out assisted phase separation of milk samples for the LIS. It was created upon the cooperation with Fikarová et al., the research group behind the previously mentioned method.

3. Objectives

The main objective of this work was to develop a method of automated centrifuge-less deproteinization of milk samples by using homogeneous liquid-liquid extraction with salting-out induced phase separation and using the Lab-In-Syringe automation technique for this task. This development was aimed to lead to a method that could be applied to sample preparation and the extraction of pharmaceutical analytes and offering the possibility of direct coupling to HPLC.

Individual objectives were set as follows:

- Study of different salting-out agents by offline experiments with photographic documentation.
- Study of different solvents for protein denaturation and homogenous liquid-liquid extraction, focussing on the deproteinization process, by offline experiments with photographic documentation.
- Determination of an optimal ratio between milk, solvent, and salting-out agent by offline experiments with photographic documentation.
- Testing two different Lab-In-Syringe configurations (syringe pump upright and upside-down) and choosing the better suited one including the development and programming of an automated protocol for an intended method.
- Optimization of the instrument layout by online experiments.
- Optimization of a cleaning protocol by online experiments.
- Determination of the optimal ratio of milk, solvent, and salting-out agent in the automated system, taking to account prior offline experiments.
- Determination of the optimal values of programable variables being primarily stirring rate and phase separation time by online experiments.

Critical discussion on individual results, novel insights, suitability for HPLC coupling and troubleshooting.

4. Materials and methods

4.1. Reagents and solutions

Ultrapure water (18.2 MΩ cm^{-1}) was supplied by a Merck Millipore purifying system (Burlington, Massachusetts) and used throughout this project.

All chemicals used were of analytical grade if not stated otherwise, purchased from Sigma Aldrich (Darmstadt, Germany).

A diluted astraphloxine solution of approx. 12.5 µmol/L was prepared by diluting 5 µL of astraphloxine stock solution, approx. 25 mmol/L, in 10 mL of water. The astraphloxine stock solution was pre-prepared for various usage in the laboratory and stored at 4°C.

For offline testing of phase separation, aqueous dilutions of acetonitrile (MeCN) and propan-1-ol (PrOH) of 120 mL each were prepared. Each solvent was diluted with water to 50% (v/v) and 67% (v/v) concentration inside laboratory flasks. To each of these four solutions, 300 µL of the diluted astraphloxine solution was added, giving the solutions a light-pink colour. Since the solutions were used for one test only, there was no need for storage. For protein denaturation and precipitation, pure MeCN, PrOH, and acetone (ACE) were used throughout the development of the method.

To test the ability to induce phase separation by salting-out, highly concentrated solutions (salting-out solutions) were prepared. Used concentrations were calculated based on the nominal saturation of the compounds at 20 °C [67] and the preparability coefficient, discussed in section 5.1.1. Values are presented in Table 1**.** Data on the solubility of selected compounds and concentrations of prepared salting-out solutions [67]. Namely, 1.1 mol/L $NaSO_4$, 1.7 mol/L $ZnSO_4$, 2.3 mol/L $MgSO_4$, 4.9 mol/L NaCl, 6.7 mol/L ammonium acetate, 2.0 mol/L sucrose, 1.3 mol/L trisodium citrate, and 1.4 mol/L disodium tartrate water solutions. In addition, two mixed solutions of 1.2 mol/L $MgSO_4$ with 2.4 mol/L NaCl (mixed solution A) and 1.9 mol/L $MgSO_4$ with 1.0 mol/L NaCl (mixed solution B) were prepared that corresponded to 1:1 and 4:1 weight ratios. The calculated amounts of these substances were weighted with a precision of two decimals (10 mg) and were dissolved in a beaker in approx. 25 mL of water. This process was accelerated by magnetic stirring, gentle heating, and ultrasonication. After cooling down to room temperature, they were transferred into a 50 mL volumetric flask and topped with water up to the mark. So-prepared solutions were stored in 50 mL clear polypropylene falcon vials, closed tightly, and at laboratory temperature.

Table 1. Data on the solubility of selected compounds and concentrations of prepared salting-out solutions [67].

Compounds	**Na_2SO_4 anh.**	**$ZnSO_4$. $7H_2O$**	**$MgSO_4$ anh.**	**NaCl anh.**	**NH_4Ac anh.**	**sucrose**
Solubility [g/50 mL]	9.8	48.3	17.6	17.9	74.0	100.0
Preparability coefficient	0.80	0.50	0.80	0.80	0.35	0.35
Mass concentration [g/50 mL]	7.8	24.1	14.0	14.3	25.9	35.0
Molar weight [g/mol]	142.04	287.56	120.36	58.44	77.08	342.30
Molar concentration [mol/L]	**1.1**	**1.7**	**2.3**	**4.9**	**6.7**	**2.0**
Compounds	**Na_3Cit anh.**	**Na_2Tar anh.**	**1:1 $MgSO_4$: NaCl (w/w)**		**4:1 $MgSO_4$: NaCl (w/w)**	
Solubility [g/50 mL]	21.3	16.7	8.8	8.9	14.0	3.6
Preparability coefficient	0.80	0.80	0.80	0.80	0.80	0.80
Mass concentration [g/50 mL]	17.0	13.3	7.0	7.1	11.2	2.9
Molar weight [g/mol]	258.07	194.05	120.36	58.44	120.36	58.44
Molar concentration [mol/L]	**1.3**	**1.4**	**1.2**	**2.4**	**1.9**	**1.0**

Abbreviations: anh. – anhydrous, Cit. – citrate, Tar. – tartrate.

The following solutions were prepared for testing of their ability to clean the syringe from any precipitated protein or milk fat remains in automated deproteination: 50 mmol/L sodium dodecyl sulphate (SDS) in water, 50 mmol/L SDS in 50% (v/v) isopropanol (iPrOH), and a 1 mol/L $NaHCO_3$ aqueous solution. For the SDS solutions, 144 mg of the SDS was dissolved in about either 8 mL of water or 50% iPrOH, stirred slowly to avoid foaming, topped up to the mark in a 10 mL volumetric flask. For the $NaHCO_3$ solution, 840 mg $NaHCO_3$ was dissolved in about 8 mL of water, transferred to a 10 mL volumetric flask, and filled up to the mark. In addition, pure ethyl acetate (EtAc), PrOH, and iPrOH were used as cleaning agents. Prepared solutions were stored in 15 mL clear polypropylene falcons, closed tightly, at laboratory temperature.

A 50% (v/v) milk solution from locally purchased semi-skimmed milk (1.5% fat content) was used for all experiments. The milk solution was prepared fresh daily and any unused solution was discarded.

4.2. Instrumentation

The Lab-In-Syringe (LIS) systems were set up in two configurations shown in Figure 5 and Figure 6, respectively, and tested for automated milk deproteinization. In the first configuration, the syringe pump was used in upside-down orientation, while in the second configuration, the syringe pump was used in the usual [52], upright orientation.

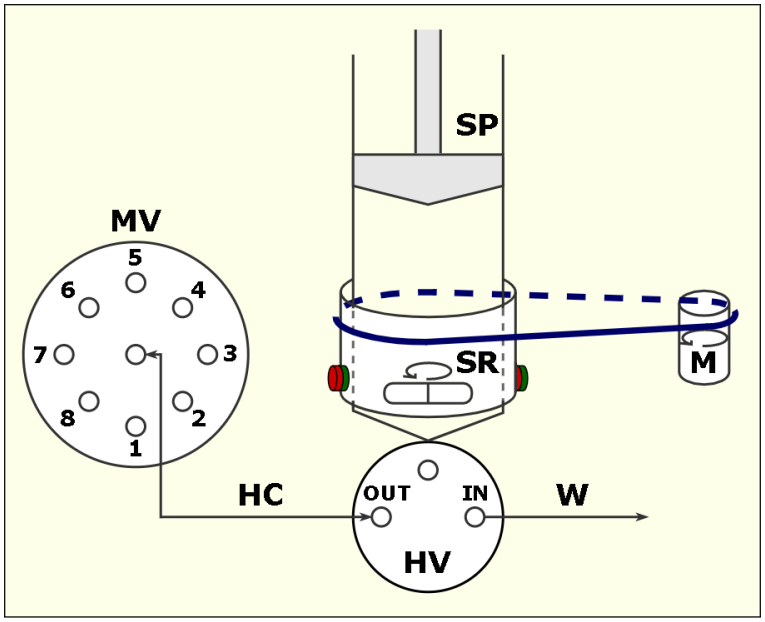

Figure 5. Diagram of used Lab-In-Syringe system, configuration with upside-down orientation of the syringe pump. HC: holding coil, HV: syringe head valve with depicted IN and OUT ports, M: rotary motor, MV: multiposition valve with depicted eight lateral ports, SP: syringe pump composed of piston and glass syringe, SR: stirring ring, W: waste output.

In both setups, the LIS system featured an automated syringe pump of 6 cm stroke length with a two-port head valve (type Cavro XLP) from Tecan Inc. (Männedorf, Switzerland) equipped with a 5 mL glass syringe and an 8-position rotary selection valve (Vici Valco, Schenkon, Switzerland). Head valve ports were named IN and OUT, relating to Sequential injection analysis (SIA) techniques where the syringe pump is used. Designation IN refers to the aspiration of a carrier solution into the syringe and OUT to discharge towards a detector through the holding coil.

Head valve IN port was connected to waste for direct discharge of the syringe content during cleaning, making this step more efficient. Later, during the development of the method, the head valve position IN port was assigned to the collection of the organic phase, as shown in Figure 6. The head valve position OUT was connected to the central port of the selection valve via a 15 cm long holding coil (PTFE, 0.8 mm i.d., approx. 75 µL) that allowed the aspiration of water, buffer, air, cleaning agent, milk, salt solution, and solvent positioned on the lateral ports 2 to 8 into the syringe void. Similarly, it allowed the discharge of any waste from the syringe through the lateral port 1.

To enable in-syringe mixing of all solutions, a 10 mm long magnetic stirring bar (coated with PTFE, 3 mm in diameter) was placed inside the syringe void. Rotation was forced by a stirring ring that was placed onto the syringe barrel. This ring held two neodymium magnetics to align and levitate the stirring bar inside [53]. The stirring ring was driven via a rubber band (4.5 cm in diameter) by a DC rotary motor featured from a pulse-width modulated computer fan that was controlled by a simple analogue circuit and activated by relay contact via the FIALab instrument.

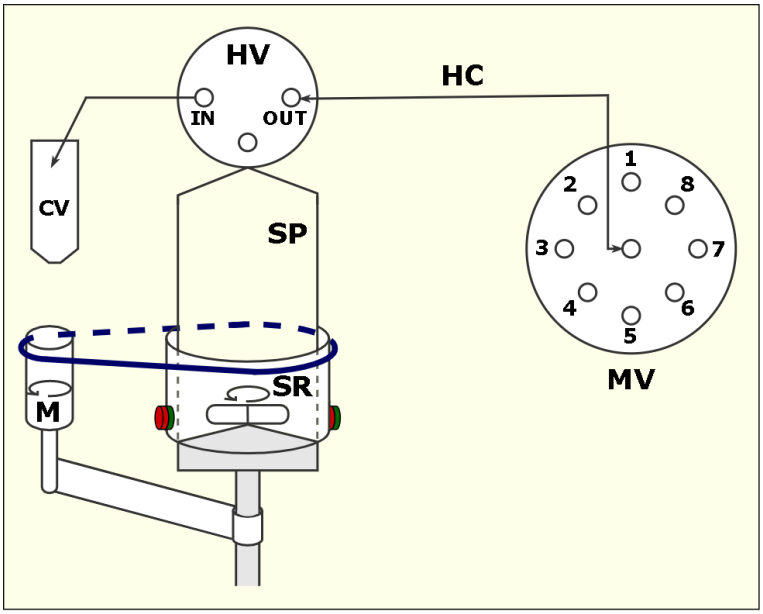

Figure 6. Diagram of used Lab-In-Syringe system configuration for the upright orientation. CV: collection vial, HC: holding coil, HV: syringe head valve with depicted IN and OUT ports, M: rotary motor mounted onto syringe piston, MV: multiposition valve with depicted eight lateral ports, SP: syringe pump composed of piston and glass syringe, SR: stirring ring.

For the upright position of the instrument, a support mounted onto the syringe piston was prepared to allow the motor and stirring ring to move alongside the stirring bar, as depicted in Figure 6. The stirring ring, motor support, and a pully-wheel head for the stirring motor were produced by 3D printing using a DeltiQ FDM printer from Trilab company (Hradec Králové, Czech Republic) from PLA filament.

All LIS instrumentation was controlled by FIALab software (FIALab Inc.) and used methods are given in the appendix (Appx. 1 and 2).

The stirring rate was measured via a contactless laser tachometer (Mextech, DT-2234C) and using a reflective strip that was stuck onto the stirring motor.

4.3. Operational protocols for offline experiments

The following experiments were performed manually and allowed limiting the experimental variables for the following experiments on automated deproteination.

4.3.1. Offline study of salting-out induced phase separation

First, 5 mL of solvent solution was transferred into graduated 25 mL glass cylinders, numbered for recognition, and values read from the graduation marks on the cylinders were noted down. Into each cylinder, a different salting-out solution was added stepwise by pipetting. After each addition, the solutions in the cylinders were shaken thoroughly for approx. 30 seconds and the total volume and the volumes of possibly separated phases were noted down after another 60 seconds. In addition, photographs were taken for documentation. The whole procedure was carried out with all solvent solutions.

Collected data were processed via MS Excel software into spreadsheets. To evaluate the efficiency of the salting-out reagents, theoretical residual solvent content in the water phase, and vice versa, were calculated using Equation 1 and 2, respectively.

$$R_S = \frac{V_{aq} - V_{theo}}{V_{aq}} \cdot 100\% \qquad \text{Eq. 1}$$

$$R_W = \frac{V_{org} - V_{theo}}{V_{org}} \cdot 100\% \qquad \text{Eq. 2}$$

Here, R_S is the residual solvent content in the aqueous phase and R_W is the residual water content in the organic phase, both calculated as the percentage of the difference between the actual and theoretical volume of opposite phase, abbreviated as V_{aq} or V_{org} and V_{theo}, respectively.

