Charles University
Faculty of Pharmacy in Hradec Králové

DIPLOMA THESIS

Spectrophotometric determination of chlorhexidine in mouthwash employing Lab-In-Syringe automated ion-pair extraction and back-extraction

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Hradec Králové 2018

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Acknowledgements

I would like to express my deepest gratitude to my supervisor Dr. Burkhard Horstkotte, Ph.D., M.Sc. for his patient guidance, advice and assistance during my research and writing. I would also like to thank doc. PharmDr. Hana Sklenářová, Ph.D. for her useful corrections of this thesis.

Finally, a special thanks belong to my family and friends for their great support throughout my studies.

Abstract

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Title of the diploma thesis: Spectrophotometric determination of chlorhexidine in mouthwash employing Lab-In-Syringe automated ion-pair extraction and back-extraction

In this work, a magnetic stirring-assisted dispersive liquid-liquid micro-extraction (MSA-DLLME) automated by the Lab-In-Syringe technique is presented. MSA-DLLME is based on mixing the sample and an immiscible solvent by the action of a magnetic stirrer for the dispersion of the solvent into fine droplet to enhance the extraction process. In Lab-In-Syringe, the magnetic stirrer is placed inside a syringe void, which is used as extraction chamber. To allow the use of an extraction solvent lighter than water, the syringe was turned upside down in this work. This method was designed for the determination of chlorhexidine in commercial mouthwash samples.

To allow the extraction of chlorhexidine, an ion-pair complex with the reagent methyl orange needed to be formed. After the extraction into an organic solvent, the aqueous solution was exchanged and the analyte was back-extracted into an acidic aqueous acceptor to yield higher selectivity. Spectrophotometric detection was used throughout.

Experimental parameters including type of extraction solvent, extraction times, volumes and, stirring rate were optimised. As extraction solvent, 1-octanol was chosen due to its highest extraction capacity of all tested solvents. Based on the experiments made, the extraction and back-extraction times were set to 30 s at a stirring rate of 1470 rpm.

Volumes of 1-octanol and methyl orange reagent were established at 250 µL and 50 µL, respectively. As back-extractant 500 µL of 0.125 mol/L hydrochloric acid was chosen.

The method performance was evaluated by the analysis of commercial mouthwash. Recovery values were below 100 %, requiring further studies on potential interferences.

Abstrakt

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Název diplomové práce: Spektrofotometrické stanovení chlorhexidinu v ústní vodě s využitím extrakce iontového asociátu a zpětné extrakce automatizované v systému Lab-In-Syringe

Práce popisuje disperzní kapalinovou mikroextrakci pomocí magnetického míchadla (MSA-DLLME), která je automatizována technikou Lab-In-Syringe. MSA-DLLME je založena na míchání vzorku s nemísitelným rozpouštědlem pomocí magnetického míchadla za účelem rozptýlení rozpouštědla na jemné kapky, což vede k podpoře extrakčního procesu. Magnetické míchadlo je umístěno uvnitř rezervoáru pístového čerpadla, který je použit jako extrakční komora. Pro umožnění použití extrakčního rozpouštědla, které je lehčí než voda, bylo v této práci pístové čerpadlo otočeno ústím dolů. Tato metoda byla navržena a optimalizována pro stanovení chlorhexidinu v komerčních vzorcích ústní vody.

Aby byla extrakce umožněna, bylo potřeba vytvořit iontový pár chlorhexidinu s činidlem methyloranž. Po extrakci iontového páru do organického rozpouštědla byla k roztoku přidána kyselina chlorovodíková, do které byl analyt zpětně extrahován za účelem dosažení vyšší selektivity. V celé práci byl jako detekční systém použit spektrofotometr. Experimentální parametry, které byly v průběhu práce optimalizovány, zahrnovaly typ extrakčního rozpouštědla, extrakční čas, použité objemy i rychlost míchání. Jako extrakční rozpouštědlo byl vybrán 1-oktanol díky své extrakční kapacitě, která byla nejvyšší ze všech testovaných rozpouštědel. Na základě provedených pokusů byl čas pro extrakci a zpětnou extrakci nastaven na 30 sekund a rychlost míchání na 1470 ot/min. Objem rozpouštědla 1-oktanolu a činidla methyloranž byly stanoveny na 250 µL a 50 µL. Jako rozpouštědlo pro zpětnou extrakci bylo zvoleno 500 µL kyseliny chlorovodíkové o koncentraci 0,125 mol/L.

Metoda byla hodnocena analýzou komerčních ústních vod. Výtěžnost nebyla stoprocentní, což vyžaduje další studie potenciálních interferencí.

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List of abbreviations

DLLME	dispersive liquid-liquid micro-extraction
FIA	flow injection analysis
HF-LPME	hollow-fibre liquid-phase micro-extraction
LIS	Lab-In-Syringe
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MO	methyl orange
MSA–DLLME	magnetic stirring-assisted dispersive liquid-liquid micro-extraction
PTFE	polytetrafluoroethylene
SDME	single-drop micro-extraction
SIA	sequential injection analysis

1. Introduction

For the pharmaceutical industry, the analytical methods are very important to secure quality and safety of products. Chlorhexidine is an antiseptic drug widely used in mouthwash. Although it has a relatively low toxicity, its pharmaceutical effect and side effects depend on the administered dose. Therefore, it is essential to find reliable analytical methods for its quantification and control in commercial products. Several techniques have been developed for chlorhexidine determination of which high-performance liquid chromatography (HPLC) is the most commonly used. However, HPLC can be costly in purchase and operation [1].

Liquid-liquid extraction (LLE) is a separation method based on a different solubility of an analyte in two different phases. This technique had been known for many years and it is still widely used for sample pre-treatment [2]. Throughout the years, the aim was to miniaturise LLE mainly to reduce waste generation. Several approaches have been developed not only leading to micro-extraction techniques but also approving higher extraction efficiency and selectivity. These techniques include single-drop micro-extraction and dispersive liquid-liquid micro-extraction (DLLME).

In this thesis, determination of chlorhexidine in mouthwash by DLLME was studied. For automation of this method, the flow technique Lab-In-Syringe (LIS) was used. LIS is a simple technique, which uses the void of an automated syringe pump as an extraction chamber. In this work, the syringe was turned upside-down to allow the use of an extraction solvent lighter than water. The method was finally applied to real mouthwash samples to study its applicability.

2. Objectives

1. Set-up of a Lab-In-Syringe system to perform dispersive liquid-liquid micro-extraction of chlorhexidine
2. Choice of a suitable organic extraction solvent to use for extraction of the analyte and study of the effect of sample buffering
3. Optimization of the experimental conditions including the volume of extraction solvent, the concentration of reagent, and the extraction and separation times
4. Rearrangement of the system for DLLME and dispersive back-extraction using an acidic back-extractant
5. Optimization of the corresponding experimental conditions including the acidity and volume of the acceptor and the back-extraction time
6. Study of the methods performance by calibrations and repetitions
7. Testing the analysis of mouthwash samples including spiked samples
8. Discussion of the results

3. Theory

3.1 Model analyte - chlorhexidine

For this study, chlorhexidine was used as a model analyte. Chlorhexidine is a cationic chemical compound which belongs to the biguanidic family [1]. Its chemical name is 1, 1-hexamethylenebis[5-(p-chlorophenyl) biguanide]. The structure of chlorhexidine is given in **Figure 1** [3]. Chlorhexidine is the most stable in the form of salts such as acetate, digluconate or hydrochloride. Chlorhexidine digluconate is the most frequently used form for having the highest solubility in water from the given compounds [4].

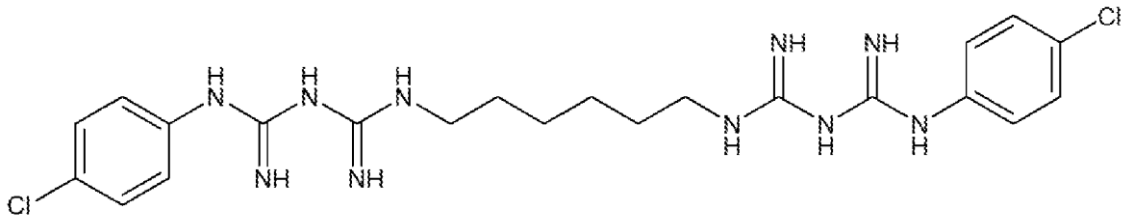

Figure 1 *Chlorhexidine CAS 55-56-1* [3]

Chlorhexidine is a disinfectant and an antiseptic. It kills microorganisms or inhibits their reproduction or metabolism. Its spectrum of activity is broad. It is active against Gram-positive and Gram-negative bacteria, fungi, and yeasts as well as against some kind of viruses [1]. The mechanism of action is associated with its positive charge where the cationic component attaches to negatively charged carboxyl groups of proteoglycans in the cell membrane and causes cell lysis [1,2]. For these properties, it is used in dentistry to eliminate orals pathogens and in veterinary and human medicine for antisepsis of wounds, skin, and cleansing equipment [1].

In dental care, chlorhexidine and its salts are widely used in mouthwash because they are most effective for inhibition of dental plaque and prevention and treatment of gingivitis. It is used in various concentrations ranging from 0.02 to 0.3 % w/V

in the final product. The mechanism of action and also the side effects seem to be dose-dependent. At very low concentrations (0.02 - 0.06 %), chlorhexidine shows bacteriostatic behaviour, while at higher concentrations (0.12 – 0.2 %), it is bactericidal [5].

In general, chlorhexidine has a low toxicity for humans but as every pharmaceutical drug, it has several side effects. The most common side effects are discoloration of teeth and other areas of the oral cavity, increased calculus formation, bitter taste, and interference with taste after using the mouthwash. When ingested, it can cause gastrointestinal irritation, vomiting, and dizziness [1,4]. All taken into consideration, the Food and Drug Administration suggested a use of chlorhexidine at concentrations from 0.12 to 0.2 % for oral mouthwash applications of 10 to 15 mL for only a limited time [6].

For the pharmaceutical industry, well-developed analytical methods are very important for the determination of chemical compounds in pharmaceutical products to guarantee its quality. The official determination of chlorhexidine salts described in the European Pharmacopoeia [7] is a titration with 0.1 mol/L perchloric acid with the potentiometric end-point determination. However, the most used method is a high-performance liquid chromatography which is also described in the United States Pharmacopoeia [8].

To simplify the process several other methods were developed. Calatayud and co-workers did a study on a simplified determination of chlorhexidine, based on the formation of an ion pair between chlorhexidine and an anionic dye using flow injection analysis with turbidimetric detection. Several dyes were tested for this purpose: thymol blue, bromocresol green, bromocresol purple, and methyl orange [9]. Another study describes ion-pair formation for the extraction of chlorhexidine into an organic solvent. As detection system, spectrophotometry was used [10].

In this work, the possibility to use automated liquid-liquid micro-extraction of chlorhexidine as an ion pair with methyl orange into an organic solvent and back-extraction of a dye into an aqueous acceptor phase for spectrophotometric determination was tested.

3.2 Liquid-liquid extraction techniques

3.2.1 Traditional liquid-liquid extraction

Liquid-liquid extraction (LLE) is a simple analytical technique used for the separation of an analyte (solute) from a liquid mixture using an extraction solvent. The extraction process is based on a distribution of the solute between two immiscible liquids – an aqueous and an organic phase. The aqueous phase (polar) is mostly represented by the initial mixture (i.e. the sample) while the extraction solvent is usually a hydrophobic organic compound (non-polar). If the sample is a hydrophobic phase and the acceptor phase is aqueous, the term "reverse extraction" is generally used.

When mixed, the analyte partially transfers from one phase to another [11]. The extent of the transfer depends on a solubility of the analyte in both phases which is described by a distribution coefficient. The distribution coefficient K_d is an equilibrium constant and it is defined by the analyte concentration in the organic phase $[X]_{org}$ divided by the analyte concentration in the aqueous phase $[X]_{aq}$ after the equilibrium is attained, as shown in the following equation:

$$\boldsymbol{K_d = [X]_{org}/[X]_{aq}}$$

The passage of the analyte from one phase to another appears only on the surface area between the aqueous and organic phase. To enlarge this area of contact, the whole system is traditionally shaken in a separating funnel, which promotes the dispersion of both phases. This significantly increases the efficiency of the extraction process.

The choice of the extraction solvent is also crucial for the efficiency of the technique. The solubility of the solute in the acceptor solvent must be much higher than in the donor phase and moreover, the donor itself and other components of the mixture should be nearly insoluble in the solvent to achieve a high clean-up [2].

LLE is one of the oldest techniques still widely used in sample pre-treatment to concentrate the analyte as well as to remove unwanted contaminants, which could interfere with further measurements (sample matrix removal). Its advantages include simplicity, wide applicability, commercial availability of the required high purity organic solvents, and use of low-cost apparatus such as separating funnels [11].