4.3.2. Offline study of milk deproteinization

For the study of protein precipitation in offline experiments, 5 mL of 50% (v/v) milk was pipetted into graduated 25 mL glass cylinders, numbered for recognition, and values read from the graduation marks on the cylinders were noted down. The first cylinder was kept as the control. In the next step, pure solvent was pipetted into the remaining cylinders with increasing volume, thus, creating a series of increasing solvent-to-milk ratio samples. The cylinders were shaken thoroughly for approx. 30 seconds and left undisturbed for another 60 seconds. Volumes of possibly formed phases were noted down and photographs were taken.

To induce phase separation, a solution of chosen salting-out reagent was pipetted stepwise into the samples of the former series. The cylinders were then sealed by hand and shaken thoroughly for approx. 30 seconds and left undisturbed for another 60 seconds allowing layer formation.

After each addition, the total volume and the volumes of possibly separated phases were noted down and photographs were taken for documentation.

Collected data were processed via MS Excel software. To evaluate the phase separation of different ratios, recovery of the solvent was calculated according to Equation 3.

$$Solvent\ recovery = \frac{V_{final}}{V_{initial}} \cdot 100\% \qquad \text{Eq. 3}$$

Here, V_{final} is the volume of the organic phase after separation and $V_{initial}$ is the volume of organic solvent added for deproteinization.

4.4. Operational protocols for online experiments

In the following, the methods are covered that were executed on the LIS system described in section 2.2. for automated deproteination.

4.4.1. Lab-In-Syringe cleaning protocol

The cleaning protocol written for FIALab software is shown in the appendix as part of the sample preparation operational protocol (Appx. 1) and as separate operational protocol for the syringe cleaning (Appx. 2).

The protocol of automated deproteination started with the thorough cleaning of the syringe void to remove remains of sample, solvent, salt, and precipitated proteins. For this, water was aspirated for an initial rinse and was discharged to waste after 5 seconds. The stirring was activated the whole time, creating a vortex to improve the cleaning. Then, the same step was done with a cleaning agent, followed by another rinse with water. Any of these steps could be repeated multiple times based on the requirements. In the end, the holding coil and the syringe were filled with air, and any remaining water was pushed out by the air. The dead volume of the syringe had to be left filled with water which was acceptable.

Throughout the optimization, different cleaning agents and different volumes were tested. To evaluate the cleaning properties, photographs of the syringe were taken before the first rinse with water and after the last cleaning cycle. For instrument shutdown after use, the input tubes on the multiposition selection valve were placed into a flask with water. Afterwards, an excess volume of water, calculated using Equation 4, was aspirated into each tube, as depicted in Figure 7.

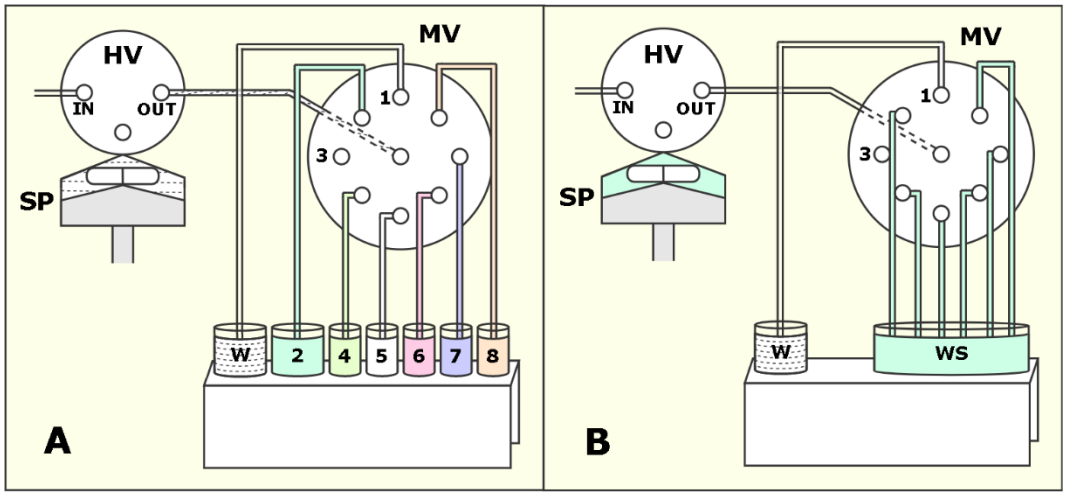

Figure 7. A: Diagram of instrument setup after salting-out induced phase separation and deproteinization of milk. **B:** Diagram of instrument ready for shutdown. HV: syringe head valve with depicted IN and OUT ports, MV: multiposition valve with depicted eight lateral ports, SP: syringe pump, W: waste container, WS: water for storage, 1: lateral port for waste, 2-8: used reagents connected to lateral ports.

Dispersion, width (0.8 mm i.d.), and the length of the tubes were considered in this calculation, so the previous solution is completely replaced by water before shutdown.

$$V_{clean} = 1.5 \cdot \pi \cdot r^2 \cdot l \qquad \text{Eq. 4}$$

Here, V_{clean} stands for the used volume of water, 1.5 is the cleaning factor to account for the dispersion between water and the previous solution, *r* is the inner radius of the tubes, and *l* is the length, different for each tube.

Finally, the syringe is rinsed five times with 2000 µL of water, assisted by activated stirring, before shutdown.

4.4.2. Lab-In-Syringe operational protocol

The operational protocol describing the method, written for FIALab software, is shown in the appendix (Appx. 1) and depicted in Figure 8.

First, 250 µL undiluted milk was aspirated into the syringe. This step was followed by the aspiration of water for dilution. This volume could be used also to aspirate a buffer solution to reach an optimal pH for analyte stabilization, yet this work was to study system and method setup and was not intended for the extraction of specific analytes. Here, the water allowed cleaning of the holding coil to prevent protein precipitation inside the holding coil in the following step. To separate the holding content from the next solution, a segmentation air bubble of small volume (30 µL in our case) was aspirated. Afterwards, the organic solvent was aspirated to denature and precipitate the proteins. Simultaneously, the stirring was activated to assure homogenous mixing of the solvent and the sample. Then, a second air bubble of 100 µL was aspirated, exceeding the holding coil in volume. Thus, all the solvent was driven into the syringe void.

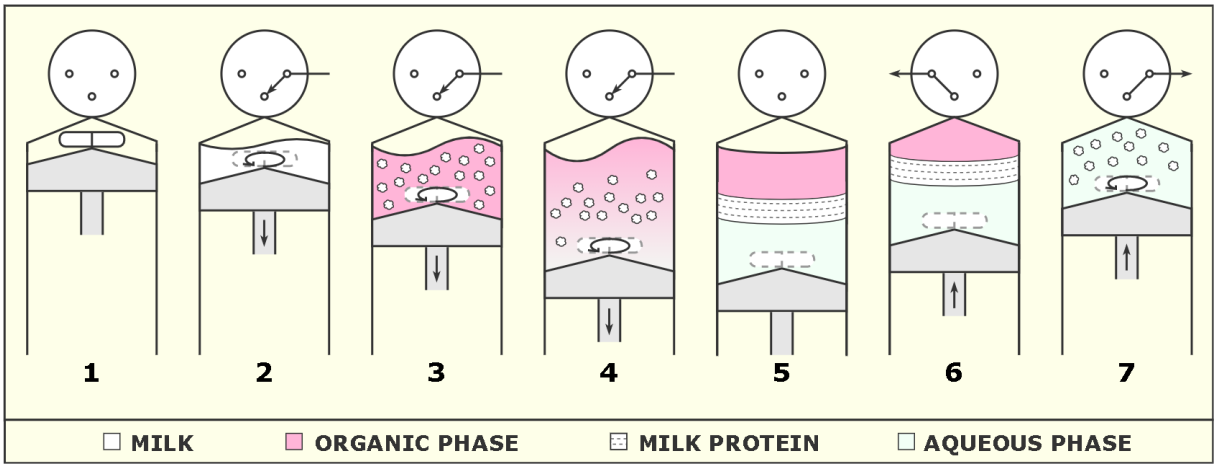

Figure 8. Diagram of automated deproteinization and salting-out induced phase separationd of milk in the LIS system, configuration 2. 1: empty syringe pump, 2: aspiration of milk, 3: aspiration of organic solvent denaturing and precipitating milk proteins, 4: aspiration of salting-out solution, 5: salting-out induced phase separation, 6: dispensation of organic phase, 7: discharge of aqueous phase to waste.

The content of the syringe was left with activated stirring to support the protein precipitation. In the next step, phase separation was induced. For this, the salting-out solution was aspirated with a reduced flow rate due to the high viscosity of this solution. The salting-out solution remaining in the holding coil was driven into the syringe void by a third air segment. The stirring was activated to assure homogenous mixing of the syringe content. As all the salting-out solution was aspirated into the syringe, the stirring was deactivated to enable layer formation. The content of the syringe remained still for a certain time, hereafter called separation time. After the phase separation, the air was dispensed from the syringe to the waste. The organic phase was collected on the head valve IN port into an Eppendorf vial for visual evaluation, i.e., if remains of precipitated protein would be recognizable. With activated stirring, the aqueous phase and protein layer were finally discharged to waste.

Considering that in the end the configuration with the upright orientation of the syringe pump was used, the protocol and Figure 8 are describing only this configuration. The differences to the upside-down orientation of the syringe pump were that waste was assigned to head valve port IN. Also, the order of dispensation of the phases was reversed. The reasons behind these decisions are discussed in section 5.2.1.

5. Results and discussion

5.1. Offline experiments

To prove the concept of centrifuge-less salting-out induced deproteinization of milk, it was decided to carry out preliminary experiments. This way, a basic understanding of the procedure, later to be automated, was obtained. Also narrowing down a wide range of possible salting-out reagents and organic solvents to the ones that fitted best for later application to the LIS system.

Setting the experiments offline, i.e., outside the LIS system, was time-saving and provided us with a simple way to evaluate the experiments. It ought to be mentioned that all the gathered values were read by the naked eye from the graduation marks on the cylinders. The residual contents and the recoveries were only calculated, not analytically tested.

5.1.1. Selection of the salting-out reagent

The salting-out reagents were selected based on research literature on the salting-out phenomenon used for sample treatment [1,36,41,68,69]. Regarded were the availability and the price of the compounds, the molar solubility of the compounds in water, the known capacity for salting out, mostly deduced from the Hofmeister series, the possibility of forming insoluble complexes, e.g., with sample matrix, as well as buffering capacity. For example, carbonate and phosphate, although highly soluble in water and with high salting-out capacity, were not studied as they would precipitate milk-calcium, changing the pH in the range from moderately acidic to moderately basic pH, i.e., if the method should later serve for analyte extraction. Moreover, carbonate could even lead to gas formation if later the method would be applied to the extraction of analytes at acidic pH. In the end, Na_2SO_4, $ZnSO_4$, $MgSO_4$, NaCl, ammonium acetate, sucrose, trisodium citrate, and disodium tartrate were selected. Since the salting-out reagents could not, in contrast to a manual procedure, be added in the form of a solid substance into a flow manifold such as the LIS system, highly concentrated solutions were used instead.

By definition [70], the water solubility is given as weight per volume of water, while for reproducible preparation, weight or mol of substance per final volume of solution would be more practical. The molar concentration also gives a better understanding on the effective salting-out capacity of the individual compounds. Therefore, the nominal water solubility of the compounds was multiplied by preparability coefficient in a range from 0.35 to 0.8, allowing dissolution into 50 mL of the final solution, therefore known molar concentrations, summarized in Table 1. Highly soluble compounds were multiplied with a higher factor to lower the mass below 50 g.

Therefore, it could be dissolved in the 50 mL volumetric flask. Anyway, even the use of a factor of 0.35 left us with highly concentrated solutions. For example, the solubility of ammonium acetate is 74 g in 50 mL of water, which corresponds to approx. 9 mol/L. The molar concentration of our solution was 6.7 mol/L, thus a solution of approximately 74% saturation. Higher concentrated solutions were impractical as a slight change in temperature would lead to the precipitation of a part of the salt. Even so, preparation of the solutions required significant time due to the already high concentrations. Due to overnight precipitation at room temperature, trisodium citrate and disodium tartrate solutions were found impractical and not used in the experiments. Moreover, they show a strong buffer capacity and at this stage, we consider that this would limit the general applicability of the method, e.g., in case that a change in pH might be required. Instead, based on the literature [7,36,68] and preliminary experiments on the induction of phase separation, two mixed solutions of MgSO4 with NaCl were tested of molar ratios of 1:2 (mixed solution A) and 2:1 (mixed solution B) were tested. Also, both salts are often used in combination in QuEChERS protocols [7].

To study the capacity of the selected salting-out reagents to induce phase separation, a solvent solution served as the initial phase. For this, 50% and 67% solutions of both acetonitrile (MeCN) and propan-1-ol (PrOH), coloured by astraphloxine, were used. These mixtures were used since deproteination protocols often use a 1:1 or 1:2 ratio of sample to denaturing solvent [19,43]. Two different solvents were selected to widen the possibility of achieving phase separation, where one was protic (PrOH), and one was aprotic (MeCN). Due to the high lipophilicity of astraphloxine, the dye would preferably be present in the organic phase upon phase separation that would facilitate photographic documentation of the observations. The aim was to select a salting-out solution that would induce phase separation at low concentration and yielding an aqueous phase with solvent content and an organic phase with water content close to 0%. Residual solvent content in the aqueous phase in the range from -5% to 5% was considered effective. Lower values indicate insufficient salting-out effect and presence of water in the organic phase, while higher values indicate possible binding of the solvents by salt ions, thus increasing solvent residuals. The experiment was carried out according to the procedure described in 4.3.1. Data in the form of a spreadsheet, containing the volumes of separated phases and residual contents are shown in the appendix (Appx. 3 - 6). With initial lower additions of the salting-out solution, from 0.25 to 0.75 mL, phase separation was not induced in any of the solvent solutions. Therefore, only additions of the salting-out solution from 1 mL to 5 mL were introduced in Figure 9 and 10. Missing columns in the figures indicate the absence of phase separation. Small columns at the bottom axis indicate phase separation with content of -20% and -50%, or lower, for MeCN and PrOH, respectively. Exact values can be observed in the appendix (Appx. 3 - 6).

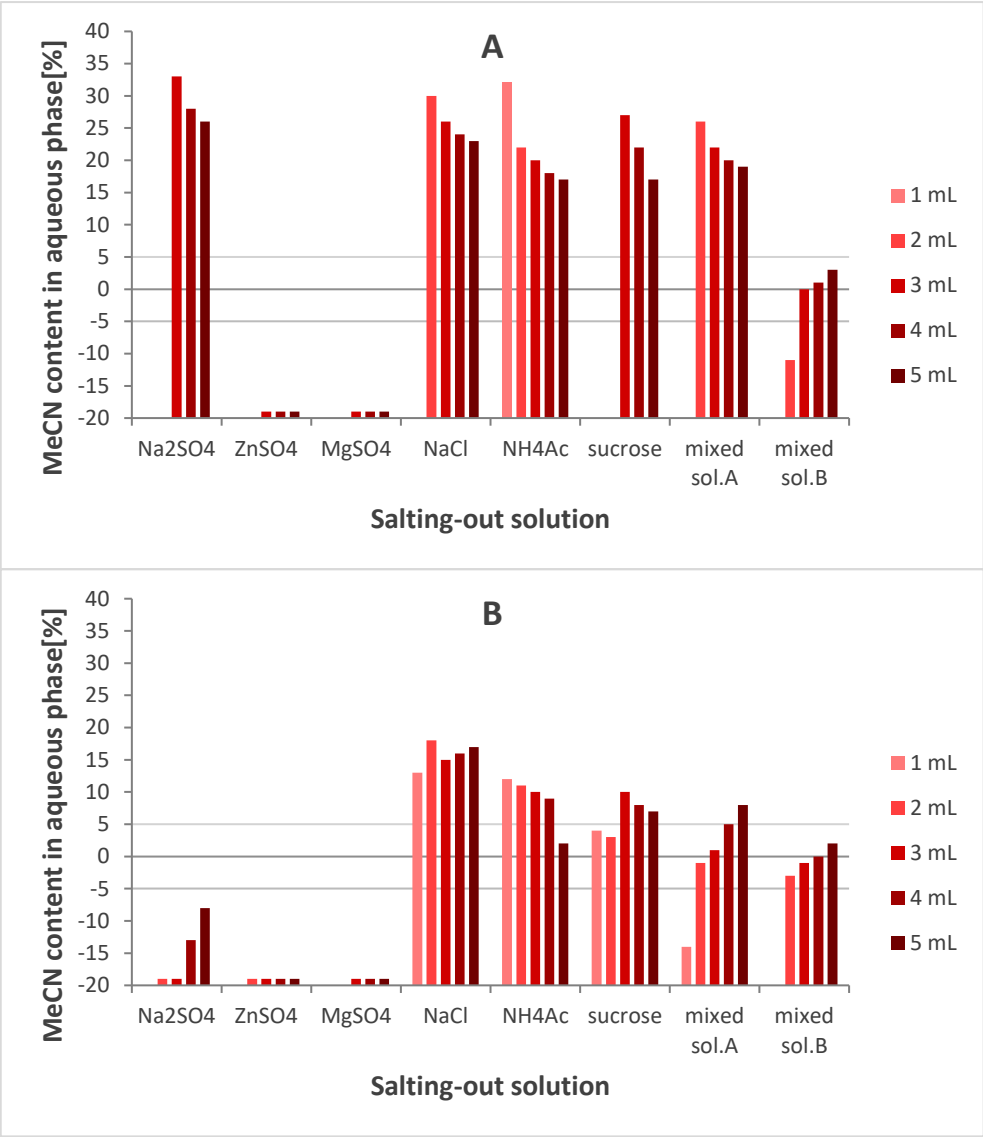

Figure 9. Effect of increasing volume of eight salting-out solutions on MeCN residual content in **A:** 50% MeCN solution, **B:** 67% MeCN solution. Small columns at -20% mark indicate phase separation with MeCN content of -20% or lower. All input values are present in Appx. 3 and 4.

It was found that when MeCN solutions were used, all the salting-out solutions were able to induce phase separation. As expected, the higher content of MeCN in the initial solution (2:1 ratio) was prone to earlier phase separation, i.e., at the addition of less salt solution, and more salting-out reagents induced effective phase separation. Also, the solvent content in the aqueous phase was lower in all the salting-out solutions. The use of NaCl and ammonium acetate led to phase separation with high MeCN residual contents. The sucrose solution and the mixed solution A also provided phase separation, even effective in higher MeCN concentration. The effect of the mixed solution B was superior to others as it led to effective phase separation, even in various volume ratios, as shown in Figure 9.

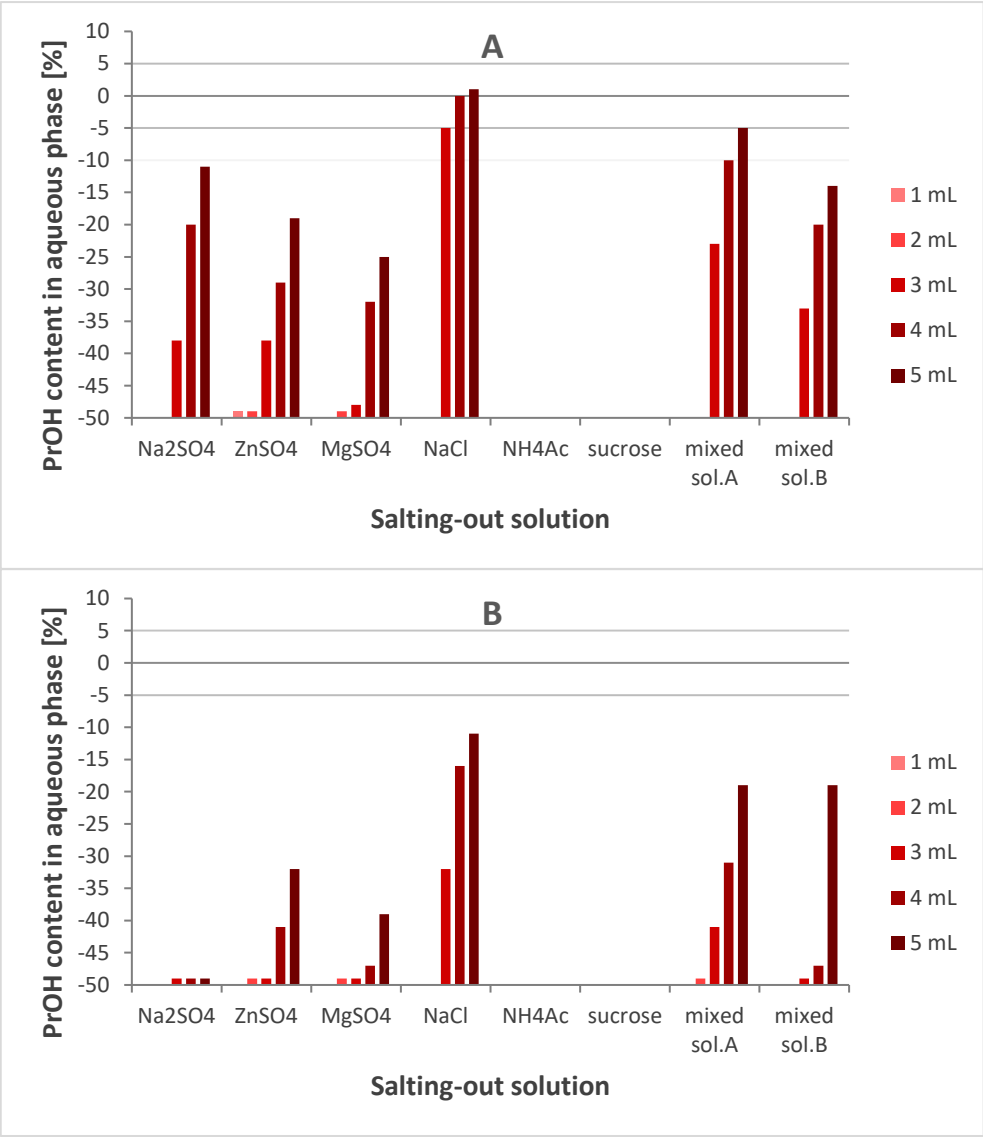

Figure 10. Effect of increasing volume of eight salting-out solutions on PrOH residual content in **A:** 50% PrOH solution, **B:** 67% PrOH solution. Small columns at -50% mark indicate phase separation with MeCN content of -50% or lower. All input values are present in Appx. 5 and 6.

When PrOH solutions were used, effective phase separation was achieved only exceptionally. Results comparable to the use of MeCN were achieved only with 50% PrOH initial solution and NaCl salting-out solution, see Figure 10. Except in this case, the phase separations resulted into PrOH content of negative percentages, which indicates presence of water in the organic phase. This was unsurprising because PrOH is a protic solvent and able to form hydrogen bonds, thus can dissolve a certain amount of salt and, consequently, even if the aqueous phase is close to being salt-saturated, some water [40]. The degree of this difference was, however, unknown to us. Nevertheless, PrOH was still studied for protein denaturation in the following experiments, alongside MeCN as well as acetone (ACE) since the present experiment was focused mainly on the effect of the salting-out solution.

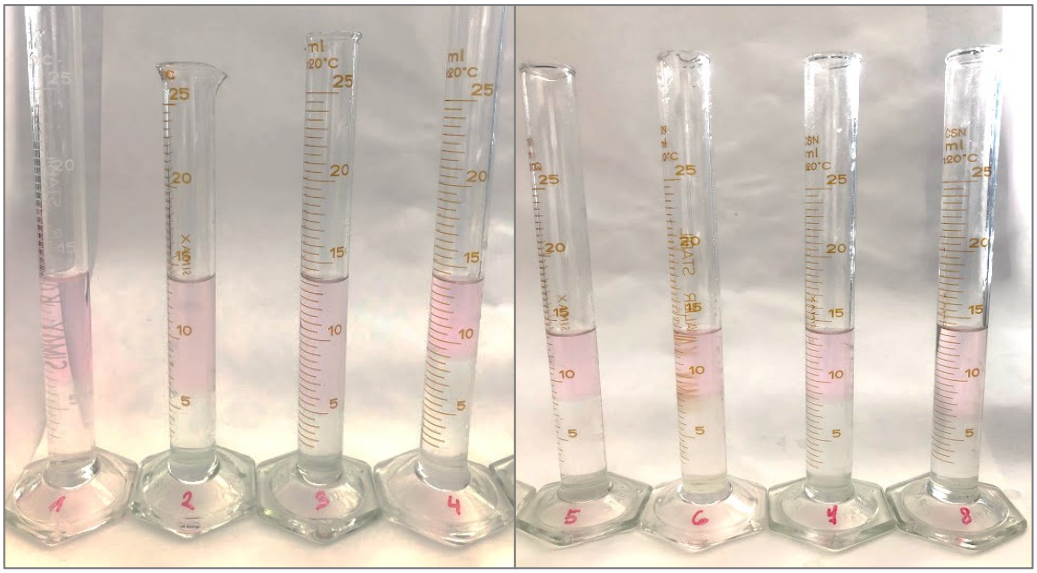

Figure 12. Photograph of salting-out induced phase separation of 67% MeCN solution coloured by astraphloxine. State after addition of 4 mL of salting-out solutions, numbered from 1 to 8. 1: Na_2SO_4, 2: $ZnSO_4$, 3: $MgSO_4$, 4: NaCl, 5: NH_4Ac, 6: sucrose, 7: mixed sol. A, 8: mixed sol. B.

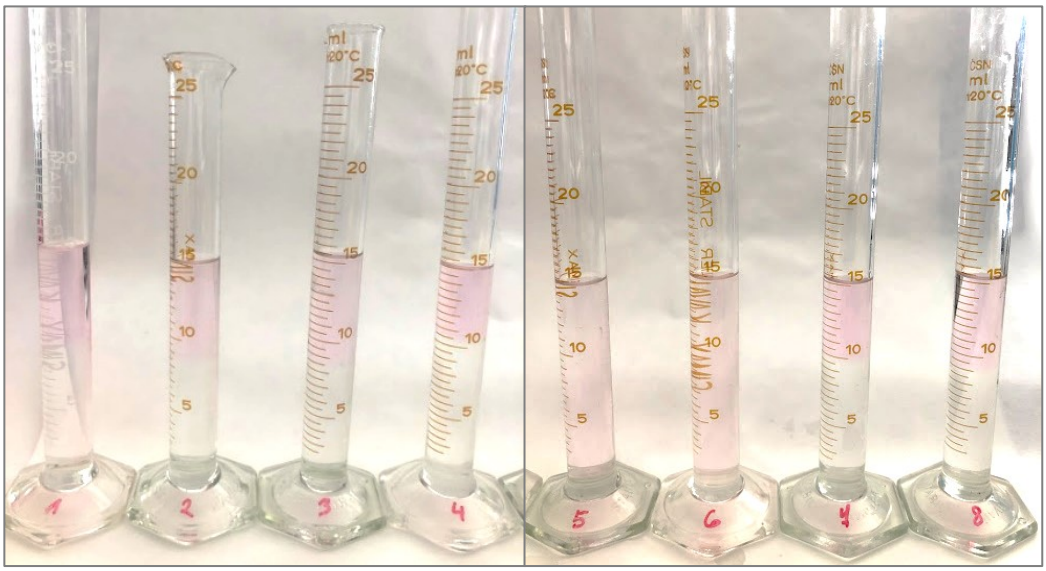

Figure 11. Photograph of salting-out induced phase separation of 50% PrOH solution coloured by astraphloxine. State after addition of 5 mL of salting-out solutions, numbered from 1 to 8. 1: Na_2SO_4, 2: $ZnSO_4$, 3: $MgSO_4$, 4: NaCl, 5: NH_4Ac, 6: sucrose, 7: mixed sol. A, 8: mixed sol. B.