However, extraction procedures use large volumes of organic solvents, which is not only costly but also dangerous when working with carcinogenic or highly flammable chemicals [12]. Another problem, which can occur especially in samples that contain surfactants or fatty materials, is the formation of an emulsion observed by white colouration of the solution within the separating funnel and lack of a distinct boundary between the aqueous and organic phase e.g. due to the presence of surfactants [11]. In addition, LLE is time and labour-consuming and difficult to automate.

3.2.2 Miniaturisation of liquid-liquid extraction

The use of large amounts of sample and solvents, their cost, toxicity, and waste disposal difficulties led to the development of various miniaturised variations of LLE – summarized by the term liquid-phase micro-extraction. In recent years, several approaches were described and divided into three main categories: single-drop micro-extraction (SDME), dispersive liquid-liquid micro-extraction (DLLME) and hollow-fibre liquid-phase micro-extraction (HF-LPME) [13].

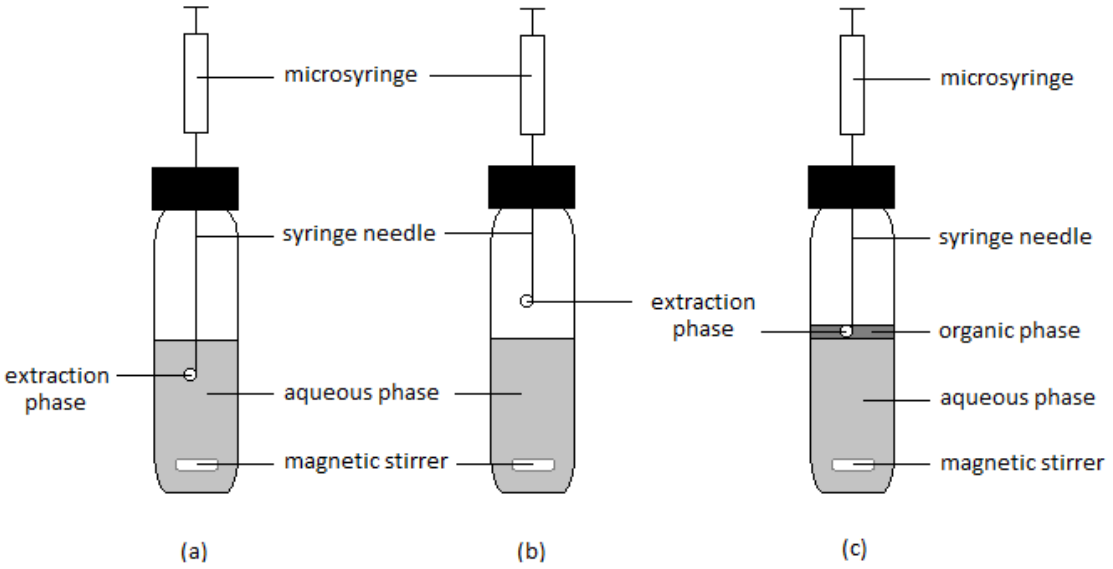

Figure 2 *Different approaches for SDME: (**a**) direct immersion-SDME, (**b**) headspace-SDME, (**c**) liquid-liquid-liquid micro-extraction. Modified from* [14].

SDME was first reported by Liu and Dasgupta [15] and by Jeannot and Cantwell [16] in 1996. It uses a single drop (typically 1 – 3 µL) of a water-immiscible extraction solvent rather than tens of millilitres needed in traditional LLE, which makes it an environmentally friendly technique. The micro-drop kept on the end of a needle is either directly immersed in the analysed sample – direct immersion-SDME or it is placed with an air gap above the sample solution where the air is denoted headspace phase – headspace-SDME allowing to extract only volatile or semi-volatile analytes. It is also possible to use a three-phase approach called liquid-liquid-liquid micro-extraction, where the analyte is extracted from aqueous sample to organic solvent layer and then back-extracted by aqueous micro-drop at the same time. The three approaches are presented in **Figure 2**. After extraction, the micro-drop is withdrawn back into the syringe and processed for further analysis [18,21].

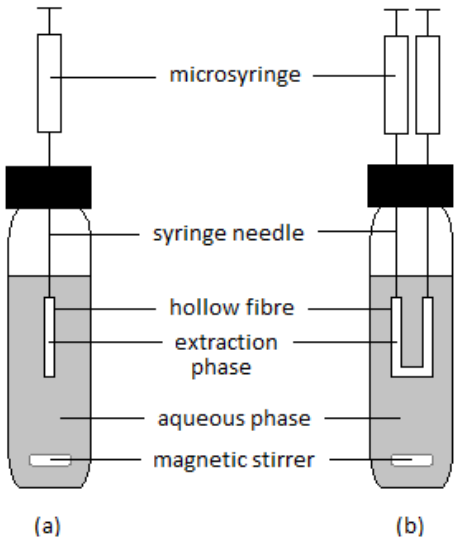

Figure 3 *A schematic diagram of HF-LPME set-up. Hollow fibre is fixed* ***(a)*** *to the end of one syringe or* ***(b)*** *to the tips of two syringes creating a u-shape. Modified from* [14].

The disadvantage of SDME is the low stability of the droplet. Therefore a modification, hollow-fibre liquid-phase micro-extraction (HF-LPME) was presented for the first time in 1999 by Pedersen-Bjergaard and Rasmussen [17]. In this method, the extraction solvent is allowed to be soaked into a porous hollow fibre, which is directly fixed to the

end of one micro-syringe (**Figure 3a**) or fixed at the tips of two micro-syringe needles creating a U-shaped configuration (**Figure 3b**) [18]. When working with HF-LPME, it is possible to use two approaches. In a two-phase system, the hollow fibre and its pores are filled with the same organic extraction solvent and immersed in the aqueous sample solution while in a three-phase system, the acceptor solution placed inside the fibre is hydrophilic whilst organic hydrophobic solution is located only in the fibre pores. In other words, the analytes are extracted from the aqueous sample into the aqueous acceptor solution through a thin layer of organic solvent [18,21]. The hollow fibre does not only protect the extraction solution from detachment but it also stops particles and macromolecules from penetrating the pores and interfere with the extraction [18].

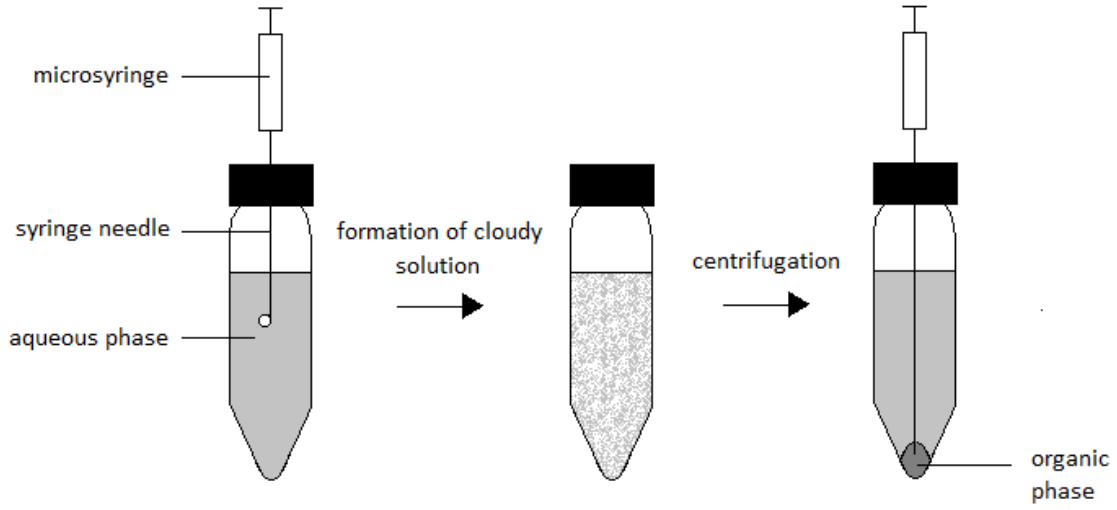

Figure 4 *Stages of DLLME. Modified from* [14].

In 2006 Rezaee and co-workers [19] presented a new micro-extraction method with significantly reduced extraction time. The method is called dispersive liquid-liquid micro-extraction (DLLME) and it is based on an extraction using only a few microlitres of extraction solvent with the addition of a 10-100 times higher volume of a so-called dispersive solvent or disperser. By a rapid injection of the mixture into the sample containing the analyte, the extraction solvent is dispersed in the form of fine droplets

and a cloudy solution is formed [13]. Thanks to the enlarged surface area between both of the phases, the equilibrium of the extraction process is achieved rapidly. As the next step, the cloudy solution is centrifuged to separate the extraction phase for easy recovery by a syringe needle. The process is shown in **Figure 4**.

There are a few requirements for this method to work. Similarly to the traditional LLE, the extraction solvent must be immiscible with the sample solution. Conversely, the dispersive solvent has to be miscible with both of the phases [14]. The advantages of DLLME are simplicity, low cost, low extraction time and high reproducibility. On the other hand, the dispersive solvent increases the solubility of the analyte in the aqueous phase which reduces efficiency.

Since DLLME was first described, several alternative methodologies have been developed in which the dispersive solvent is replaced by kinetic energy e.g. ultrasound-assisted DLLME [20], air-assisted DLLME [21], magnetic stirring-assisted DLLME [22] and more. Magnetic stirring-assisted DLLME, used in this work, is further described in chapter 3.3.4. For its automation, the technique Lab-In-Syringe was used, introduced in the following text.

3.3 Flow techniques

3.3.1 General characteristics and automation

In analytical chemistry, there are two basic approaches, which can be used to handle laboratory tasks such as reaction-based assays. The first is traditionally called the batch chemistry as it is originally performed in beakers or vials. This way, the most of analytical operations, for instance, sample preparation, measurements, and calculation of the results are often performed manually, which can be labour-intensive and time-consuming.

The other way to approach analytical operation is to use flow techniques. Flow techniques are tools to automate classical laboratory tasks. A simple flow system consists of a pump, valve, mixing coil, and detector, all connected by flexible tubing. The principle operation is based on the injection of a sample into a stream of a suitable liquid carrier. After injection, the sample is mixed and is allowed to react with reagents present in the carrier by the dispersion occurring in the tubing system (manifold). The reaction product is then transported by the flow to the detection cell [23].

By using flow techniques, a large number of samples can be analysed with a minimum of human intervention. Moreover, the automated instrument can treat more samples per hour than a human employee, which saves time and labour costs. Another major advantage is the elimination of human mistakes. Well-designed analyser often yields higher reproducibility of measurement over a longer period of time because all samples are handled in exactly the same way and for the same time. Automated instruments also enable to process samples without risking contact with the analyst, which is useful for handling toxic chemicals or to avoid contamination. However, the role of human analysts is still important as they are fully responsible for the data quality and calibration and validation of the methods [24].

Since its first appearance, flow techniques have gone through a variety of changes. The first technique based on flow operation was developed by L. Skeggs in 1952 [25]. His work described air-segmented (continuous) flow analysis, a method in which the

samples introduced into the tubing system are separated by air bubbles present in the carrier flow. Throughout the years, several other techniques were developed such as flow injection analyses (FIA) [26], sequential injection analysis (SIA) [27] or most recently Lab-In-Syringe (LIS) [28], which are described in the following chapters.

3.3.2 Flow injection analysis

Flow injection analysis (FIA) is often denoted the first generation flow technique and was first presented by J. Růžička and E. H. Hansen in 1975 [26]. The simplest flow injection instrumentation (illustrated in **Figure 5**) consists of a pump, an injection valve, a mixing coil and a detector, all connected by flexible tubing [23].

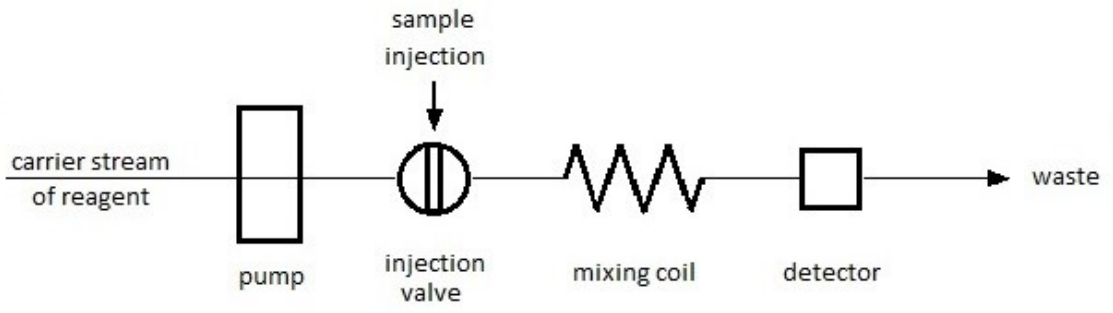

Figure 5 *Schematic diagram of a basic FIA set-up. Modified from* [29].