As mentioned before, the mixed solution B induced effective phase separation in both MeCN to water ratios (2:1 and 1:1) of initial solutions. Both $MgSO_4$ and NaCl were proven effective in salting-out procedures. This can be explained by strong salting-out properties of the used salts. It was described that the contribution of ions to the salting-out effect follows the Hofmeister series [1,34]. As depicted in Figure 2, the used ions are categorized as the kosmotropes (order-making). These ions have positive effect on the arrangement of water molecules when solvation shells are re-formed. In addition, anions attribute more to the total salting-out effect than cations due to strong solvation properties and larger ionic radii [40].

Specifically, the used anions, SO_4^{2-} and Cl^-, have high negative charge density and the possibility to form hydrogen bonds with water molecules. On the other hand, Na^+ and Mg^{2+} tend to form stronger hydration bonds and can polarize anions, further stabilizing the solvation shells [40]. Therefore, Na_2SO_4 is often chosen for salting-out procedures [68,69], but by our experiments, it was proven ineffective. The mixed solution B has a higher concentration of SO_4^{2-} anions in comparison to the mixed solution A, thus providing sufficient power to form solvation shells with water molecules (Figure 1). Other ions help to further dry out the organic phase and stabilize the aqueous phase. The results of the separate use of $MgSO_4$ or NaCl further support this explanation, as they were ineffective.

The use of sucrose solution in 67% MeCN solution led to phase separation with residual content around 105%, as observable in Figure 9. Nevertheless, it was considered impractical due to the high viscosity of the solutions and quick recrystallization when exposed to the air.

In the continuation of this thesis, it was found that the use of $(NH_4)_2SO_4$ solution provided effective phase separation and might be of interest. The use of $(NH_4)_2SO_4$ was pointed out by many articles about salting-out assisted liquid-liquid extraction (SALLE) [35,68], unfortunately $(NH_4)_2SO_4$ was not purchased to be tested as the ammonium cation is relatively well soluble in solvents and shows a strong buffer capacity and was therefore not considered.

Based on the results of these experiments, the mixed solution B was selected for application in the following experiments.

5.1.2. Milk deproteinization

As the first step of the deproteinization, MeCN, PrOH, and ACE were tested for the ability to denature and precipitate the proteins in a 50% milk solution. The solvents were selected based on the literature research and HPLC compatibility [7,19,32,43]. For salting-out and phase separation in the second step, the mixed solution B was used. Considering that the ratio of the reagents is an essential factor for the deproteinization [32,43], four ratios of the solvent to the milk solution treated with 0.25 to 5.0 mL of the mixed solution B were tested.

The aim of this experiment was to achieve salting-out induced phase separation with a compact layer of precipitated proteins and calculated recovery of the organic solvent close to 100%. A recovery in the range from 90% to 110% was considered effective. The experiment was carried out according to the procedure described in section 4.3.2. Data in the form of a spreadsheet containing the volumes of separated phases and residual contents are shown in the appendix (Appx. 7).

When ACE was used for precipitation, protein agglomerates were observed that were dispersed throughout the entire solution volume, indicating imperfect denaturation. After the addition of the mixed solution B, phase separation was not achieved in any tested ratio, observable in Figure 13A. Therefore, ACE was considered unsuitable for our application and was not formed into the diagram.

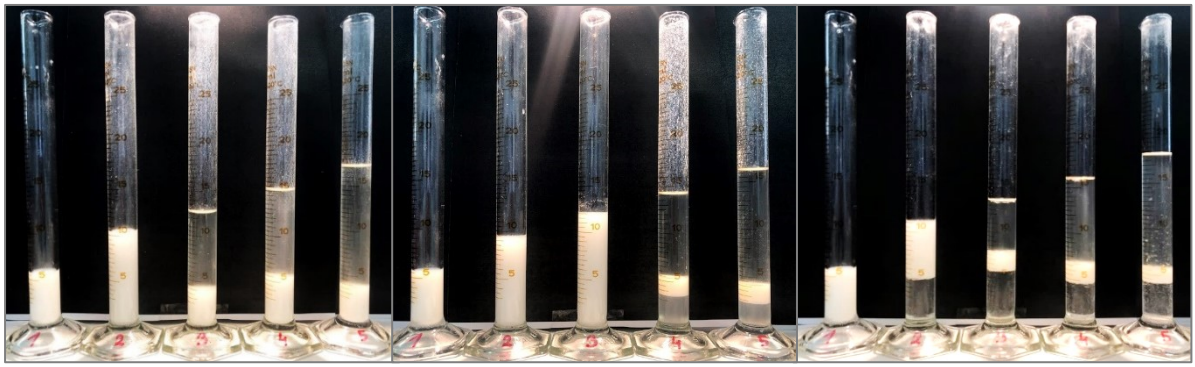

Figure 13. Photograph of salting-out induced phase separation and deproteinization of 50% milk solution. **A:** ACE used for deproteinization, 3 mL of mixed solution B added for salting-out. **B:** PrOH used for deproteinization, 4 mL of mixed solution B added for salting-out. **C:** MeCN used for deproteinization, 4 mL of mixed solution B added for salting-out. Cylinders numbered 1: control sample, 2-4: increasing solvent to milk solution volume ratio (2.5/5.0, 5.0/5.0, 7.5/5.0, 10.0/5.0 mL).

With PrOH, phase separation and formation of a compact layer of precipitated proteins were observed. The phase separation was achieved only with higher volumes of the mixed solution B added (3 and 4 mL) in three, PrOH to the milk solution volume, ratios as shown in Figure 14. The recovery value was increasing with the higher volume of the mixed solution B added with values higher 100 % throughout. In other words, the organic layer volume exceeded the initial volume of PrOH, indicating a high water content in the organic phase for reasons discussed in the previous section. Effective phase separation, i.e., yielding a recovery close to 100 %, was not achieved in any solvent to sample ratio.

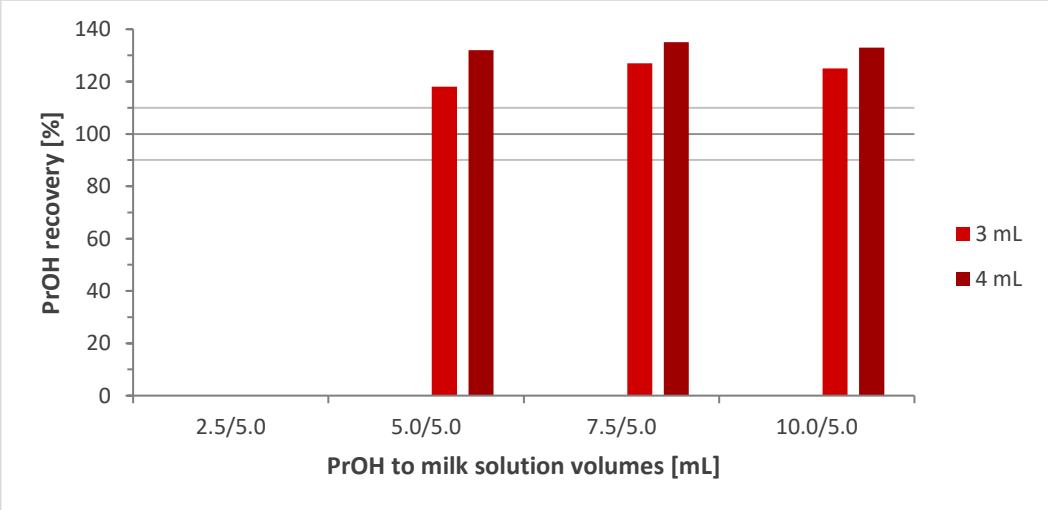

Figure 14. Effect of increasing volume of the mixed salting-out solution B on PrOH recovery in four different PrOH to milk solution ratios. All input values are present in Appx. 7.

Using MeCN, the proteins precipitated and formed a compact layer. Subsequential addition of mixed solution B led to phase separation in ratios of 3:2 (7.5/5.0 mL) and 2:1 (10.0/5.0 mL) of MeCN to milk solution, see Figure 15. Phase separation was achieved even with lower volumes of the mixed solution B (1 and 2 mL), effective only with the use of 3 or 4 mL. Unlike with PrOH, the recovery was decreasing with the gradual addition of the mixed solution B, approaching the desired 100% recovery.

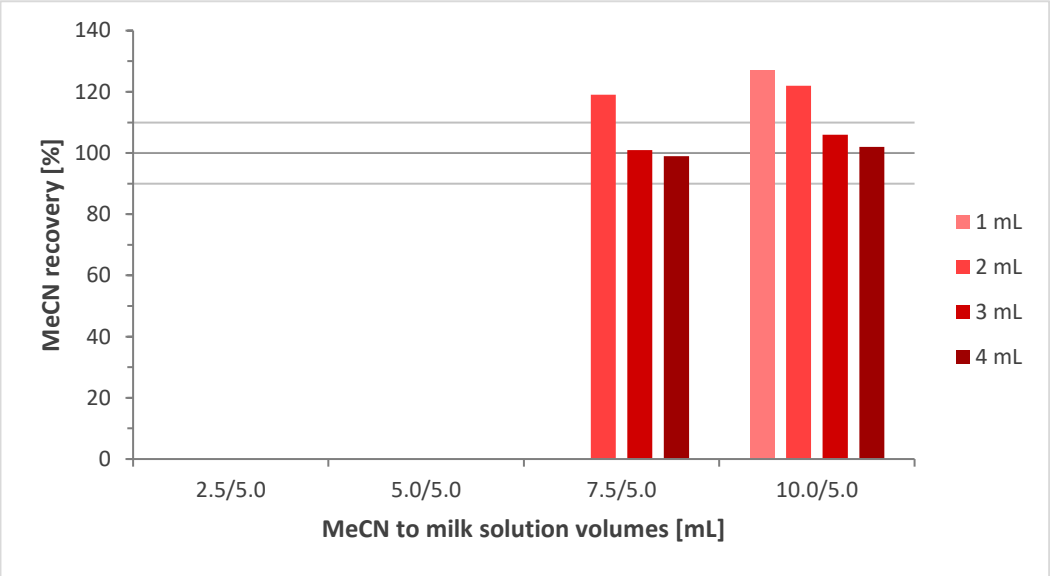

Figure 15. Effect of increasing volume of the mixed salting-out solution B on MeCN recovery in four different MeCN to milk solution ratios. All input values are present in Appx. 7.

By this experiment, MeCN was proven optimal not only for the salting-out induced phase separation but also for the denaturation and precipitation of the milk proteins. Overall, the number of solvents that are of interest for salting-out procedures is moreover limited because the solvent should also show compatibility with the intended instrumental technique for which the current work was developed, that is HPLC. Solvents of lower water solubility, e.g., ethyl acetate (EtAc) or butanol, are also less effective in the extraction of polar analytes, as are many analytes of today's interest, e.g., pharmaceuticals using in veterinary medicine, thus possible to find in milk [14,35]. They would also most likely lead to peak distortion when being injected into HPLC.

Effective phase separation was achieved only with higher MeCN to milk solution ratios (3:2 and 2:1). That is unsurprising since when lower solvent to sample ratios of 1:2 [36], 1:5 [35] and 1:6 [68], described in former articles on SALLE, are used, high amounts of salts in solid form are added. Leading to salt concentrations of approx. 20%, 28% and 30% (w/v), respectively. This is impossible to achieve in our procedure where an additional volume of concentrated salting-out solution further increases the proportion of the aqueous phase. Therefore, the use of higher ratios of solvent is necessary. This was also observed by former article [69] where low concentration of salt, approx. 8%, is used to induce phase separation of solvent and sample, in the ratio of 3:1.

However, higher ratios such as 3:1 would undesirably lower the preconcentration factor in our method. Therefore, the highest tested ratio was 2:1.

In conclusion, the minimal volume of MeCN needed to achieve phase separation was 7.5 mL to 5.0 mL of the sample volume. Thus, ideal ratios of MeCN to the milk solution observed in this experiment were 3:2 and 2:1 (v/v). For effective phase separation, 3 mL of the mixed solution B had to be added to either 12.5 mL or 15.0 mL, representing salt (NaCl and $MgSO_4$ combined) concentration of approx. 6% (w/v) and 5% (w/v), respectively.

5.2. Operational optimization of automated deproteination

To assure the optimal conditions for the automated method, basic operation protocols were prepared. Two different setups of the instrument, based on previous applications, were tested, leading eventually to the best-suited configuration for milk and possibly also biological sample treatment. Before the optimization experiments of the phase separation, the establishment of an effective cleaning protocol was needed. This way, the results of the optimizations could be compared without any bias.

5.2.1. Optimization of the instrument layout

The first protocol was programmed for an upside-down orientation of the syringe pump, the configuration depicted in Figure 5. Initial tests were performed to demonstrate the viability of an automated deproteinization. Down-scaled volumes of reagents were used based on the effective ratios from the offline experiments in section 5.1.2. Using a 5 mL syringe, the volume of milk was 250 µL, diluted with 250 µL of water and treated with 1000 µL of MeCN and 500 µL of the mixed solution B. The principle aim for testing an upside-down syringe orientation was to keep the stirring mechanism simple and to minimize the dead volume inside the syringe that is typical for upright orientation [52]. This is because the stirring bar remains at the bottom of the syringe, i.e., at the syringe inlet and air would form a cushion inside the syringe void above the liquid content that would allow dispensing the entire liquid content from the syringe void at emptying. Consequently, the configuration with the syringe pump in the upside-down orientation would allow more effective cleaning.