The pump generates a continuous unidirectional flow of a carrier containing the reagent, which is pumped into an injection valve, where a well-defined volume of sample is injected. Then the solution passes through a mixing coil to a detection flow cell. The most common detectors used in FIA are optical detection systems i.e. spectrophotometry.

At the very moment the sample is injected, it creates a sample concentration zone of the rectangular shape shown in **Figure 6a**. As the sample moves down the system, it disperses into the carrier stream and the concentration zone changes its form. The dispersion is caused by two processes: friction and diffusion. First, the friction occurs due to laminar flow [24]. Friction causes that the sample at the tubing inner walls moves

far more slowly than the sample in the centre of the tubing, which results into a parabolic shape of the concentration zone presented in **Figure 6b** [29]**.** Simultaneously, diffusion appears due to the concentration gradient between sample and carrier. There are two types of diffusion: radial and axial. However, in narrow tubing axial diffusion is insignificant whereas radial is much more important [24]. By this, analyte molecules close to the walls can diffuse more towards the centre of tubing and thus move faster. On the contrary, it also makes the leading molecules diffuse towards the walls which slow them down. This is how the flow profile shown in **Figure 6c** arises. The dispersion continues with time and the concentration zone reaches a symmetrical distribution shown in **Figure 6d** [24,29].

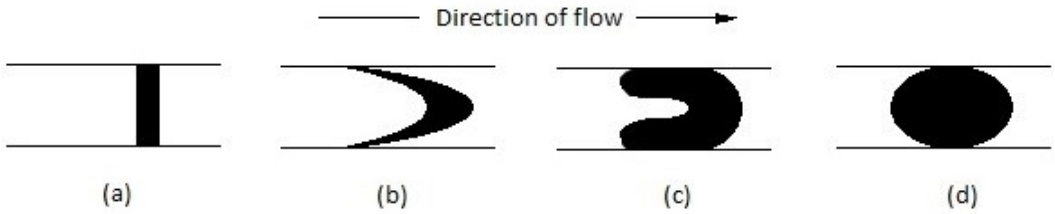

Figure 6 *The effect of friction and diffusion on a shape of concentration zone: **(a)** no dispersion, **(b)** dispersion by friction, **(c)** dispersion by friction and radial diffusion, **(d)** dispersion only by diffusion* [24].

Precise sample injection, repeatable timing, and controlled dispersion are the most important characteristics of FIA. The dispersion is controlled by three variables: sample volume, flow rate, and tubing parameters such as length, diameters, and coiling [24]. In case that all these factors are held constant, the dispersion is reproducible, so all the samples are handled in exactly the same way. Owing to that, the chemical reaction does not have to reach equilibrium state before the sample reaches the detector, which allows reducing the time of the performed assays significantly [29].

3.3.3 Sequential injection analysis

One of the disadvantages of the continuous flow of FIA is a high consumption of reagents and a large production of waste. Therefore in 1990, J. Růžička and G. D. Marshall proposed a variation of FIA called sequential injection analysis (SIA) [27].

A typical apparatus of SIA consists of an automatic bidirectional pump or a syringe pump, a valve, a holding coil, a reactor, a detection system, and finally a computer. A typical SIA system is presented in **Figure 7**. The simple injection valve of FIA is replaced by a multi-position selection valve, which is connected to various sample and reagent containers, detector, and the syringe pump via suitable tubing [29].

SIA is a flow technique based equally on sample injection, controlled dispersion, and reproducible timing. However, unlike FIA, SIA uses discontinuous bidirectional flow and the dispersion is controlled by means of flow programming, which in effect reduces the consumption of reagents and the production of waste and which is beneficial particularly when working with expensive or toxic chemicals [30].

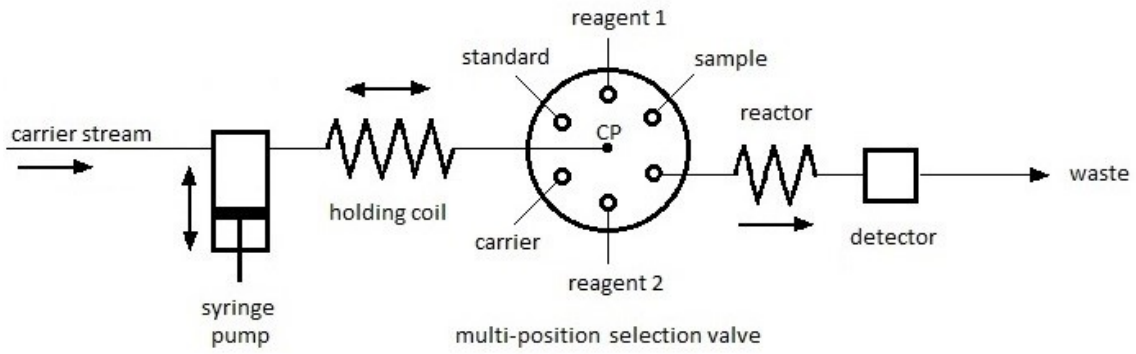

Figure 7 *Schematic diagram of SIA system. Modified from* [29]. *CP - central port*

The principle of SIA is described in **Figure 8**. First, all solutions, reagents and sample are introduced sequentially into the system by aspiration of well-defined volumes from the selection valve through its central port into the holding coil [29]. After aspiration, the sample and reagent zones are pushed further upstream and disperse into one another,

which lead to a formation of a reaction product zone. The holding coil needs to be large enough to allow a creation of the reaction product without the zones reaching the syringe. The multi-position selection valve then switches to the detector position and the flow is reversed pushing the zones through a reaction coil to the detection system. The choice of detector depends on a type of reaction; the most frequently used detection system is UV-VIS spectrophotometry.

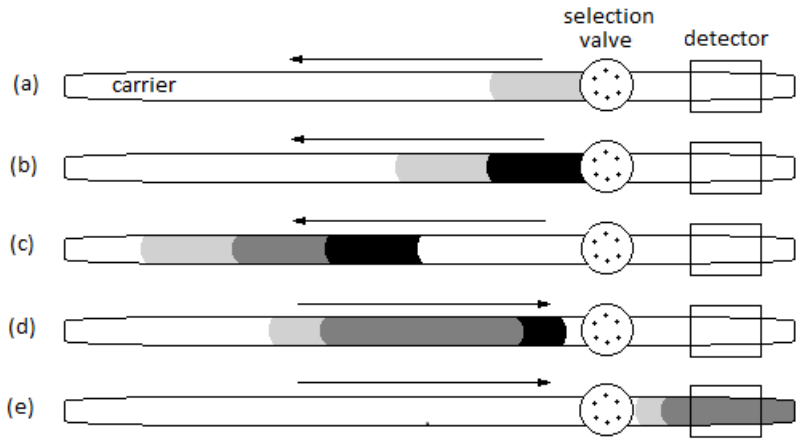

Figure 8 *Principle of SIA: (**a**) sample injection, (**b**) reagent injection, (**c**) injection of a carrier, dispersion, and creation of a reaction product, (**d**) flow reversal towards the detector, and (**e**) detection of product. Modified from* [23].

Apart from lower reagent consumption and waste production, SIA has several other advantages compared to FIA and related flow techniques. The manifold is easily adapted to another analytical procedure just by altering the flow program. SIA is also fully automated and completely computer controlled. However, when compared to FIA, the injection frequency of samples is typically reduced due to required steps of solution aspiration and syringe re-filling [8,13].

3.3.4 Lab-In-Syringe

Lab-In-Syringe (LIS) is a relatively new approach first described by Maya and co-workers in 2012 [28], who used it for the automation of DLLME. This flow technique uses the syringe void of a SIA system as a reaction chamber in which various analytical steps are carried out. Thanks to the piston, the syringe is size-adaptable and yet sealed, which contributes to precise and reproducible measurements.

In the same year, Maya et al. presented a fully automated in-syringe DLLME system in which the spectrophotometric detection also takes place inside the syringe [31]. However, in both of these papers, a large volume of dispersive solvent is required to initiate the dispersion process.

The first to use an in-syringe magnetic stirring-assisted DLLME was Horstkotte et al. in 2013 [32]. Instead of a dispersive solvent, a magnetic stirring bar placed inside the syringe was used for initiation of dispersion. The entire procedure is shown in **Figure 9**.

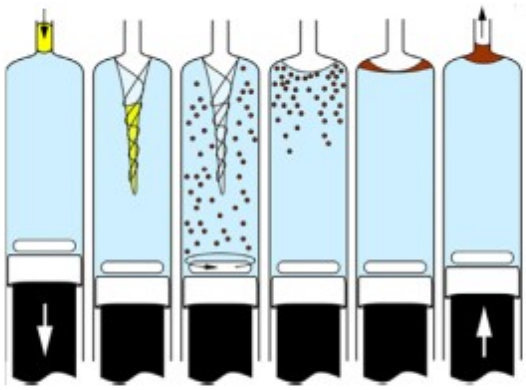

Figure 9 *Scheme of MSA-DLLME: After aspiration, the solvent is dispersed into fine droplets by rotation of the stirring bar placed within the syringe. When the stirring stops, the solvent droplet, enriched with the analyte, float, coalescence, and create an organic upper layer. Finally, by means of the piston, the solvent is then pushed out of the syringe towards the detection cell* [32].

To rotate the magnetic stirrer inside the syringe, a specially developed magnetic driver (presented in **Figure 10**) was placed onto the syringe and the rotating magnetic field was obtained. The stirring rotation was activated by a motor connected to the bottom ring of the driver by a rubber band [32].

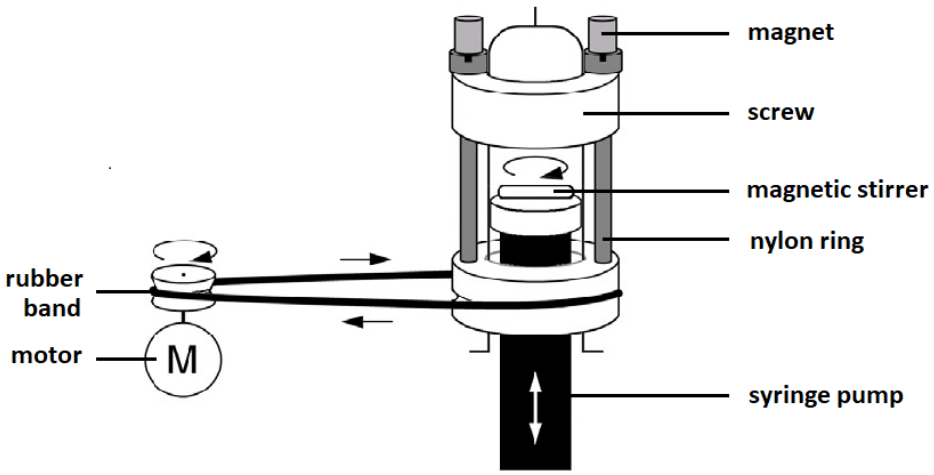

Figure 10 *Magnetic stirring bar driver, consisting of two magnets, two screws and two nylon rings, placed onto the syringe barrel* [32].

Using in-syringe MSA-DLLME enables the analysis of solutions of variable volumes and viscosities. Step-by-step addition of solutions is possible and moreover, the analysis time and waste production are reduced. On the other hand, the system shows a large dead volume due to the space required for the stirring bar inside the syringe. Moreover, it is best suited for solvents lighter than water. To use extraction solvents of higher density than water and to reduce the dead volume, the syringe can be turned upside-down, so that the liquid is forced out emptying the syringe by the air cushion remaining inside the syringe [33].

3.4 Principle of the applied reaction

The extraction is based on an ion-pair formation between chlorhexidine and methyl orange (MO). Chlorhexidine is protonated and positively charged, while MO contains a sulphonic acid group, which is negatively charged above pH 4. When mixed together, they form a yellow ion-pair complex. The complex is lipophilic and can be extracted into an organic solvent such as 1-octanol. After addition of an acid such as hydrochloric, the N-atom in the MO molecule ($pK_a = 3.4$) becomes protonated and the complex appears red (below pH 3) and its solubility in water increases, which allows the back-extraction of the ion-pair components into an acidic aqueous phase. The chemical structures of both forms of MO are presented in **Figure 11.** The colour intensity of the back-extract is measured spectrophotometrically at a wavelength 508 nm against a reference of 585 nm. It ideally corresponds to the concentration of the analyte in the sample solution [34].

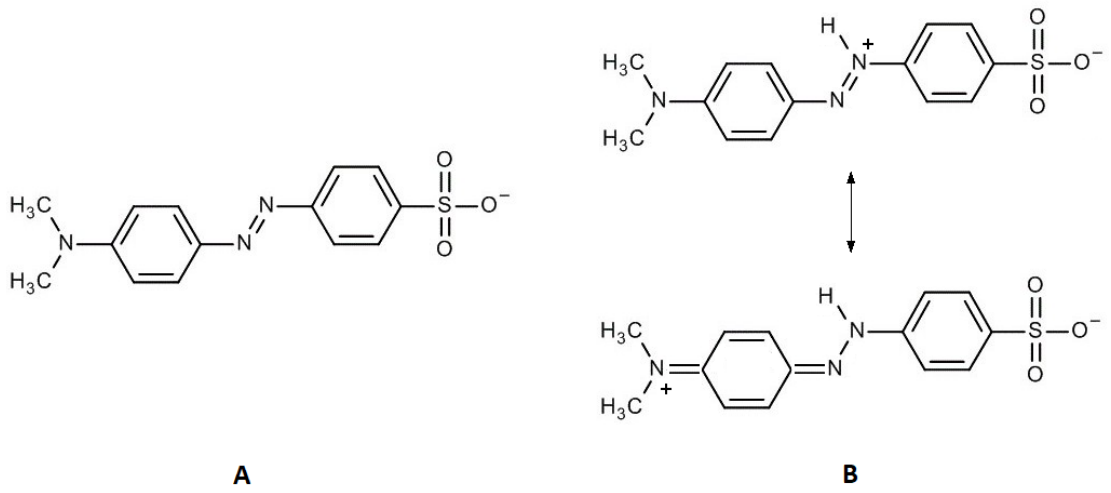

Figure 11 *Structure of MO: **A** – yellow form; **B** – red form.*

4. Materials and methods

4.1 Reagents, samples and solutions

For cleaning the syringe and flow system before each extraction, water of bi-distilled quality (provided by a MilliQ system) and isopropyl alcohol were used. Water of bi-distilled quality was also used for preparation of all solutions. An analytical balance was used for weighting of reagent and analytical standard.

Chlorhexidine was obtained from Sigma-Aldrich (Product No. 282227, ≥ 99.5 %) and used for a preparation of an aqueous stock solution of a concentration of 1 g/L. The stock solution was prepared once at the beginning of this experiment and was stored in a fridge at 4 °C at all times. All standard solutions were prepared newly every day before starting the measurements from the stock solution by appropriate dilution. Conditions for preparation of standard solutions used for calibration are listed in **Table 1**.

Table 1 *Parameters for six standard solutions used in chapter 5.4*

Concentration of standard solution [mg/L]	**Prepared volume of standard solution [mL]**	**Used volume of 1 g/L stock solution of chlorhexidine [µL]**
0.5	50	25
1.0	50	50
1.5	50	75
2.0	50	100
2.5	50	125
3.0	50	150

All chemicals are summarized including their quality and provider in **Table 2**.

An anionic dye, methyl orange (MO) was purchased from Roanal (Budapest, Hungary). At start of this work, a stock solution of 1 g/L concentration was prepared by dissolution of 20 mg of MO in a 20 mL volumetric flask in water. From this primary stock solution, various MO solutions were prepared by dilution with water and were used as reagents.

As extraction solvents, pure 1-octanol, 1-hexanol, amyl acetate, methyl benzoate, chloroform, and toluene were tested.

A variety of sodium citrate buffer solutions of different pH values ranging from 3 to 7 were used to study the effect of the extraction pH. The concentration of citric acid in all buffer solutions was 100 mmol/L. Each solution was prepared by mixing 0.96 g of citric acid and approximately 20 mL of distilled water. Then the pH was adjusted by the addition of a sodium hydroxide solution (1 mol/L) while stirring the solution using a magnetic stirrer. Finally, the solution was transferred to a 50 mL volumetric flask and made up to the final volume using distilled water.

As the back-extraction solvent, solutions of hydrochloric acid (Sigma-Aldrich, p. a.) of different concentrations were tested. All solutions were prepared by diluting a 2 mmol/L stock solution with water.

As real samples, commercial mouthwash products of following trademarks were used: Chlorhexil, Rebi-Dental Mouthwash, Colgate Max White, Listerine, and Parodontax. These trademarks were chosen randomly according to the local supermarket assortment. To test the selectivity of the method, samples without chlorhexidine were also used. The content of chlorhexidine digluconate in each product and other contained substances are listed in **Table 3**. Before measurement, all samples were diluted 1:1000 into 100 mL volumetric flasks with water.

Table 2 *Overview of used chemicals*

Name	**Company**	**Purity**
1-hexanol	Sigma-Aldrich Prague, Czech Republic	p.a.
1-octanol	Sigma-Aldrich Prague, Czech Republic	p.a.
Amyl acetate	Sigma-Aldrich Prague, Czech Republic	p.a.
Citric acid	Penta, Chrudim, Czech Republic	p.a.
Chlorhexidine	Sigma-Aldrich Prague, Czech Republic	p.a., ≥ 99.5 %
Chloroform	Sigma-Aldrich Prague, Czech Republic	p.a.
Hydrochloric acid	Sigma-Aldrich Prague, Czech Republic	p.a.
Isopropyl alcohol	Sigma-Aldrich Prague, Czech Republic	> 99 %, FG
Methyl benzoate	Sigma-Aldrich Prague, Czech Republic	p.a.
Methyl orange	Roanal, Budapest, Hungary	p.a.
Toluene	Sigma-Aldrich Prague, Czech Republic	p.a.
Sodium hydroxide	Penta, Chrudim, Czech Republic	p.a.

Table 3 *List of used mouthwash samples and its composition*

Brand name of mouthwash product	**Content of chlorhexidine digluconate**	**Other substances claimed on the package**
Chlorhexil	0.12 % w/V	Chamomilla recutita, Bisabolol, Krametia triandra, Commiphora myrrha, Allantoin, Xylitol
Rebi-Dental Mouthwash	0 %	Aqua, glycerine, sodium monofluorophosphate, polysorbate 20, PEG 40 hydrogenated castor oil, sodium benzoate, sodium saccharin, 2-bromo-2-nitro-1,3-propanediol, aroma, triclosan, menthol, benzyl alcohol, limonene, CI 42090
Colgate Max White	0 %	Aqua, glycerine, propylene glycol, sorbitol, tetrapotassium pyrophosphate, polysorbate 20, tetrasodium pyrophosphate, zinc citrate, PVM/MA copolymer, aroma, benzyl alcohol, sodium fluoride, sodium saccharin, CI 42051
Listerine	0 %	Aqua, alcohol, sorbitol, poloxamer 407, benzoic acid, sodium saccharin, eucalyptol, methyl salicylate, aroma, thymol, menthol, sodium benzoate, CI 47005, CI 42053
Parodontax	0.06 % w/V	Sodium fluoride (0.0553 %), aqua, propylene glycol, sorbitol, PEG-40 hydrogenated castor oil, aroma, methylparaben, propylparaben, sodium saccharin, eugenol

4.2 Set-up of the system

For all measurements, a stand-alone automated syringe pump from FIAlab Inc. company (Bellevue, WA) was used. The syringe pump (shown in **Figure 12**) was equipped with a 2.5 mL syringe and had a 9-port selection valve replacing the typical head valve. The syringe was turned upside-down to facilitate sample discharge and keeping the extraction solvent of lower density than water for later back-extraction as well as to allow emptying the syringe pump completely. Each position of the selection valve was connected to a required solution or the detection flow cell by PTFE tubing of 0.8 mm id. The positioning of the solutions is depicted in **Figure 13**.

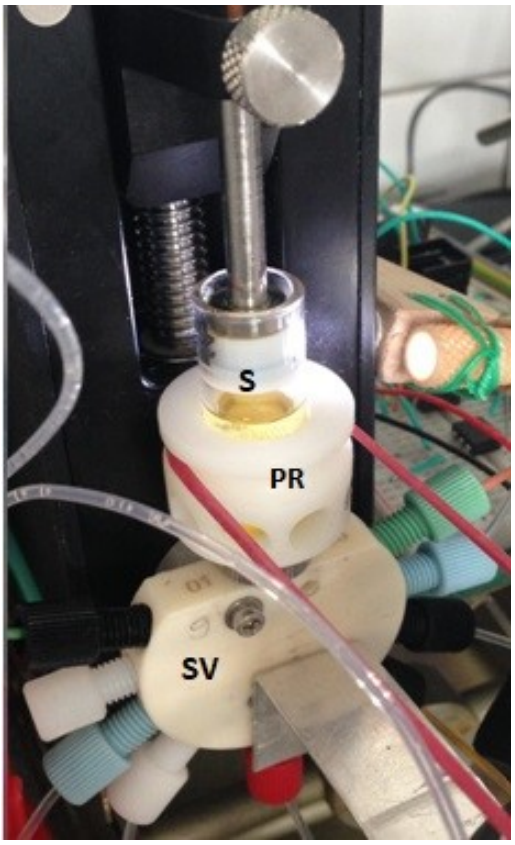

Figure 12 *The used LIS system: A syringe pump (S) was connected to a 9-port selection valve (SV). A plastic ring (PR) was placed onto the syringe barrel and connected to a motor (not shown) via rubber band. A LED light was fixed to the system for observation of the syringe content through the holes in the plastic ring.*

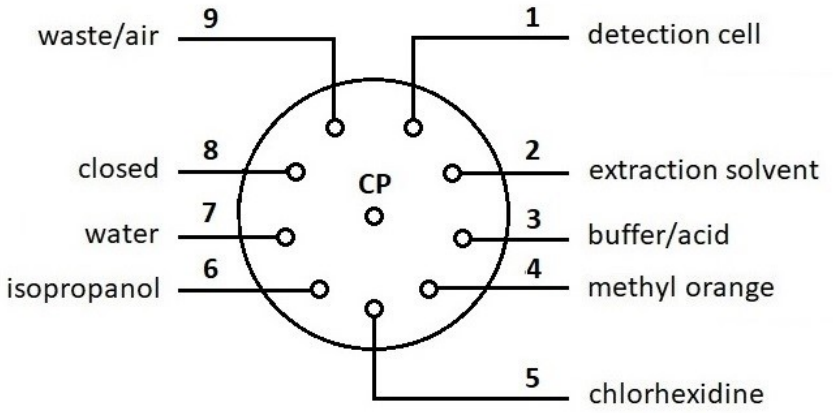

Figure 13 *Selective valve positions on the head valve of the used syringe pump. CP - central port*

To achieve homogeneous mixing inside the syringe, a magnetic stirring device was used. This device consisted of an electric motor made from a computer ventilator, a plastic ring holding two neodymium magnets (4 diameter x 4 mm length) situated opposite to each other creating a magnetic field, and a small PTFE-covered magnetic stirring bar (10 mm diameter) placed inside the syringe. The ring was placed onto the syringe´s barrel and driven by the motor by a rubber band. When the motor was switched on, the ring started to rotate around the syringe barrel and the magnetic bar inside the syringe was equally forced to rotate with it. Control of motor activation and deactivation was enabled by software via a relay board also allowing adjustment of the rotation speed. The magnetic stirring system is given in **Figure 14**.

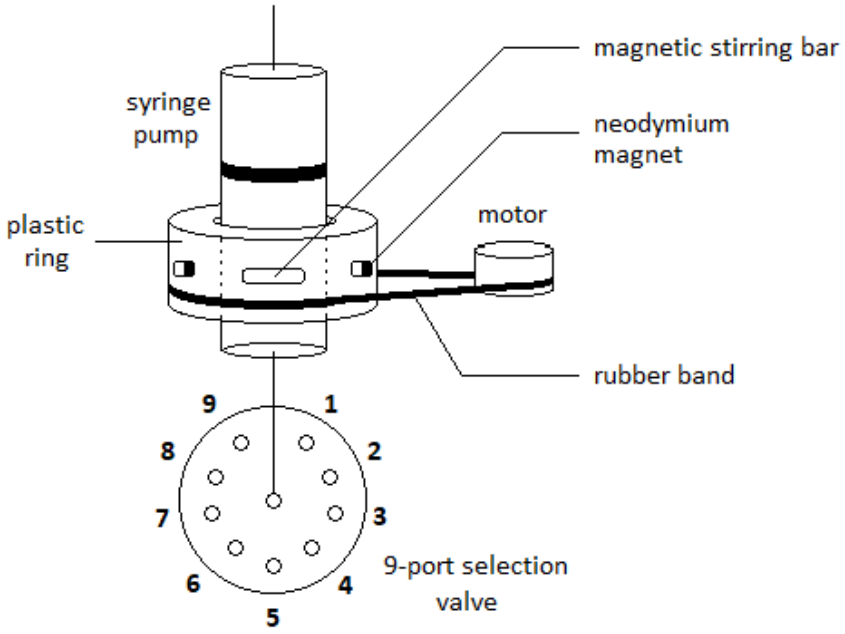

Figure 14 *Scheme of magnetic stirring system placed onto a syringe pump.*

As detection system, an USB2000 miniature fibre-optic spectrophotometer from OceanOptics Inc. (Dunedin, FL) was used.