On the other hand, the aqueous phase would be the first one to be pushed out of the syringe followed by the organic phase. Thus, it could be possibly devalued by a contamination of the tubes by the aqueous phase and precipitated proteins through which the organic phase would have to be pushed out. Therefore, it was decided to restrict waste discharge to the lateral port 1 on the multiposition valve and use the head valve port IN for the collection of the organic phase. This allowed dispensing the organic phase directly from the syringe without transfer through a contaminated holding coil.

During discharge of the aqueous phase, it was observed that the proteins tend to agglomerate on top of the syringe outlet and the stir bar. As it was of the utmost importance to obtain a clear organic extract without protein particles as it would be the case in manual procedures after centrifugation, it was finally decided to turn the instrument, i.e., to use the syringe pump in the upright orientation, the configuration is depicted in Figure 6 and observable in Figure 16.

In later time, the extract was intended to go to an injection valve connecting the HPLC and the LIS system that would be connected to one of the lateral ports of the selection valve and the waste would be dispensed through the head-valve IN port.

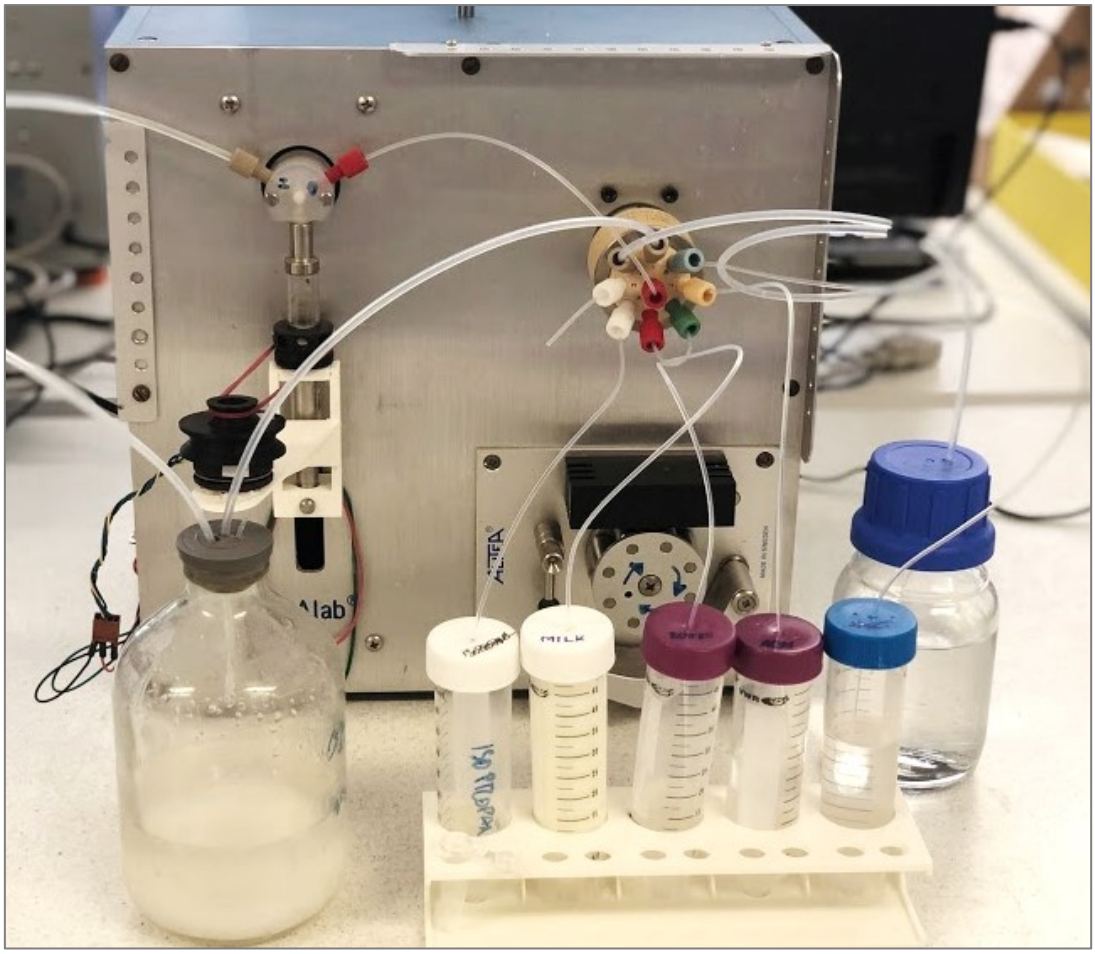

Figure 17. Photograph of final instrument layout for automated LIS salting-out induced phase separation and deproteinization of milk. Same setup is depicted by Figure 6.

Consequently, the organic phase was dispensed first, improving the purity. On the other hand, a larger dead volume was implied as liquid remained in this configuration inside the syringe after emptying – approx. 300 µL - due to the gap needed for the stirring bar, as shown in Figure 17. This required a longer cleaning protocol and an adaptation of the stirring system, as explained in 5.2.2 and shown in Figure 6, respectively. The tube from the head valve port IN was shortened to lower the volume lost during organic phase collection.

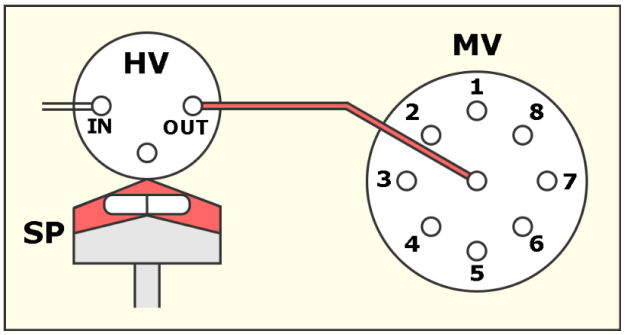

Figure 16. Diagram depicting dead volume (red filling) of used LIS system setup. HV: syringe head valve with depicted IN and OUT ports, MV: multiposition valve with depicted eight lateral ports, SP: syringe pump.

5.2.2. Optimization of the cleaning

The nature of the milk sample matrix posed a challenge in the cleaning of the system. It is a water-based emulsion of organic and inorganic molecules, including fat and protein. For possible further application of our method, the collected organic phase had to be without any protein contamination, similar to centrifuge-based manual protocols [35,36,68]. Also, thorough cleaning is essential considering the quantification of an analyte present in the sample to avoid cross-over contamination between samples.

During the first test runs, the cleaning was done according to the procedure described in 4.4.1, with 500 µL of PrOH as the cleaning agent and 750 µL of water. Each cleaning step was repeated twice. Using this initial protocol, it was observed that sometimes proteins agglomerated on the syringe piston and the stir bar, which was observable after the cleaning. Perceptible progress was achieved by the addition of an extra 5 seconds with activated stirring before discharge, forcing the protein flakes to be carried by the vortex into the waste. Nevertheless, a minimal residue of unknown origin was left behind. We considered the residue could be more protein flakes or even milk-fat droplets.

With that in mind, six cleaning agents were tested in total. To keep the method as environmentally friendly as possible, only water-miscible organic solvents were used for cleaning, namely, EtAc, PrOH, and iPrOH. On the other hand, defatting of milk samples is often done with highly hydrophobic solvents such as hexane [71]. The cleaning agents were chosen based on the literature research [72-75] and availability. In addition, a detergent was also used in the cleaning protocols in previous works [76], two 50 mmol/L solutions of SDS were tested alongside with 1 mol/L $NaHCO_3$ solution, as a basic solution with pH of approx. 10.2.

For the testing, the cleaning protocol consisted of three rinses with 1000 µL of water, followed by three applications of 750 µL of the cleaning agent. Each cleaning agent was tested right after the SALLE procedure to set up identical conditions for comparison. A subsequent washing step with water was done after the evaluation, so only the cleaning properties of the individual solutions were compared. Verbally described results were summarized in Table 2.

Due to the complexity of the milk matrix, the use of an organic solvent for cleaning was of our interest as the tested solvents can dissolve both organic and inorganic compounds. The log P values of the solvents were in the range from 0.71 for EtAc, representing the most hydrophobic solvent, to 0.16 for iPrOH, representing the most hydrophilic solvent. In between was PrOH with a log P value of 0.33 [77]. The cleaning properties of iPrOH showed to be superior, and no residues were observed (Figure 18) as oppose to EtAc and PrOH, as described in Table 2.

These results correlate with the possibility that more hydrophilic contaminants were eliminated with initial water rinses and visible debris is rather hydrophobic.

Table 2. Testing of cleaning agents for Lab-In-Syringe system.

	Observed status of the syringe void before water rinse
	Shapeless agglomerates were covering the entire stir bar. Some particles of approx. 2 mm in diameter were stuck to the glass syringe barrel and the piston.
Cleaning agent	**Observed status of the syringe void after cleaning**
EtAc, pure	Flat agglomerates > 1 mm were stuck on the stir bar. Even smaller bead-like particles were stuck to the barrel.
PrOH, pure	Two large agglomerates of approx. 3 mm in diameter were stuck to the stir bar and some smaller particles were also observed all around.
iPrOH, pure	No observable debris.
1 mol/L $NaHCO_3$	Shapeless agglomerates of sizes in the range of 1 to 4 mm were present on the stir bar as well as the piston.
50 mmol/L SDS in 50% iPrOH	Flat agglomerates > 1 mm were stuck on the stir bar.
50 mmol/L SDS in water	No observable debris.

Most of the milk protein is casein, which forms soluble salts in pH > 4.6, its isoelectric point [78]. However, this applies to casein micelles in untreated milk and might not have any effect on milk proteins denatured by a combination of organic solvent and salt. Nevertheless, to increase pH during the cleaning, which should increase the protein solubility, 1 mol/L $NaHCO_3$ solution was tested. However, cleaning was not improved as oppose to the solvents since various debris was left behind, as described in Table 2.

As mentioned above, detergents are often used for their amphiphilic properties. Chosen SDS is an anionic surfactant with the 12-carbon hydrophobic tail and highly hydrophilic negatively charged sulphate group used throughout many sections of chemistry. These chemical and physical properties make SDS very effective even in low concentrations, we decided to use 50 mmol/L concentration, which exceeds the critical micelle concentration of 8.2 mmol/L (at 25 °C) [77]. The water solution was superior to the 50% iPrOH solution and no debris was left behind as with iPrOH, as commented in Table 2.

In summary, iPrOH and SDS water solution removed all the protein particles. However, we were worried that the SDS might contaminate the organic phase and eventually be injected into HPLC once the methodology would be applied to this end. Contamination with SDS would be a severe problem due to a dynamic coating effect on RP-HPLC columns that would intermediately show a negative charge and change the interaction of analyte and stationary phase [79]. More washing steps with water would have to be added to lower this probability, but it would make the cleaning procedure much more time-consuming. In contrast, the use of iPrOH would not pose this thread.

Even if some iPrOH would remain in the syringe after cleaning, it would not disturb the method of deproteination, phase separation, or HPLC separations. Therefore, the iPrOH was chosen as the cleaning agent for our method.

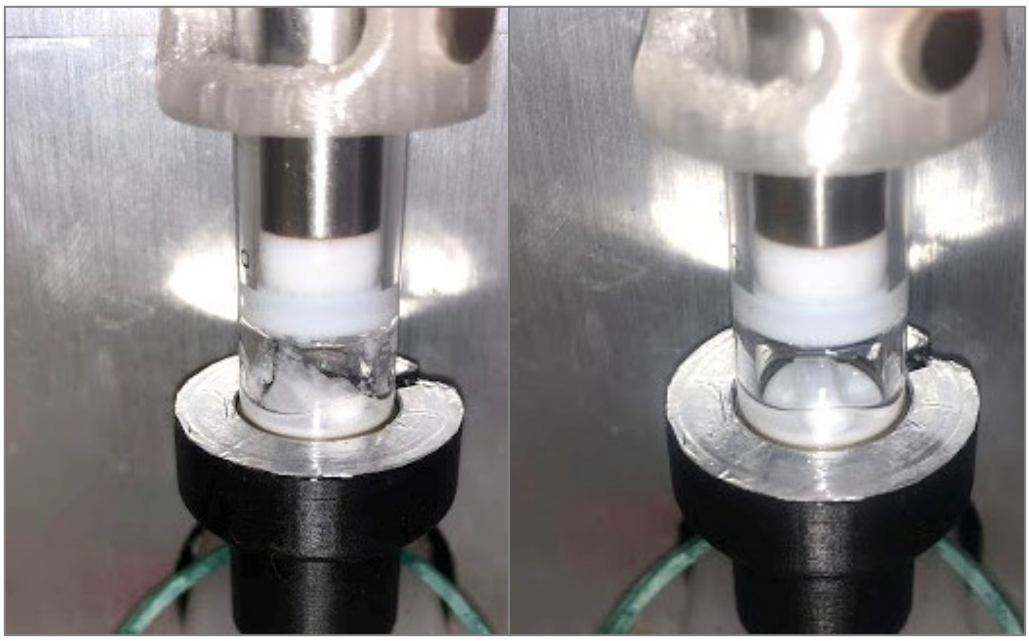

Figure 18. Photograph of cleaning optimization **A:** before use of iPrOH as cleaning agent, **B:** after use of iPrOH as cleaning agent.

From the observation, a highly effective way to get rid of the protein agglomerates was to use a higher volume of water, so the proteins were carried out by a stronger vortex. So, it was decided to increase the volume of water to 1500 µL for both initial and terminal rinses. All cleaning steps were repeated three times.

Due to the holding coil, the upright syringe setup, and the stir bar inside the syringe, a significant dead volume had to be considered. While the holding coil was emptied entirely, i.e., filled with air, the dead volume of the syringe had to be filled with water as an inert solvent at the end of the cleaning protocol. The final cleaning protocol written for FIALab software is shown in the appendix as part of the sample preparation operational protocol (Appx. 1) and as separate operational protocol for the syringe cleaning (Appx. 2).

5.3. Optimization of salting-out induced phase separation

With optimized instrument setup and effective cleaning protocol, the salting-out induced deproteinization was studied. Based on the results of the offline experiments, the volumetric ratios were studied further to find the most effective combination of salt solution, sample, and solvent volumes. Other variables, such as the stirring rate and the separation time were studied, too.

It ought to be mentioned that the recoveries were calculated only from the volumes read out by the naked eye from the graduation marks on the syringe body, with a precision of approx. 50 µL. The purity of the organic phase was not analytically tested.

5.3.1. Optimization of the volume ratios

All previous test runs using the LIS system were carried out with 250 µL of milk, 250 µL of water, 1000 µL of MeCN, and 500 µL of the mixed solution B. Even though the protein precipitation and the phase separation occurred, the recovery of the solvent after separation was not evaluated. Similar to the offline experiments, various ratios of the reagents were tested directly inside the LIS system. The aim was to achieve the protein precipitation and the phase separation with a volume of the organic layer as close as possible to the solvent input volume, i.e., solvent recovery of 100%.

Based on the offline milk deproteinization results (Figure 15), two volume ratios, 1000/500 µL and 750/500 µL of MeCN to the diluted milk, 2:1 and 3:2 (v/v), respectively, were tested. In 1:2 and 1:1 (v/v) ratios, the phase separation was not achieved in the offline deproteinization experiments. The phase separation was induced by the mixed solution B ranging from 200 µL to 600 µL. The experiment was carried out according to the procedure described in section 4.4.2.

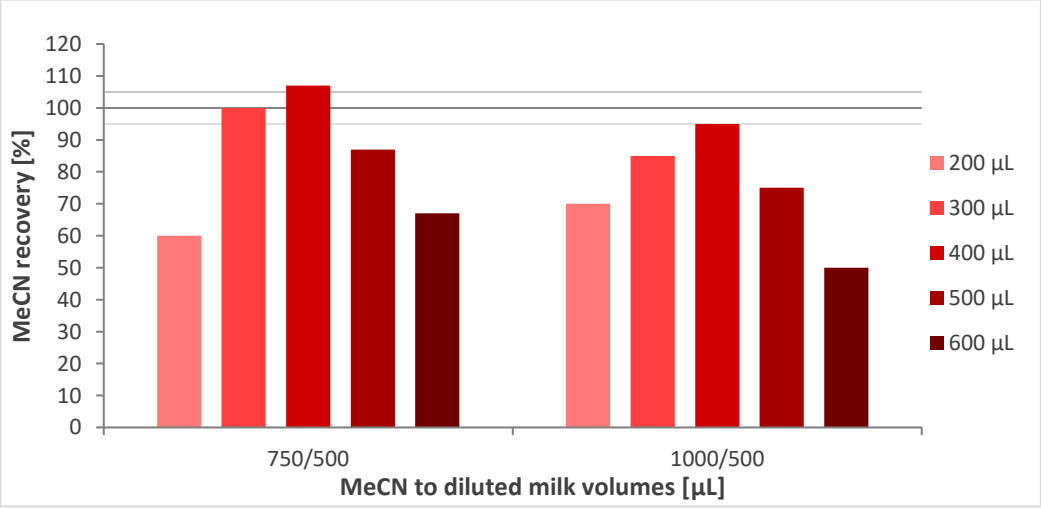

Figure 19. Effect of increasing volume of the mixed salting-out solution B on MeCN recovery in two different MeCN to diluted milk ratios.

When 1000 µL of MeCN was used, 95% recovery was achieved with 400 µL of the mixed solution B (Figure 19 and 20A). For the second ratio, 750/500 µL, even better phase separation, with 100% recovery, was achieved with 300 µL of the mixed solution B (Figure 19 and 20B).

The tests showed that the recovery of solvent was not improved by using larger volumes of the mixed solution B. That was expected since the same results were observed in the offline deproteinization experiments and in researched literature [35,36,68]. This is because a small part of the solvent dissolves in the aqueous phase that cannot be fully saturated or oversaturated with salt in a flow system (no solid salt can be used but only salt solution). Nevertheless, a certain concentration of the mixed solution B had to be achieved for the phases to separate.

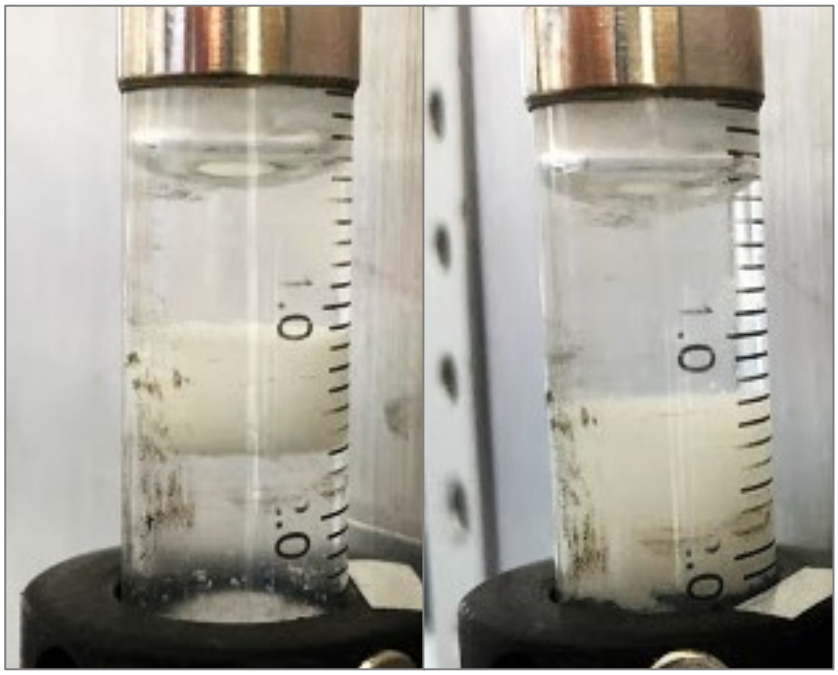

Figure 20. Photograph of salting-out induced phase separation and deproteinization of milk. MeCN to diluted milk to mixed solution B volume ratios of **A:** 750:500:300 µL and **B:** 1000:500:400 µL.

By these results, the optimal ratio of the reagents for the milk deproteinization was 500 µL of diluted milk, followed by 750 µL of MeCN and 300 µL of the mixed solution B, see Figure 20B. Note that the results should serve as a practical guide for the future progression of this work using the developed methodology for the extraction of analytes of environmental interest or pharmaceutical origin from milk samples. In case of an application of this method, further optimization will be based on achievable recovery values and preconcentration factors of the analytes of interest evaluated by online-coupled liquid chromatography. This, however, would have exceeded the aims and extends of this thesis.

5.3.2. Optimization of mixing and phase separation time

When the mixed solution B was aspirated into a mixture of the milk and MeCN after the protein denaturation, the stirring was applied to facilitate homogenous mixing. Initially, the content of the syringe was stirred only during the aspiration of the salting-out solution. Then, three quick stirs were done to facilitate layer formation, which was achieved by an on/off switching for 2 seconds. It was observed that these quick stirs were not affecting the mixing nor the layer formation. Any application of the stirring was ineffective because vortex creation was blocked due to the dense protein layer that formed immediately on the boundary between organic and aqueous phases.

Therefore, it was decided to prolong the initial stirring. It was left on for additional 5 seconds after the aspiration of the salting-out solution was completed, providing thorough mixing of the reagents. As before, the stirring was then deactivated so the phase separation could succeed.

The aim of the time optimization was to find an optimal balance between adequate phase separation and the time efficiency of the method. The separation time in the range from 60 seconds to 180 seconds was tested. Every 30 seconds, the volume of the organic phase was read from the syringe graduation and noted down, then the recovery of the solvent was calculated. The experiment was carried out according to the procedure described in section 4.4.2. The volumes were 250 µL of milk, 250 µL of water, 750 µL of MeCN, and 300 µL of the mixed solution B.

It was observed that a 100% recovery of MeCN was achieved after 150 seconds (Figure 21). It must be noted that the volumes were read by the naked eye with a precision of approx. 50 µL. Considering the aim of this method, it was decided that 150 seconds was sufficient.

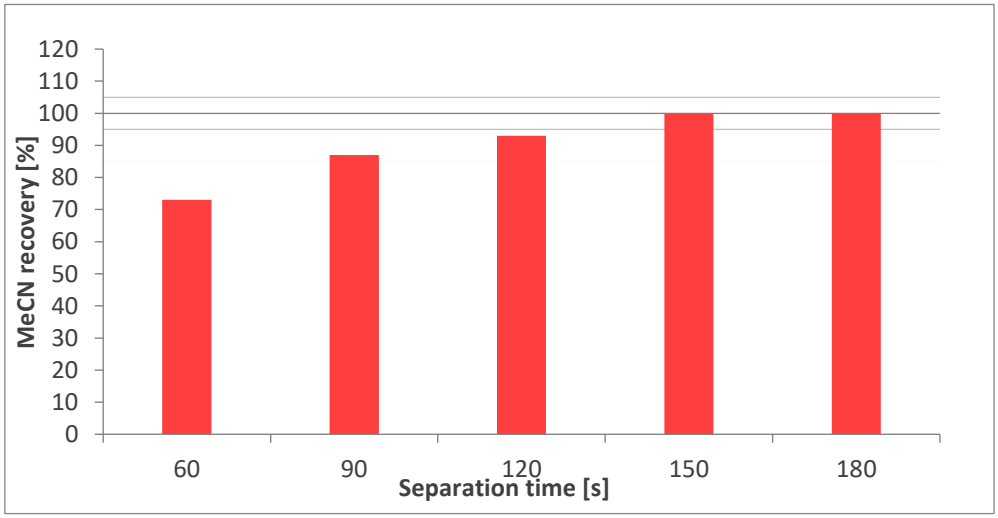

Figure 21. Effect of increasing separation time on MeCN recovery.

Besides the phase separation time, the stirring rate was also a relevant factor for the formation of a dense protein layer as desired. A sufficiently high stirring rate was essential for homogeneous mixing of the reagents and thorough cleaning. It should be pointed out that the rate was controlled by an analogue circuit and that is was possible to use only one stirring rate throughout the entire procedure.

In a range from 800 rpm to 1200 rpm, it was observed that that the separation time became longer with increasing stirring rate. With lower stirring rates, either the mixing was not homogeneous or the cleaning was not sufficient. Contrariwise, the rates above 1400 rpm caused a formation of dense dispersion, and the phase separation was not achieved in an acceptable time. In SALLE technique [35,36,68,69] , thorough mixing is provided by vigorous shaking of the vial or vortexing it (2000-4000 rpm). Although the principle of the mixing by a vortex and our in-syringe stirring is similar, the velocity had to be significantly lower in our method. Cited methods depend on the subsequential use of a centrifuge to form separate layers of separated phases by centrifugal force. In contrast, layer formation in our method depended only on the separation time and did not require centrifugation but to the cost of using a salting-out solution. On the other hand, that also allowed to reduce the extractant. That is a great advantage of our method, but the efficiency of the mixing, evaluated by analyte recovery, would be necessary if our method is applied. At this point, a stirring rate of 1000±200 rpm was considered optimal. The range of 400 rpm is mentioned because the stirring rate can be slightly affected by the tension and the state of the used rubber band.

6. Conclusions

A method for automated deproteinization and salting-out induced phase separation of milk samples using the LIS technique has been designed, tested, studied, and optimized. It presents a novel automated approach of using a highly concentrated salt solution to induce phase separation of a homogenous mixture of milk sample and water-miscible solvent. Furthermore, mixing was done by in-syringe stirring technique and no centrifugation for separation of precipitated protein from the extract was needed.

In the process of development, offline experiments were performed, from which subsequent results were gathered. From eight tested salting-out solution, the mixed solution of 2 mol/L MgSO4 with 1 mol/L NaCl was proven superior in terms of inducting phase separation and drying out the organic phase, which corresponds to the use of similar combinations of these salts in QuEChERS protocols. It was found that an exact volume of the salting-out solution must be added to the sample-solvent mixture as the efficiency of phase separation decreases with excessive volumes. Under optimized conditions, recoveries of MeCN of 100±5% were achieved during offline deproteination experiments. This result was one of several important findings that were taken into account in posterior automated sample treatment.

For Lab-In-Syringe, two instrument setups, for syringe orientation upright and upside-down, were compared. It was concluded that the upright orientation is better suited for our aims as it allowed discharge of the organic phase after the phase separation first avoiding secondary extract contamination. The syringe was cleaned effectively using the order of cleaning steps with water, iPrOH, and again water. From six tested agents it was found out that iPrOH is the best-suited one. In addition, cleaning with higher volume of water, 1500 µL, presents an important step as a stronger vortex is created by in-syringe stirring, overall improving the cleaning.

Offline obtained experimental results allowed straightforward in-syringe automation and optimization of the procedure yielding a viable automated method. Milk samples of 500 µL could be treated with 750 µL of MeCN, which resulted in efficient proteins precipitation. By addition of 300 µL mixed salting-out solution, phase separation and formation of a compact protein layer were achieved. Recovery of the organic phase was close to 100 %.

These results prove that a fully-automated, miniaturized, economic, environmentally friendly, and quick method for the preparation of milk samples can be performed with the use of LIS technique. Furthermore, an online coupling to HPLC is currently proven as a continuation of this work that will lead to a fully-automated method for the analysis of sulfonamide antibiotics in milk samples.

7. Shrnutí

Hlavním cílem této práce bylo vyvinout metodu pro automatizovanou deproteinizaci vzorků mléka pomocí homogenní kapalinové extrakce (HLLE) se separací fází navozenou vysolováním. K provedení této metody bez užití centrifugy a k její automatizaci byla využita technika Lab-In-Syringe (LIS). Cílem bylo také, aby vyvinutá metoda mohla být použita pro přípravu vzorků, extrakci farmaceutických analytů a k přímému spojení HPLC.