As light source, a deuterium-halogen lamp (OceanOptics) was used, which was connected by fibre optics of 1 mm core diameter to flow cell made of PTFE with a Z-shaped flow channel of 1.6 mm id and 1 cm path-length (fibres and cell from FIAlab) and further, by another optical fibre to the USB2000.

Through the entire study, absorbance was measured at three wavelengths: 420 nm, being the maximum absorbance of the acidic, yellow form of MO in the organic phase, 465 nm for the yellow form in water, and 508 nm for the basic and red form in the aqueous phase. The system also used a reference wavelength for reduction of unspecific effects, which was set to 585 nm.

The entire system was controlled by the FIAlab software version 5.6, which was also used for data acquisition. For posterior data evaluation, the software MS Excel was used.

4.3 Operation methods

At the beginning of each working day, all solutions including water were prepared in sufficient amount and placed in the correct positions on the selection valve and the waste bottle was emptied. The instrument and the software were started and dark and reference scans were performed on the USB2000 spectrophotometer.

Every morning the entire system was cleaned using the following cleaning method. The cleaning method started by emptying the syringe and aspirating a specific volume of each solution from every position of the selection valve to fill the tubes with the respective solutions. The aspirated volume was adjusted according to the length of each tube. Then the content of the syringe was discharged to waste and the syringe was cleaned once using isopropyl alcohol and twice using water. While aspirating these solutions, the magnetic stirrer was activated to improve the cleaning process before the syringe content was pushed through the detection cell to waste.

The extraction method was adjusted during the thesis according to the experimental findings while the main characteristics remained unchanged. The method is given as **Appendix 1**.

Within the method, the spectrophotometer settings were defined such as the appropriate wavelengths (see chapter 4.2) as well as other variables. The method started with cleaning of one of the tubes, an optional part of the method, which could be activated by variable setting if needed. This part was activated only when one of the solutions was replaced during the experiment, e.g. the sample. Another optional part was performing a reference scan after filling the detection cell with water. This was followed by cleaning of the syringe once with isopropyl alcohol and twice with water, which is similar to the cleaning method described above but what was performed before each analysis.

After the cleaning procedure, solutions were aspirated in the following order: buffer (if used), extraction solvent, MO reagent, and sample, standard or water (used for blank measurement). Before aspirating the sample, the stirrer was activated to promote solvent dispersion into droplets and to perform the extraction. After an appropriate extraction time, the stirring was deactivated for phase separation. Finally, the entire syringe content was pushed through the detection flow cell for absorbance measurement.

After optimisation of analyte extraction, the method was modified by adding the required steps for analyte and MO back-extraction (see **Appendix 2**). For this, only the aqueous phase was discharged to waste after phase separation and the sample was washed by aspirating water, activating the stirrer for about 1 s, and then dispensing the water to waste. As a next step, a well-defined volume of hydrochloric acid was aspirated and the stirrer was activated to perform the back-extraction of the ion-pair components into the acidic aqueous phase. After deactivation of the stirrer and an appropriate time allowing for phase separation, both phases were pushed through the detection cell and absorbance was measured.

5. Experiments, results and discussion

The work was done in two steps. In the first, formation and extraction of the ion-pair complex to an organic phase were studied and the extraction parameters including the pH, the extraction time, the volume of the reagent, and the stirring rate were adjusted. Furthermore, a suitable extraction solvent was chosen and its volume was optimised. In the second step, back-extraction to an acidic aqueous phase and corresponding parameters were studied. Eventually, the method was applied to real samples.

5.1 Set-up of system/operation

The system was set-up and the operation method was written as it is described in chapter 4.2 and chapter 4.3, respectively. Significant system adjustments were not done during the study as the same configuration was used as for another experimental work, running in parallel to this thesis. Simple adjustments included changing solutions and adjusting the stirring rate.

5.2 Experiments without back-extraction

5.2.1 Study of pH

The effect of pH on the formation of a MO-chlorhexidine ion pair was studied. To adjust the pH of the reaction, a sodium citrate buffer was used. Nine different solutions were prepared going up in the pH scale in increments of 0.5 ranging from 3 to 7 as described in chapter 4.1.

The experiment was done with three repetitions both with water as a blank solution as well as with a 20 mg/L chlorhexidine standard. First, 100 µL of 0.2 g/L MO reagent was aspirated followed by 300 µL of amyl acetate, 200 µL of the citrate buffer, and 1 mL of water respectively chlorhexidine solution. Amyl acetate was chosen as an extraction solvent due to its similarity to n-butyl acetate, a solvent used in a paper which described an assay of nitrogen-containing drugs based on a similar reaction as used in this thesis [34].

The extraction time was set to 60 seconds, a value which is higher than the typical values used in previous works performing DLLME by LIS [32,35]. The separation time was set to 20 s.

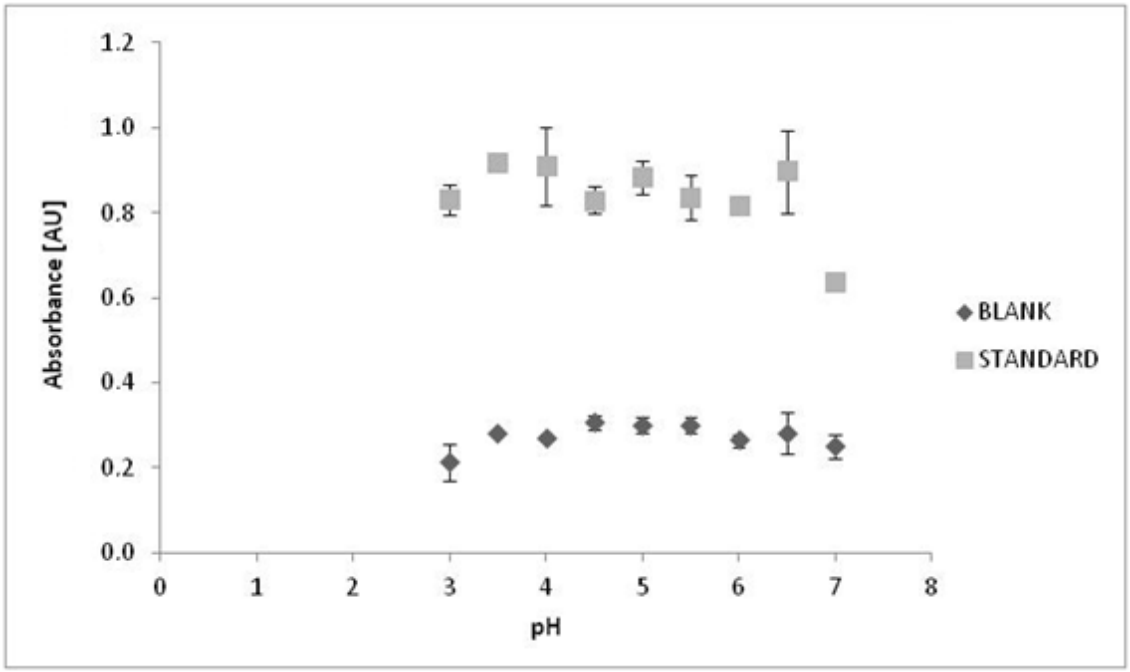

Figure 15 *The effect of pH on extraction of an ion-pair complex chlorhexidine-MO*

The maximum absorbance in the studied range was observed at pH 4. At lower pH, MO molecules become protonated on its N-atom, which makes it more soluble in water therefore less extractible into an organic phase as a chlorhexidine-MO complex. Chlorhexidine is a compound able to get protonated twice with pK_b values of 2.2 and 10.3 [36], therefore, in the range of pH 3.2 to 9.3, the analyte can be considered single positively charged. As shown in **Figure 15**, the decrease of signal at pH can be therefore explained rather by the lower extraction capacity of MO (pK_a 3.4) while at pH 7, the signal decrease is rather due to an inhibition of the extraction by the citrate itself, being at this pH triple negatively charged. As the effect in the studied pH range was small and the real concentrations in mouthwash required high dilution, we therefore omitted the use of buffer at this stage. However, the study of an acetate buffer instead of citrate buffer could have resulted later advantageous considering that the found analyte recovery was low.

5.2.2 Study of extraction solvent

To find a suitable extraction solvent, six organic compounds namely 1-hexanol, 1-octanol, methyl benzoate, amyl acetate, toluene, and chloroform were studied. Each solvent was tested individually by performing four in-syringe extractions with 1.0 mL of water as a blank solution and four extractions with 1.0 mL of a chlorhexidine standard with a concentration of 12.5 mg/L. Solvents were aspirated in a volume of 0.3 mL. For all experiments, 0.025 mL of MO with a final reagent concentration of 0.5 g/L was used. The stirring rate was set to 1260 rpm. The extraction time was set to 60 seconds. The phase separation time was set to 10 seconds for all solvents except 1-octanol for which it was increased to 20 seconds due to its higher viscosity and lasting phase separation. Measurements were done in the organic phase aiming for the yellow, extractable form of MO at 420 nm.

As it can be seen in **Figure 16**, the highest standard signal was obtained when 1-hexanol was used as an extraction solvent. Unfortunately, the blank signal was unacceptably high using hexanol indicating that this solvent is capable of dissolving also the MO reagent itself.

A similarly high standard signal was measured using 1-octanol. Compared to 1-hexanol, 1-octanol is more hydrophobic which can explain why the blank signal was significantly lower as the reagent MO alone is hardly extracted. However, having used a longer phase separation time for 1-octanol might have had also an effect on the results, in particular it can be seen that the blank signal shows a significantly better reproducibility for 1-octanol than for 1-hexanol.

The tests using amyl acetate and methyl benzoate yielded lower standard signals but clear distinction between blank and standard signal was possible for these solvents, too. Chloroform and toluene did not show any significant extraction capacity for the MO-chlorhexidine ion pair, likely for being too non-polar and not able to form hydrogen bonds.

Based on these experiments 1-octanol was chosen for further measurements. It has to be pointed out that even with absorbance values higher than the typical usable range of up to 1 AU for 1-octanol, 1-hexanol, and methyl benzoate, the results were significant and

reproducibility was acceptable in all cases yet repetition of the study with a lower standard concentration could be of interest. For following experiments, the used chlorhexidine standard concentration was decreased to 6 mg/L and further diluted in-syringe to 1.5 mg/L.

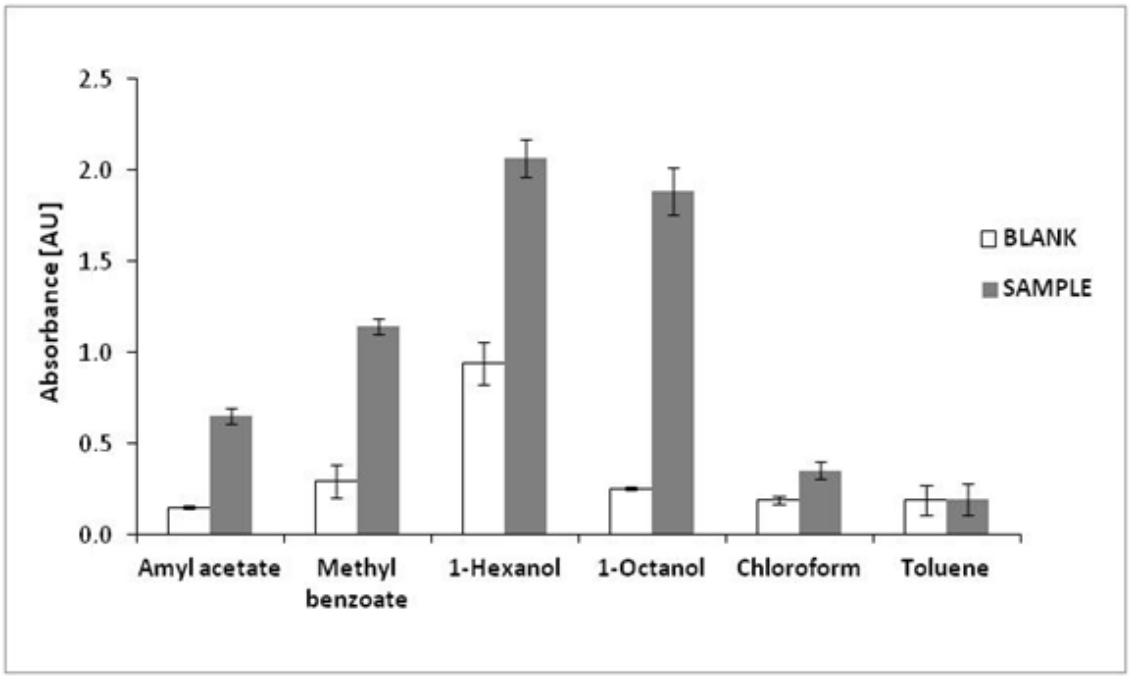

Figure 16 *Absorbance of organic phase at 420 nm after extraction of MO-chlorhexidine ion-pair complex using different extraction solvents.*

5.2.3 Study of extraction time and stirring rate

In order to study the extraction time and stirring rate, the following extraction times were tested: 5 s, 10 s, 20 s, 40 s, 80 s and 160 s. Each extraction time was tested using two different stirring rates: 930 rpm and 1470 rpm. The aim of this experiment was to find the shortest time required for quantitative extraction as well as to evaluate the effect of the stirring rate.

The stirring rate was adjusted via potentiometer on the controlling relay board. Eventually, two approximate stirring rates were possible to use, both possible to activate via auxiliary ports of the syringe pump and operation method. The first one of 930 rpm

was the lowest stirring rate possible to use without the dispersion of the extraction solvent and used for extract washing. The second stirring rate was set to 1470 rpm, the highest value at which the stirring bar would rotate at constant speed. Further increase of the stirring rate caused regular detachment of the rubber band from the fast-rotating motor, which forced the stirring bar to bounce and slow down.

Each experiment was performed in triplicate using 1 mL of water as a blank solution and 250 µL of 6 mg/L chlorhexidine as a standard diluted furthermore in-syringe with 750 µL of water yielding likewise 1 mL.

As a first step, the 350 µL of 1-octanol and 50 µL of 250 mg/L MO were aspirated. After activation of the stirrer, sample respectively water was aspirated followed by extraction and phase separation. The phase separation time was set to 30 s. The measurement was done in the organic phase at 420 nm omitting back-extraction.

From **Figure 17** and **Figure 18**, it can be seen that for both stirring rates, the blank signals remained approximately constant. On the other hand, the standard signals increased in both cases with the extraction time following saturation behaviour. Furthermore, the accuracy was increasing with the extraction time. This indicates that the longer the solution is mixed, the more analyte is extracted into the organic phase.

For a stirring rate of 930 rpm it was found that beyond an extraction time 30 s, the absorbance did not increase more than 3 %.

Using a stirring rate of 1470 rpm, the absorbance values for standard were higher than using 930 rpm. Moreover, the saturation occurred within the first 15 s proving a higher efficiency of extraction at this stirring rate. Taking all results into consideration, a stirring rate 1470 rpm and an extraction time of 30 s were chosen for further experiments.

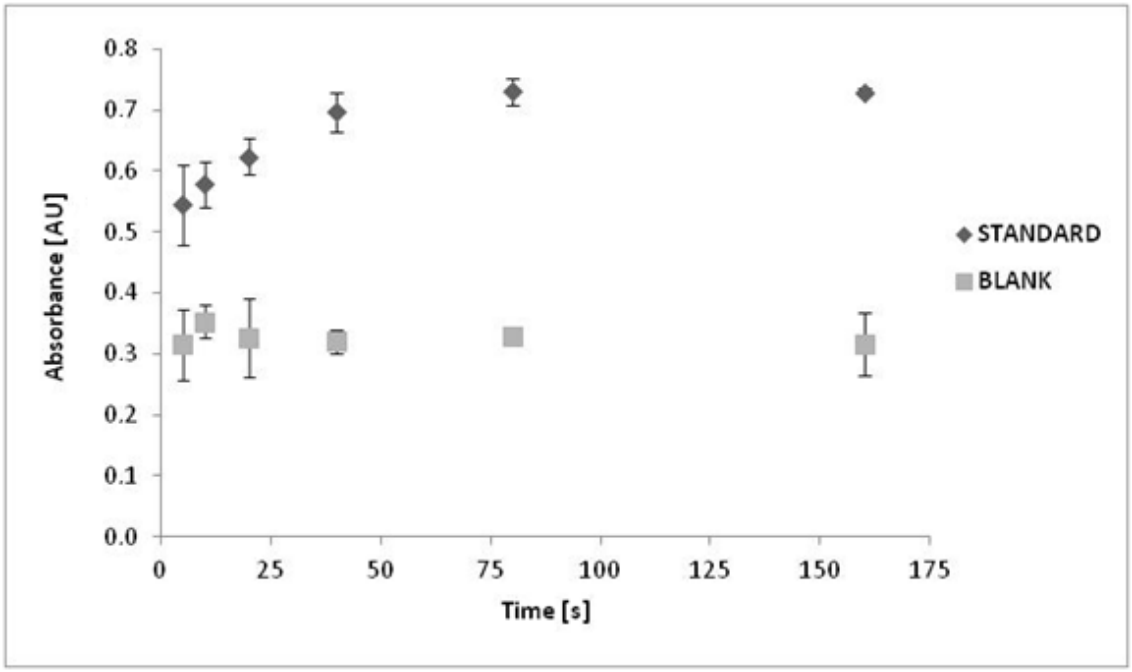

Figure 17 *Absorbance at 420 nm measured for different extraction times in standard and blank solutions while stirring rate was set to 930 rpm*

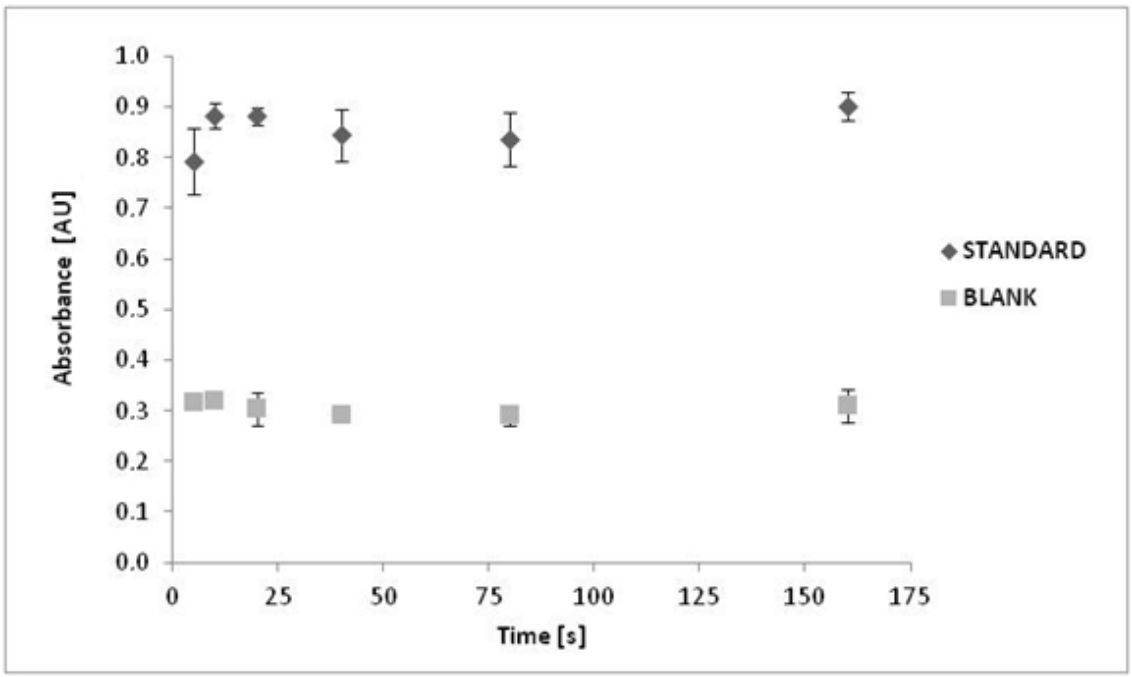

Figure 18 *Absorbance measured at 420 nm for different extraction times in standard and blank solutions while stirring rate was set to 1470 rpm*

5.2.4 Study of volumes of 1-octanol and methyl orange reagent

Based on the previous experiments, 1-octanol had been chosen as extraction solvent. The aim was now to find the lowest volume of 1-octanol required to achieve quantitative extraction to minimize the amount of organic solvent released to waste and furthermore to lower the dilution of the analyte. For this purpose, three different volumes were tested: 350 µL, 250 µL, and 150 µL. A series of in-syringe extractions were performed using three times 1 mL of water as a blank and three times 250 µL of chlorhexidine of a concentration 6 mg/L (diluted in syringe by 750 µL of water) as a standard. This series was repeated three times, each time using a different volume of a 250 mg/L MO reagent: 25 µL, 50 µL and 100 µL. Both extraction and phase separation times were set to 30 s. The stirring rate was set to 1470 rpm.

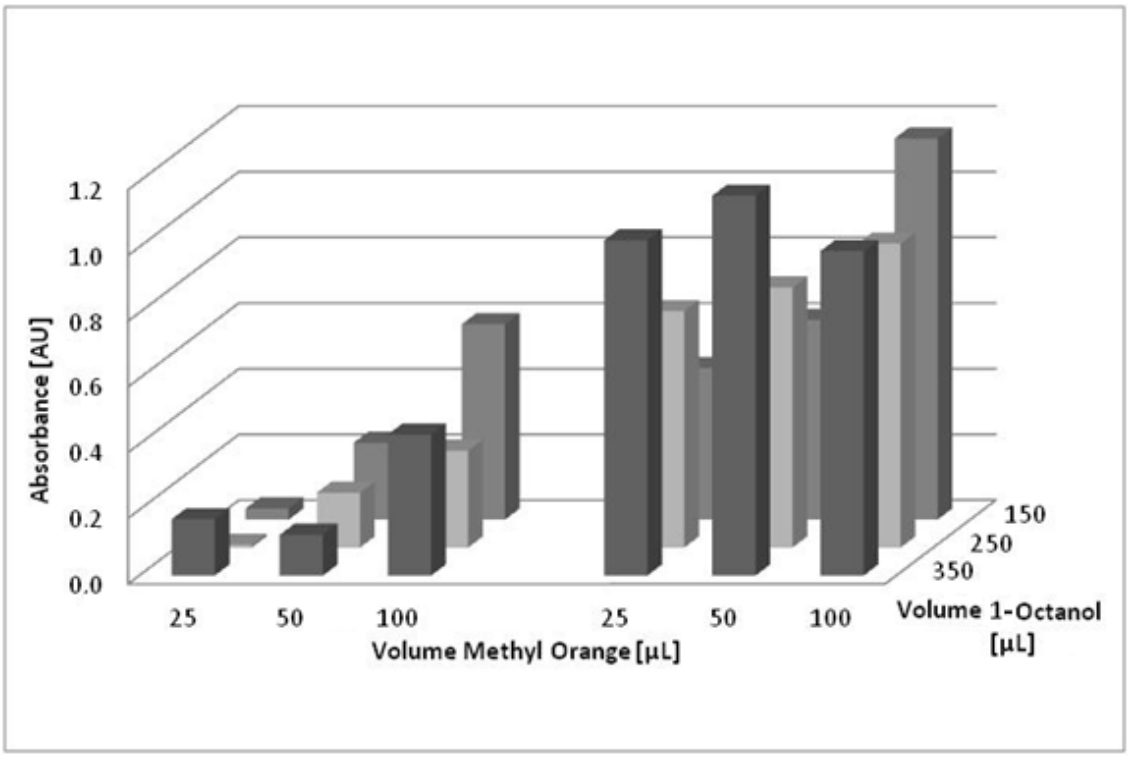

Figure 19 *Absorbance measured at 420 nm in dependency of 1-octanol volume and volume of MO solution for blank (left) and 6 mg/L chlorhexidine standard solution (right)*

It can be seen from **Figure 19** that both blank and standard signals increased with the used volumes of MO although some results did not follow this trend, which also hindered the use of the data for experimental design optimisation. It must be understood that the final goal was back-extraction of MO into an aqueous acceptor so the signal height of the standard was not of primary interest, which is strongly influenced by the volume of 1-octanol (dilution effect) but the difference between blank and standard signal. In addition, a volume of 25 µL was considered to be very small to be aspirated with high repeatability. As for the volume of solvent, using a larger volume implies more organic waste while a smaller volume could be insufficient to achieve good dispersion, efficient extraction and could imply an inacceptable error if some solvent is lost during sample dispersion before the intended back-extraction. Therefore, a volume of the MO reagent of 50 µL and a volume of 1-octanol of 250 µL were finally chosen.

5.3 Experiments including back-extraction

Beyond this point, back-extraction was performed to achieve better selectivity of the method for chlorhexidine. The following experiments, therefore, deal with the study of the respective parameters. The method was adjusted as described in chapter 4.3.

Before back-extraction, the sample was washed with 1500 µL of water (see chapter 4.3). The used amount of water for this step was chosen as a compromise between the requirement that the organic phase had to be lifted so far that loss of solvent would be improbable at emptying the syringe after washing step and intended dilution of sample remains in the syringe after the extraction (ca. 0.35 mL) considering that a lower volume implies short time required for aspiration and dispense.