Automatizace představuje možnost, jak zrychlit, zefektivnit nebo miniaturizovat analytické metody [61,62]. Toto vede k levnějším a k životnímu prostředí šetrnějším metodám [5], jelikož jsou výrazně sníženy nároky na lidskou práci a použité materiály. Jedním z přístupů k automatizaci v analýze je užití průtokových technik. Ty se skládají z pumpy a systému tenkých hadiček, ve kterých dochází k mísení, chemickým reakcím i detekci v jednom uzavřeném systému. Příkladem těchto technik jsou dobře známé průtoková injekční analýza (FIA) [45] nebo sekvenční injekční analýza (SIA) [46], které spočívají v užití nosného proudu, jehož průtok je řízen peristaltickou nebo dvousměrným pístovým čerpadlem. Do nosného roztoku je pomocí selekčních ventilů vstřikován vzorek a další reagencia. Skrze disperzi na rozmezí jednotlivých roztoků, která je typická pro laminární proudění kapalin v tenkých trubicích jako jsou tyto, dochází k mísení a případným reakcím. Následná detekce, založená většinou na spektrometrii, je možná přímo v hadičce, která vede skrze detekční celu.

Technika nazvaná Lab-In-Syringe, která byla odvozena od SIA, prezentuje využití dvousměrného pístového čerpadla, ve kterém dochází k mísení vzorku a reagencií a případné detekci [51,52]. Jednotlivé roztoky jsou nasávány pomocí vícecestného selekčního ventilu. Mísení uvnitř prostoru čerpadla je zajištěno vloženým magnetickým míchadélkem, které je ovládáno pomocí vnějšího magnetického pole dvou neodymových magnetů [53]. Ty jsou vloženy do kroužku, který je nasazen na pumpu a otáčen skrze řemenem propojený rotor. Celý proces je řízen počítačovým softwarem, který zajišťuje přesné provedení jednotlivých úkonů. Tato technika umožňuje automatizaci a miniaturizaci složitějších metod ku příkladu disperzivní kapalinové mikroextrakce [55,56], dvoufázové kapalinové mikroextrakce [51] nebo, jako i v této práci, homogenní kapalinové extrakci [54].

Technika HLLE byla vybrána na základě dlouholetého použití v analýze pro přípravu biologických a potravinových vzorků [29,30]. Umožňuje odstranění matrice vzorku a extrakci analytů o různé polaritě na základě rozdělovacího koeficientu, logP, mezi vodnou fází a organickým rozpouštědlem. Jedná se především o s vodou mísitelná rozpouštědla, díky čemuž se snižuje negativní dopad na životní prostředí, oproti jiným, také používaným, organickým rozpouštědlům jako je ku příkladu chloroform. Tato jednofázová soustava je následně narušena změnou chemických nebo fyzikálních vlastností, které ovlivňují hydrataci rozpouštědla a analytu, což vyústí v separací fází. Mezi často využívané přístupy patří změna pH [48] nebo změna iontové síly roztoku, tj. vysolování [32]. Právě metoda vysolování je bodem zájmu pro tuto práci.

V běžných postupech pro vysolováním asistovanou kapalinovou extrakci [35-37] nebo techniku QuEChERS (z angličtiny: Rychlá, Jednoduchá, Levná, Efektivní, Robustní a Bezpečná) [7] je fázová separace indukována přidáním většího množství soli, což vede k vytvoření solí přesycené vodné fáze [1]. Tento postup ovšem není proveditelný v instrumentaci pro LIS, jelikož sůl v pevné formě nemůže být přidána. Proto jsme přišli s řešením v podobě vysoce koncentrovaného vysolovacího roztoku, který slouží k indukci fázové separace.

V procesu vývoje metody byly prováděny offline experimenty, ze kterých byly shromážděny následné výsledky. Z osmi testovaných vysolovacích roztoků se ukázalo, že směsný roztok o koncentraci 2 mol/l $MgSO_4$ s 1 mol/l NaCl je lepší z hlediska indukce fázové separace a vysušení organické fáze, což odpovídá použití podobných kombinací těchto solí v protokolech pro QuEChERS. Bylo zjištěno, že do směsi vzorku a rozpouštědla musí být přidán přesný objem vysolovacího roztoku, protože účinnost fázové separace klesá s nadměrnými objemy. Za optimalizovaných podmínek bylo během offline deproteinizačních experimentů dosaženo obnovení 100±5 % MeCN po fázové separaci. Tento výsledek byl jedním z několika důležitých poznatků, které byly vzaty v úvahu při následném automatizovaném zpracování vzorku.

Pro LIS systém byly porovnány dvě konfigurace, pro orientaci čerpadla v poloze svislé s pístem dole a obrácené o 180°. Byl vyvozen závěr, že prvně zmíněná orientace je pro naše cíle vhodnější, protože umožňovala vypouštění organické fáze jako první v pořadí po oddělení fází, přičemž se zabránilo sekundární kontaminaci extraktu. Vnitřní prostory pístového čerpadla byly účinně vyčištěny pomocí postupného čištění vodou, iPrOH a znovu vodou. Ze šesti testovaných činidel, bylo zjištěno, že iPrOH je nejvhodnější, protože bylo dosaženo kompletního vyčištění a případná rezidua iPrOH by negativně neovlivnila tuto metodu ani HPLC. Kromě toho představuje čištění s větším objemem vody, 1500 µL, důležitý krok, protože mícháním vzniká silnější víření, což zlepšuje čištění čerpadla.

Získané experimentální výsledky umožnily přímou automatizaci v LIS systému a optimalizaci postupu, čímž vznikla proveditelná automatizovaná metoda. Ke vzorku mléka o objemu 500 µl bylo přidáno 750 µl MeCN, což vedlo k účinnému vysrážení proteinů. Přidáním 300 µL směsného vysolovacího roztoku bylo dosaženo fázové separace a vytvoření kompaktní proteinové vrstvy. Dosažená efektivita fázové separace byla téměř 100 %.

Tyto výsledky dokazují, že plně automatizovanou, miniaturizovanou, ekonomickou, ekologickou a rychlou metodu přípravy vzorků mléka lze provést pomocí techniky LIS. Kromě toho se v současné době pracuje také na on-line spojení s HPLC jako pokračování této práce, což povede k plně automatizované metodě pro analýzu sulfonamidových antibiotik ve vzorcích mléka.

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9. Appendix

Appx. 1. Operational protocol for LIS automated deproteinization and salting-out induced phase separation of milk samples, written for FIALab software. It is described verbally in section 4.4.2.

```
'Deproteinization and salting-out induced phase separation

'Multiposition valve ports
'1 Waste//2 Water//3 Air//4 iPrOH//5 Milk//6 MeCN
'7 Salting-out mixed solution B//8 Buffer

'Variables definition
Variable Define New VolClean
Variable Define New VolSample
Variable Define New VolBuffer
Variable Define New VolWater
Variable Define New VolSolvent
Variable Define New VolSaltSol
Variable Define New VolHC
Variable Define New VolOrgPhase
Variable Define New TimeMixing
Variable Define New TimeExtraction
Variable Define New TimeSeparation
Variable Define New PosClean
Variable Define New RepClean

'Variables settings
VolSample = 250
VolBuffer = 0
VolWater = 250
VolSolvent = 750
VolSaltSol = 300
VolHC = 100
VolOrgPhase = 500
TimeMixing = 5
TimeExtraction = 25
TimeSeparation = 150

Contact Closure Off

'CLEANING
'Water Rinse
SyringePump Flowrate (microliter/sec) 300
PosClean = 2
RepClean = 3
VolClean = 1500
Insert File C:\Users\Obsluha\Desktop\Machian\SyringeCleaning.fia
'Cleaning
SyringePump Flowrate (microliter/sec) 150
PosClean = 4
RepClean = 3
VolClean = 750
Insert File C:\Users\Obsluha\Desktop\Machian\SyringeCleaning.fia
'Water Rinse
SyringePump Flowrate (microliter/sec) 500
PosClean = 2
RepClean = 3
VolClean = 1500
Insert File C:\Users\Obsluha\Desktop\Machian\SyringeCleaning.fia

'SAMPLE PREPARATION
'Aspirate Sample
SyringePump Valve Out
SyringePump Flowrate (microliter/sec) 100
Multiposition Valve port 5
SyringePump Aspirate (microliter) VolSample
SyringePump Delay Until Done
Delay (sec) 1

'Aspirate Water
SyringePump Flowrate (microliter/sec) 150
SyringePump Valve Out
Multiposition Valve port 2
Contact Closure On
SyringePump Aspirate (microliter) VolWater
SyringePump Delay Until Done
```

```
'Aspirate Segmentation bubble
Multiposition Valve port 3
SyringePump Aspirate (microliter) 30
SyringePump Delay Until Done
Contact Closure Off

'PROTEIN PRECIPITATION
'Aspirate Solvent
Multiposition Valve port 6
Contact Closure On
SyringePump Aspirate (microliter) VolSolvent
SyringePump Delay Until Done

'Aspirate Segmentation Bubble
Multiposition Valve port 3
SyringePump Aspirate (microliter) VolHC
SyringePump Delay Until Done
Delay (sec) 3
Contact Closure Off

'SALTING-OUT INDUCED PHASE SEPARATION
'Aspirate Salting-out Solution
SyringePump Flowrate (microliter/sec) 75
Multiposition Valve port 7
Contact Closure On
SyringePump Aspirate (microliter) VolSaltSol
SyringePump Delay Until Done

'Aspirate Segmentation bubble
SyringePump Flowrate (microliter/sec) 150
Multiposition Valve port 3
SyringePump Aspirate (microliter) VolHC
SyringePump Delay Until Done

Delay (sec) TimeMixing
Contact Closure Off

'Phase separation
Delay (sec) TimeSeparation

'DISCHARGE OF PHASES
'Discharge Air
SyringePump Valve Out
SyringePump Dispense (microliter) 250

'Discharge organic phase
SyringePump Valve In
SyringePump Flowrate (microliter/sec) 100
SyringePump Dispense (microliter) VolOrgPhase

'Empty collecting tube
SyringePump Valve In
SyringePump Flowrate (microliter/sec) 150
SyringePump Aspirate (microliter) 100
SyringePump Delay Until Done

'Discharge waste
SyringePump Valve Out
Multiposition Valve port 1
Contact Closure On
SyringePump Empty
SyringePump Delay Until Done
Contact Closure Off

'Empty HC
SyringePump Valve In
SyringePump Aspirate (microliter) 200
SyringePump Delay Until Done
SyringePump ValveOut
Multiposition Valve port 1
SyringePump Empty
SyringePump Delay Until Done
```

Appx. 2. Operational protocol for cleaning of the syringe, written for FIALab software. It is used by the main operational protocol (Appx. 1) in form of file link, marked in red colour.

```
'SYRINGE CLEANING

Loop Start (#) RepClean

'Aspiration
SyringePump Valve Out
Multiposition Valve port PosClean
SyringePump Aspirate (microliter) VolClean
Contact Closure On
SyringePump Delay Until Done
Delay (sec) 5

'Empty HC
Multiposition Valve port 3
SyringePump Aspirate (microliter) VolHC
SyringePump Delay Until Done

'Discharge
Multiposition Valve port 1
SyringePump Empty
SyringePump Delay Until Done
Contact Closure Off

'Empty HC
SyringePump Valve In
SyringePump Aspirate (microliter) 150
SyringePump Delay Until Done
SyringePump ValveOut
Multiposition Valve port 1
SyringePump Empty
SyringePump Delay Until Done

Loop End
```

Appx. 3. Data from the offline selection of salting-out agent experiment. The initial solution for phase separation was 50% MeCN.

1.1 mol/L Na_2SO_4									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.2	10.5	10.7	10.9	11.8	12.7	13.6	14.5
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.2	10.5	10.7	10.9	11.8	12.0	12.5	13.5
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	0.7	1.1	1.0
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	-614%	-355%	-400%
Solvent content in aqueous phase	-	-	-	-	-	-	33%	28%	26%
1.7 mol/L $ZnSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.7	10.9	11.9	12.7	13.6	14.6
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.7	10.9	11.9	5.5	7.0	7.8
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	7.2	6.6	6.8
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	31%	24%	26%
Solvent content in aqueous phase	-	-	-	-	-	-	-45%	-29%	-28%
2.3 mol/L $MgSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.8	10.0	10.2	10.5	10.7	11.6	12.6	13.6	14.6
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.8	10.0	10.2	10.5	10.7	11.6	4.5	6.0	7.5
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	8.1	7.6	7.1
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	38%	34%	30%
Solvent content in aqueous phase	-	-	-	-	-	-	-78%	-50%	-33%
4.9 mol/L NaCl									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.9	10.1	10.4	10.6	10.8	11.7	12.6	13.5	14.5
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.9	10.1	10.4	10.6	10.8	10.0	10.8	11.8	13.0
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	1.7	1.8	1.7	1.5
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-194%	-178%	-194%	-233%
Solvent content in aqueous phase	-	-	-	-	-	30%	26%	24%	23%

6.7 mol/L ammonium acetate									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.2	10.5	10.6	10.9	11.9	12.8	13.6	14.5
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.2	10.5	10.6	8.8	9.0	10.0	11.0	12.0
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	2.1	2.9	2.8	2.6	2.5
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-138%	-72%	-79%	-92%	-100%
Solvent content in aqueous phase	-	-	-	-	32%	22%	20%	18%	17%
2.0 mol/L sucrose									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.6	9.9	10.2	10.5	10.8	11.7	12.5	13.0	14.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.6	9.9	10.2	10.5	10.8	11.7	11.0	11.5	12.0
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.5	2.0
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	-233%	-233%	-150%
Solvent content in aqueous phase	-	-	-	-	-	-	27%	22%	17%
1.2 mol/L $MgSO_4$ with 2.4 mol/L NaCl, mixed sol.A									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.1	10.4	10.6	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.1	10.4	10.6	10.8	11.0	9.5	10.3	11.3	12.4
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	2.5	2.7	2.7	2.6
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-100%	-85%	-85%	-92%
Solvent content in aqueous phase	-	-	-	-	-	26%	22%	20%	19%
1.9 mol/L $MgSO_4$ with 1.0 mol/L NaCl, mixed sol.B									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.2	10.5	10.7	10.9	11.9	12.9	13.9	14.9
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.2	10.5	10.7	10.9	6.3	8.0	9.1	10.3
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	5.6	4.9	4.8	4.6
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	11%	-2%	-4%	-9%
Solvent content in aqueous phase	-	-	-	-	-	-11%	0%	1%	3%

Appx. 4. Data from the offline selection of salting-out agent experiment. The initial solution for phase separation was 67% MeCN.