5.3.1 Study of back-extraction time

The back-extraction time was studied by testing four different times: 4 s, 8 s, 16 s and 32 s. Each measurement was repeated four times using 250 µL of chlorhexidine with a concentration of 6 mg/L, which was diluted in-syringe with 750 µL of water as a standard. First, the extraction was performed using 50 µL of a MO reagent with a

concentration of 250 mg/L and 250 µL of pure 1-octanol as extraction solvent. After extract washing, 500 µL of a 0.5 mol/L hydrochloric acid was aspirated and with the stirrer activated for the extraction time, the sample was back-extracted into the aqueous phase. The phase separation time after both extraction and back-extraction were adjusted to 40 s, based on observation of droplet floatation.

When the time was set to 4 s, which was the lowest tested time, the absorbance measured for the standard was already 0.494 AU. With increasing time, the extraction efficiency improved. As can be seen in **Figure 20**, the absorbance increased with the back-extraction time up to a value of 0.801 AU following saturation behaviour. By further increase of the back-extraction time, no gain in sensitivity could be expected. Based on this measurement, the back-extraction time was set to 30 s.

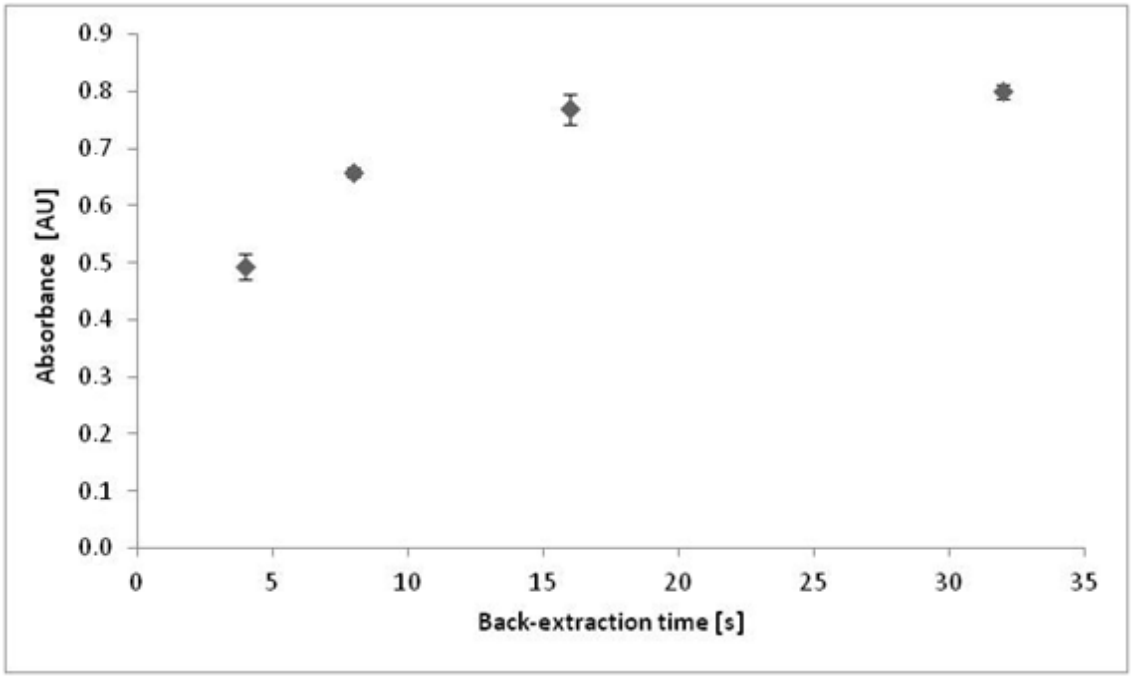

Figure 20 *Absorbance measured at 508 nm in the acidic phase (500 µL of 0.5 mol/L hydrochloric acid) after back-extraction of chlorhexidine (6 mg/L) using different back-extraction times (4 s, 8 s, 16 s and 32 s).*

5.3.2 Study of acidity of acceptor

Addition of acid into the solution in the syringe lowers the pH and protonates another N-atom in the MO molecule, which makes the whole complex more soluble in the aqueous phase. For back-extraction, hydrochloric acid was chosen as acidic acceptor. To find a suitable acid concentration, five solutions of hydrochloric acid were tested at following concentration levels: 0.03, 0.06, 0.125, 0.25, and 0.5 mol/L. The solutions were prepared by diluting a 2 mol/L stock solution of hydrochloric acid with water. Extraction and back-extraction were performed using 250 µL of 1-octanol as extraction solvent, 50 µL of MO reagent (concentration 250 mg/L) and 250 µL of a 6 mg/L chlorhexidine standard diluted in-syringe four times with water. Measurements of standard and blank solutions were repeated four times. The amount of hydrochloric acid aspirated for each measurement was 500 µL. The extraction time was set to 60 s and the back-extraction time was set to 30 s.

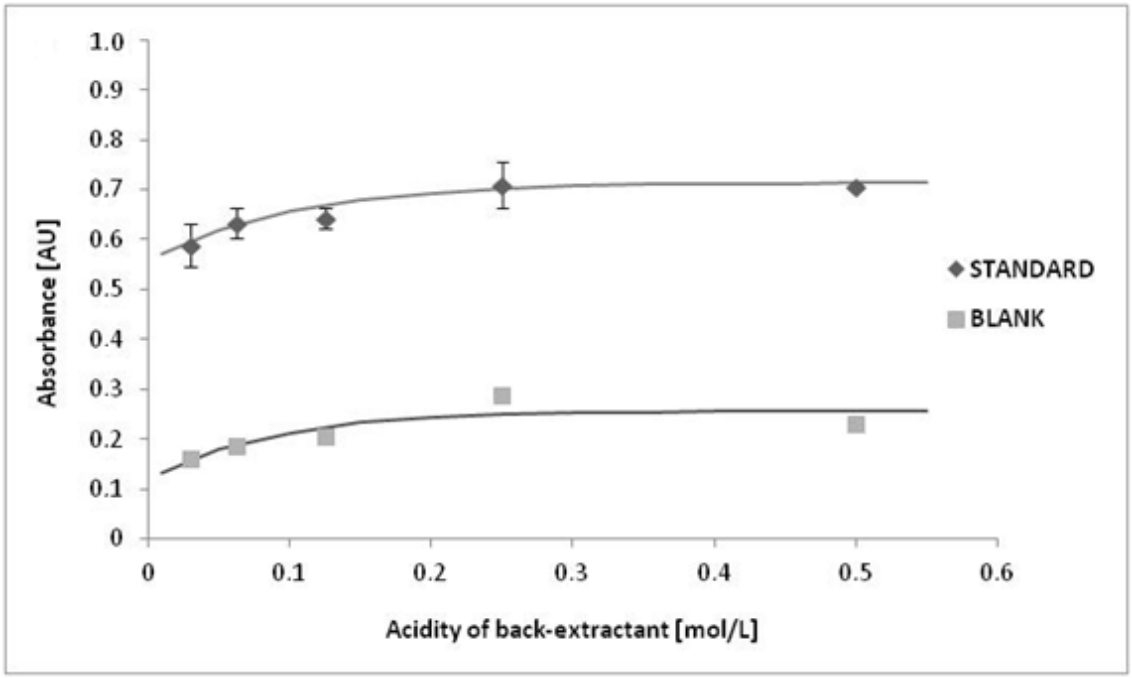

Figure 21 *Absorbance measured at 508 nm in standard and blank solution after extraction and back-extraction using a solution of hydrochloric acid of following concentrations: 0.03, 0.06, 0.125, 0.25, and 0.5 mol/L.*

The graph in **Figure 21** displays the values of absorbance measured in the aqueous phase at 508 nm, which corresponds with the concentration of the back-extracted complex. At first, the measured signal was increasing but after the concentration of hydrochloric acid reached 0.25 mol/L, the signal reached equilibrium. However, as also the blank signal increased, the acidity of back-extractant was set to 0.125 mol/L.

5.3.3 Study of standard stability

Extensive changes in stability of standard solutions could significantly alter the results. Therefore the standard stability was studied to find out whether the sample solution needs to be prepared anew before each measurement or whether it is sufficient to prepare it once and use it over longer period of time.

Three experiments were made performing in-syringe extraction and back-extraction under the same conditions as in the previous study. Three samples were tested, each prepared and stored under distinctive conditions. All samples were of a concentration 6 mg/L. Sample A and B were both prepared the previous day, approximately 24 hours before the measurement. Sample A was stored at the room temperature and sample B in a fridge at the temperature 4 °C. The third sample C was freshly prepared before the measurement.

Table 4 *Overview of tested samples and measured absorbance*

Sample	Conditions	Measured absorbance [AU]	Percentage of sample C [%]
A	prepared previous day, stored at the room temperature	0.769 ± 0.036	108.8
B	prepared previous day, stored at the temperature 4 °C	0.727 ± 0.073	102.8
C	prepared fresh the same day	0.707 ± 0.044	100.0

As can be seen in **Table 4**, chlorhexidine concentration did not change significantly over the first 24 hours even if not protected from light and stored at ambient temperature. This test, however, does not say anything about a stability change over a longer period of time, but good stability can be assumed.

5.4 Performance testing/overview

After optimisation of all parameters, a calibration was done with chlorhexidine standards in a range of 0 to 3 mg/L to evaluate the method performance such as linearity and typical repeatability etc. Standard solutions were prepared as described in chapter 4.1. Optimised parameters used for the calibration are listed in **Table 5**.

Table 5 *Optimised parameters used for calibration*

Tested parameter	Optimal value
Volume of extraction solvent (1-octanol)	250 µL
Concentration of MO reagent	250 mg/L
Volume of MO reagent	50 µL
Extraction time	30 s
Separation time	40 s
Concentration of back-extractant (HCl)	0.125 mol/L
Volume of back-extractant (HCl)	500 µL
Back-extraction time	30 s
Stirring rate	1470 rpm

First, the blank solution (1 mL of water) was measured six times. Sequentially, six standard solutions in volume 250 µL were tested, each in four repetitions. As in the previous experiments, standard solutions were further diluted in-syringe with 750 µL of water. The values are shown in **Figure 22**. It can be seen that the middle standard showed a too high value while linearity was given up to 3 mg/L. The calibration curve followed the function:

$$Signal\ [AU] = 0.456\ AU\ .\ Conc.[mg/L] + 0.292\ AU.$$

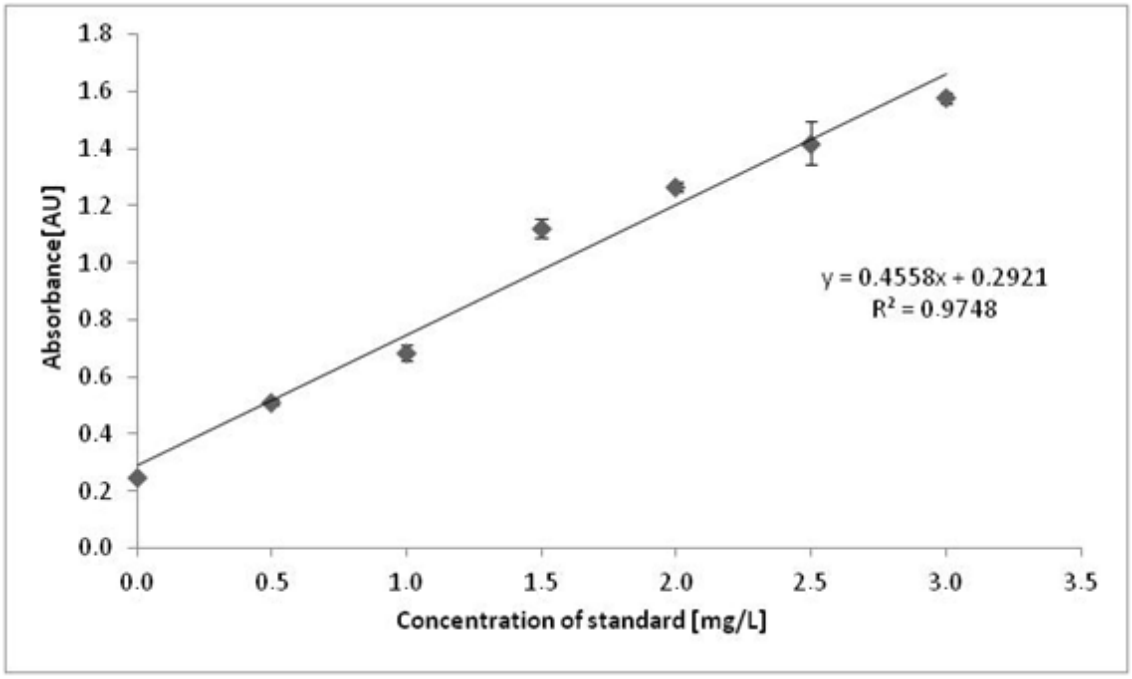

Figure 22 *Calibration curve – Absorbance measured at 508 nm in dependency of concentration of standard. Chlorhexidine standards were prepared individually outside of the system.*

The repeatability of the method was studied by six blank measurements and the standard deviation was calculated to be 0.004. The LOD and LOQ were determined as the standard deviation of blank, evaluated from six times measurement, multiplied by factor 3 and 10 to be 0.03 mg/L and 0.09 mg/L, respectively.