1.1 mol/L Na_2SO_4									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.2	10.5	10.7	11.0	11.9	12.6	13.6	14.6
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.2	10.5	10.7	11.0	3.7	5.3	6.5	7.7
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	8.2	7.3	7.1	6.9
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	19%	9%	6%	3%
Solvent content in aqueous phase	-	-	-	-	-	-44%	-19%	-13%	-8%
1.7 mol/L $ZnSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.1	10.3	10.6	10.8	11.0	11.9	12.8	13.8	14.7
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.6	10.8	11.0	3.0	4.4	5.5	6.6
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.1	0.0	0.0	0.0	0.0	8.9	8.4	8.3	8.1
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	25%	21%	20%	18%
Solvent content in aqueous phase	-	-	-	-	-	-78%	-44%	-33%	-26%
2.3 mol/L $MgSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	11.9	12.5	13.5	14.5
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	11.9	3.5	4.7	5.8
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	9.0	8.8	8.7
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	-	26%	24%	23%
Solvent content in aqueous phase	-	-	-	-	-	-	-81%	-56%	-44%
4.9 mol/L NaCl									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.7	11.0	11.9	12.7	13.7	14.7
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	5.0	6.5	7.5	8.6	10.0
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	6.0	5.4	5.2	5.1	4.7
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-11%	-23%	-28%	-31%	-42%
Solvent content in aqueous phase	-	-	-	-	13%	18%	16%	15%	17%

6.7 mol/L ammonium acetate									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.9	10.1	10.4	10.6	10.8	11.7	12.6	13.5	14.5
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.9	10.1	10.3	10.6	4.9	6.0	7.0	8.1	8.5
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	5.9	5.7	5.6	5.4	6.0
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-13%	-17%	-19%	-23%	-11%
Solvent content in aqueous phase	-	-	-	-	12%	11%	10%	9%	2%
2.0 mol/L sucrose									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.4	9.6	9.9	10.1	10.4	11.3	12.2	13.2	14.2
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.4	9.6	9.9	10.2	4.5	5.5	7.0	8.0	9.0
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	5.9	5.8	5.2	5.2	5.2
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-13%	-15%	-28%	-28%	-28%
Solvent content in aqueous phase	-	-	-	-	4%	3%	10%	8%	7%
1.2 mol/L $MgSO_4$ with 2.4 mol/L NaCl, mixed sol.A									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	3.8	5.3	6.4	7.7	9.1
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	7.2	6.7	6.6	6.3	5.9
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	7%	0%	-1%	-6%	-13%
Solvent content in aqueous phase	-	-	-	-	-14%	-1%	1%	5%	8%
1.9 mol/L $MgSO_4$ with 1.0 mol/L NaCl, mixed sol.B									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	11.9	12.8	13.8	14.8
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	5.2	6.3	7.3	8.5
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	6.7	6.5	6.5	6.3
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	0%	-3%	-3%	-6%
Solvent content in aqueous phase	-	-	-	-	-	-3%	-1%	0%	2%

Appx. 5. Data from the offline selection of salting-out agent experiment. The initial solution for phase separation was 50% PrOH.

1.1 mol/L Na_2SO_4									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	5.8	7.5	9.0
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	7.2	6.5	6.0
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	**31%**	**23%**	**17%**
Solvent content in aqueous phase	-	-	-	-	-	-	**-38%**	**-20%**	**-11%**
1.7 mol/L $ZnSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	2.5	4.5	5.8	7.0	8.4
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	8.5	7.5	7.2	7.0	6.6
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	**41%**	**33%**	**31%**	**29%**	**24%**
Solvent content in aqueous phase	-	-	-	-	**-140%**	**-56%**	**-38%**	**-29%**	**-19%**
2.3 mol/L $MgSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	4.0	5.4	6.8	8.0
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	8.0	7.6	7.2	7.0
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	**38%**	**34%**	**31%**	**29%**
Solvent content in aqueous phase	-	-	-	-	-	**-75%**	**-48%**	**-32%**	**-25%**
4.9 mol/L NaCl									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.2	10.5	10.7	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.2	10.5	10.7	11.0	12.0	7.6	9.0	10.1
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	5.4	5.0	4.9
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	**7%**	**0%**	**-2%**
Solvent content in aqueous phase	-	-	-	-	-	-	**-5%**	**0%**	**1%**

6.7 mol/L ammonium acetate									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.8	10.0	10.3	10.5	10.8	11.7	12.6	13.6	14.6
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.8	10.0	10.3	10.5	10.8	11.7	12.6	13.6	14.6
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	-	-	-
Solvent content in aqueous phase	-	-	-	-	-	-	-	-	-
2 mol/L sucrose									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.4	9.6	9.9	10.1	10.4	11.5	12.5	13.5	14.5
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.4	9.6	9.9	10.1	10.4	11.5	12.5	13.5	14.5
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	-	-	-
Solvent content in aqueous phase	-	-	-	-	-	-	-	-	-
1.2 mol/L $MgSO_4$ with 2.4 mol/L NaCl, mixed sol.A									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	6.5	8.2	9.5
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	6.5	5.8	5.5
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	**23%**	**14%**	**9%**
Solvent content in aqueous phase	-	-	-	-	-	-	**-23%**	**-10%**	**-5%**
1.9 mol/L $MgSO_4$ with 1.0 mol/L NaCl, mixed sol.B									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.1	10.4	10.6	10.9	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.1	10.4	10.6	10.9	11.0	12.0	6.0	7.5	8.8
Aqueous phase theoretical vol. [mL]	5.0	5.3	5.5	5.8	6.0	7.0	8.0	9.0	10.0
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	7.0	6.5	6.2
Organic phase theoretical vol. [mL]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Theoretical organic/aqueous phase ratio	1.0	1.0	0.9	0.9	0.8	0.7	0.6	0.6	0.5
Water content in organic phase	-	-	-	-	-	-	**29%**	**23%**	**19%**
Solvent content in aqueous phase	-	-	-	-	-	-	**-33%**	**-20%**	**-14%**

Appx. 6. Data from the offline selection of salting-out agent experiment. The initial solution for phase separation was 67% PrOH.

1.1 mol/L Na_2SO_4									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	9.8	10.0	10.3	10.5	10.8	11.8	12.8	13.7	14.7
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	9.8	10.0	10.3	10.5	10.8	11.8	3.2	4.5	5.5
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	9.6	9.2	9.2
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	-	31%	28%	28%
Solvent content in aqueous phase	-	-	-	-	-	-	-98%	-63%	-52%
1.7 mol/L $ZnSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	14.9
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	3.0	4.0	5.2	6.3
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	9.0	9.0	8.8	8.6
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	26%	26%	24%	22%
Solvent content in aqueous phase	-	-	-	-	-	-78%	-58%	-41%	-32%
2.3 mol/L $MgSO_4$									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	2.5	3.9	5.0	6.0
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	9.5	9.1	9.0	9.0
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	30%	27%	26%	26%
Solvent content in aqueous phase	-	-	-	-	-	-113%	-62%	-47%	-39%
4.9 mol/L NaCl									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	4.8	6.3	7.5
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	8.2	7.7	7.5
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	-	19%	13%	11%
Solvent content in aqueous phase	-	-	-	-	-	-	-32%	-16%	-11%

6.7 mol/L ammonium acetate									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.7	11.0	12.0	13.0	14.0	14.9
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.7	11.0	12.0	13.0	14.0	14.9
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	-	-	-	-
Solvent content in aqueous phase	-	-	-	-	-	-	-	-	-
2 mol/L sucrose									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.2	10.4	10.7	10.9	11.1	12.0	13.0	14.0	14.9
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.2	10.4	10.7	10.9	11.1	12.0	13.0	14.0	14.9
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	-	-	-	-
Solvent content in aqueous phase	-	-	-	-	-	-	-	-	-
1.2 mol/L $MgSO_4$ with 2.4 mol/L NaCl, mixed sol.A									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.3	10.5	10.8	11.0	11.2	12.2	13.2	14.2	15.2
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.3	10.5	10.8	11.0	11.2	2.8	4.5	5.6	7.0
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	9.4	8.7	8.6	8.2
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	29%	23%	22%	19%
Solvent content in aqueous phase	-	-	-	-	-	-90%	-41%	-31%	-19%
1.9 mol/L $MgSO_4$ with 1.0 mol/L NaCl, mixed sol.B									
Salting-out agent sol. vol. [mL]	0.00	0.25	0.50	0.75	1.00	2.00	3.00	4.00	5.00
Total vol. [mL]	10.0	10.3	10.5	10.7	10.9	12.0	13.0	14.0	15.0
Total theoretical vol. [mL]	10.0	10.3	10.5	10.8	11.0	12.0	13.0	14.0	15.0
Aqueous phase vol. [mL]	10.0	10.3	10.5	10.7	10.9	12.0	3.6	5.0	6.2
Aqueous phase theoretical vol. [mL]	3.3	3.6	3.8	4.1	4.3	5.3	6.3	7.3	8.3
Organic phase vol. [mL]	0.0	0.0	0.0	0.0	0.0	0.0	9.4	9.0	8.8
Organic phase theoretical vol. [mL]	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
Theoretical organic/aqueous phase ratio	2.0	1.9	1.7	1.6	1.5	1.3	1.1	0.9	0.8
Water content in organic phase	-	-	-	-	-	-	29%	26%	24%
Solvent content in aqueous phase	-	-	-	-	-	-	-76%	-47%	-34%

Appx. 7. Data from the offline milk deproteinization experiment.

Initial conditions												
Solvent to milk sol. ratio	1:2			1:1			3:2			2:1		
Solvent vol. [mL]	2.5			5.0			7.5			10.0		
Milk solution vol. [mL]	5.0			5.0			5.0			5.0		
Solvent addition												
	2.5/5.0 mL solvent to milk solution			5.0/5.0 mL solvent to milk solution			7.5/5.0 mL solvent to milk solution			10.0/5.0 mL solvent to milk solution		
	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE
Aqueous layer vol [mL]	7.5	7.6	7.5	10.3	10.4	10.2	4.0	12.9	12.5	11.9	0.2	0.1
Protein layer vol. [mL]							8.5			2.7	14.5	14.5
Total vol. [mL]	7.5	7.6	7.5	10.3	10.4	10.2	12.5	12.9	12.5	14.6	14.7	14.6
1 mL salting-out solution addition												
	2.5/5.0 mL solvent to milk solution			5.0/5.0 mL solvent to milk solution			7.5/5.0 mL solvent to milk solution			10.0/5.0 mL solvent to milk solution		
	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE
Organic layer vol [mL]										12.7		
Protein layer vol. [mL]						3.0	3.0	7.1	3.3	2.3	9.3	3.2
Aqueous layer vol [mL]	8.5	8.6	8.4	11.1	11.2	8.2	10.5	6.5	10.2	1.0	6.8	12.4
Total vol. [mL]	8.5	8.6	8.4	11.1	11.2	11.2	13.5	13.6	13.5	16.0	16.1	15.6
Recovery	-	-	-	-	-	-	-	-	-	**127%**	-	-
2 mL salting-out solution addition												
	2.5/5.0 mL solvent to milk solution			5.0/5.0 mL solvent to milk solution			7.5/5.0 mL solvent to milk solution			10.0/5.0 mL solvent to milk solution		
	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE
Organic layer vol [mL]							8.9			12.2		
Protein layer vol. [mL]						3.0	1.9	5.2	3.6	1.8	4.5	3.5
Aqueous layer vol [mL]	9.4	9.6	9.1	12.0	12.0	8.7	3.5	9.8	10.6	2.3	12.5	12.7
Total vol. [mL]	9.4	9.6	9.1	12.0	12.0	11.7	14.3	15.0	14.2	16.3	17.0	16.2
Recovery	-	-	-	-	-	-	**119%**	-	-	**122%**	-	-
3 mL salting-out solution addition												
	2.5/5.0 mL solvent to milk solution			5.0/5.0 mL solvent to milk solution			7.5/5.0 mL solvent to milk solution			10.0/5.0 mL solvent to milk solution		
	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE
Organic layer vol [mL]					5.9		7.6	9.5		10.6	12.5	
Protein layer vol. [mL]		5.0			2.9	4.0	2.1	1.9	5.5	2.0	1.5	3.8
Aqueous layer vol [mL]	10.4	5.5	10.0	12.9	4.7	8.5	5.3	4.6	9.5	4.4	4.0	13.3
Total vol. [mL]	10.4	10.5	10.0	12.9	13.5	12.5	15.0	16.0	15.0	17.0	18.0	17.1
Recovery	-	-	-	-	**118%**	-	**101%**	**127%**	-	**106%**	**125%**	-
4 mL salting-out solution addition												
	2.5/5.0 mL solvent to milk sol. ratio			5.0/5.0 mL solvent to milk solution			7.5/5.0 mL solvent to milk solution			10.0/5.0 mL solvent to milk solution		
	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE	MeCN	PrOH	ACE
Organic layer vol [mL]					6.6		7.4	10.1		10.2	13.3	
Protein layer vol. [mL]		6.1			2.9	4.2	1.7	2.1	5.9	2.4	1.8	4.0
Aqueous layer vol [mL]	11.0	5.1	10.7	13.6	4.6	8.9	6.6	4.4	9.7	5.1	3.9	13.8
Total vol. [mL]	11.0	11.2	10.7	13.6	14.1	13.1	15.7	16.6	15.6	17.7	19.0	17.8
Recovery	-	-	-	-	**132%**	-	**99%**	**135%**	-	**102%**	**133%**	-