5.5 Application to real samples

At the end, the method was tried out on the analysis of chlorhexidine in real mouthwash samples. Five mouthwash samples listed in chapter 4.1 were tested. Two of the samples indicated chlorhexidine as component while the three others did not. Each sample was diluted 1000 times outside of the system and then four times in-syringe to enable the measurement of a signal within the calibration range. The measurement parameters were set to the same values as in the previous experiment (**Table 5**). Subsequently, the samples were spiked by the addition of chlorhexidine standard to a final chorhexidine concentration of 0.6 ppm (which corresponds to approx. 1.07 ppm of chlorhexidine digluconate) and measured under the same conditions. Each sample was measured four times and the average of measured absorbance values is listed in **Table 6**, together with calculation of chlorhexidine concentration of each sample, standard deviations and the recovery. Concentrations of chlorhexidine digluconate in the samples were calculated as chlorhexidine, assuming that 1 mg/L chlorhexidine standard corresponds to an equivalent of 1.78 mg/L chlorhexidine digluconate.

In general, the quantity of chlorhexidine in the samples measured with this method was far lower than it was declared. From the recovery values it appears that there are interferences by other compounds contained in the samples even considering the high dilution factor. This is also indicated by the real measurement data recording for 0.5 mg/L chlorhexidine standard and the sample Parodontax shown in **Figure 23** and **24**. It can be seen that except for the first repetition value, which was not included in the calculations, the standard signals are stable and reproducible while noise appears on the sample signals. The difference of the first signal is likely due to the cleaning of the sample channel before each repeated measurement and remains of the sample in the syringe due to insufficient cleaning.

Table 6 *An overview of the measured and calculated values for mouthwash samples*

Mouthwash sample	Content of chlorhexidine digluconate [% w/V]	Content of chlorhexidine in the diluted sample [mg/L]	Sample diluted 1000x		Sample diluted 1000x and spiked with 0.6 ppm		Recovery [%]
			Measured absorbance [AU]	Calculated concentration [mg/L]	Measured absorbance [AU]	Calculated concentration [mg/L]	
Chlorhexil	0.12	0.676	0.380±0.061	0.193	0.499±0.087	0.454	43.5
Rebi-Dental Mouthwash	-	-	0.183±0.002	(-0.239) < LOD	0.393±0.003	0.221	76.8
Colgate Max White	-	-	0.165±0.009	(-0.279) < LOD	0.188±0.012	(-0.228) < LOD	8.4
Listerine	-	-	0.166±0.040	(-0.277) < LOD	0.398±0.009	0.232	84.8
Parodontax	0.06	0.338	0.287±0.026	(-0.011) < LOD	0.468±0.031	0.386	66.2

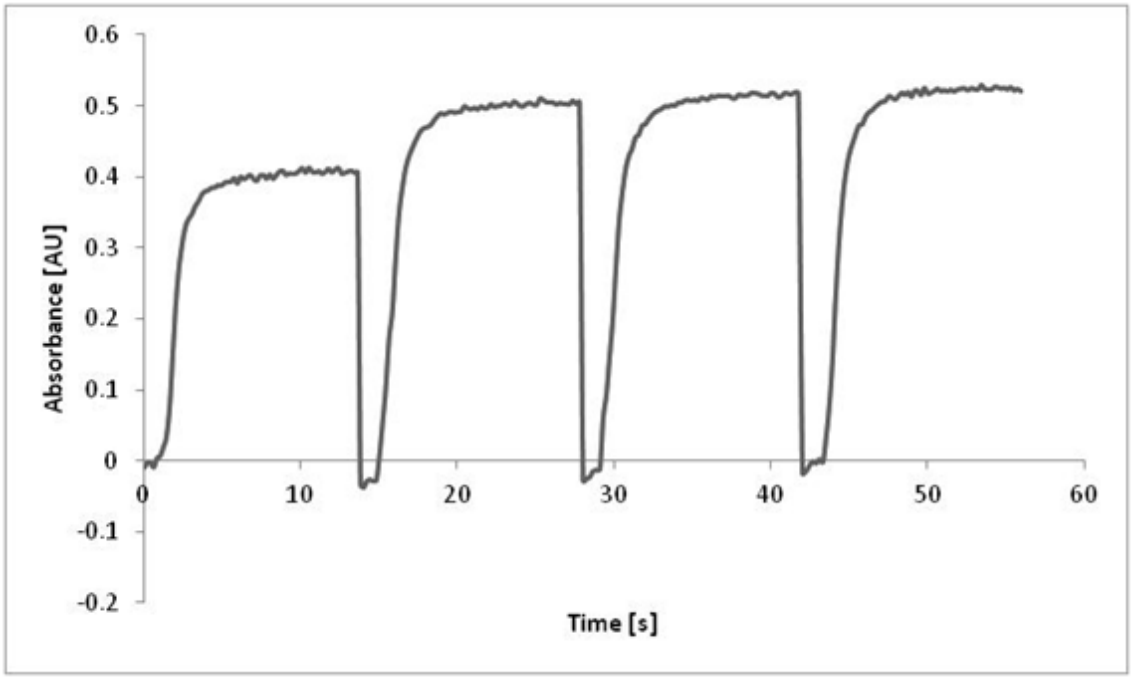

Figure 23 *Real measurement data for 0.5 mg/L chlorhexidine standard at 508 nm (first value not included in the calculation)*

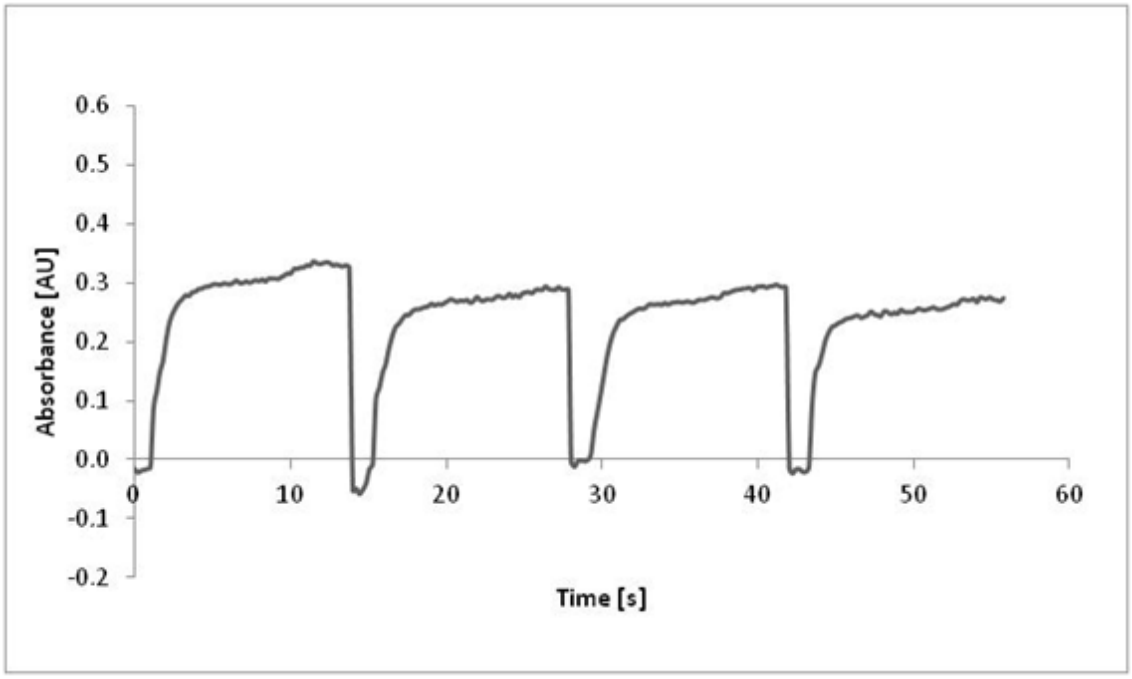

Figure 24 *Real measurement data for sample Parodontax (containing 0.06 % w/V of chlorhexidine digluconate) at 508 nm*

From the sample components, indicated on each product and listed in **Table 3**, there are several interferences which can be considered. The alcohol content of the sample is unlikely to interfere significantly as the sample was diluted highly. Colouring substances (e.g. CI 42051) are negatively charged and could be extracted along the aimed ion-complex of chlorhexidine with MO. However, as significant changes in the colour of the extracts were not observed and these substances are used in very low concentrations, their interference is unlikely. The effect could be compensated by spectrum analysis anyway.

Components which are considered possible interferences are listed in **Table 7**. The interferences could originate from other amines able to form extractable ion pairs with MO, which would have positive effect on the results. However, as low recovery was found, the most likely interferences would be other organic anions (e.g. sodium benzoate, sodium saccharin, eugenol) able to form ion pairs with the analyte either not extractable or not coloured. Here, studying these substances for their interference level and study the effect of buffer to achieve the highest selectivity for the real samples would be advisable. In addition, the change to another dye or solvent, unable to extract these different ion-pair complexes could be of interest.

Table 7 *Possible interferences in different brands of mouthwash*

Mouthwash brand name	Possible interferences
Chlorhexil	Alantoin
Rebi-Dental Mouthwash	polysorbate 20, sodium benzoate, sodium saccharin
Colgate Max White	polysorbate 20, PVM/MA copolymer, sodium saccharin, CI 42051
Listerine	benzoic acid, sodium saccharin, methyl salicylate, sodium benzoate
Parodontax	sodium saccharin, eugenol

An important effect could also be that the gluconate anion itself, present only in the samples, changes the extraction efficiency. This could be possible compensated by using either this substance as calibration standard or evaluating the effect of gluconate on the extraction for which the time planned for the thesis was not sufficient. Similarly,

the analyte recovery was between 44 % and 85 % while for one sample (Colgate Max White) only 8.4 %. Here, the PVM/MA copolymer, the only component which is not present in the other samples could have played an important role likely by encapsulation of the hydrophobic MO-chlorhexidine ion pair. It should be pointed out that former works omitting separation techniques, i.e. which were based on similar measurement principle aimed the determination of chlorhexidine in saliva or water samples and not in the presence probably high concentrations of auxiliary substances [9,10].

As the values in **Table 6** show, there is a significant difference between the samples which contain chlorhexidine and those which do not. This shows that the method is sensitive to the analyte but interfered by different sample components, which would to be tested one-by-one, which would have surpassed the aim of this thesis being the study of applicability of LIS for extraction and back-extraction of chlorhexidine from mouthwash.

It appears that the blank for the sample is far lower than for calibration standards (water), explaining the negative concentration values found for most samples.

5.6 Final discussion and outlook

It is clear that further studies are required to find out about the interference observed with the real sample matrices. In particular, it is believed that these interferences are related to organic anions forming equally strong yet water soluble ion-pair complexes with the analyte, by this inhibiting its extraction as ion pair with MO. A possibility would be here to re-evaluate the content of MO in the extraction mixture or replace it with a different anionic dye such as thymol blue. The evaluation of buffering pH would be also of interest but with e.g. a mixed real sample in comparison with a water standard. In particular, there should be a comparison between chlorhexidine and chlorhexidine digluconate as analytical standard. On the other hand, the content of alcohol, pH, or colouring of the samples should have only a minor effect due to the high dilution factor which was required to reach the linear working range of the samples. It will be also of interest for future work to test in-syringe sample dilution as the technique is potentially well-suited for preparing homogenous mixtures yet had not been tested for dilution factors in the range of 1:1000.

6. Conclusion

In conclusion, a method based on dispersive liquid-liquid micro-extraction automated by Lab-In-Syringe technique was described and its parameters optimised for the determination of chlorhexidine in commercial mouthwash. As an organic extraction solvent 250 μL of pure 1-octanol was chosen and 50 μL of 250 mg/L methyl orange was used as a reagent to form an extractable ion-pair complex with chlorhexidine.

In the first part of this thesis, the parameters of extraction itself were studied. The intention of the optimisation was to improve efficiency while saving time and reducing waste production. Extraction time and stirring rate were evaluated together as these parameters were supposed to affect each other. It was found that higher efficiency was given using stirring rate 1470 rpm than 930 rpm. Extraction time was set to 30 s as further increasing of time had not led to improvement.

In the second part, back-extraction into 500 μL of 0.125 mg/L hydrochloric acid was added as a next step to approve selectivity. Back-extraction time was set to 30 s. Times for phase separation for both, extraction and back-extraction were set to 40 s which was based on the observation of phase separation.

The concentration of chlorhexidine standard solution which was used for optimisation was first set to 20 mg/L. Throughout the experiment, it was found that the measured absorbance signal for this concentration was too high which affects the accuracy of the measurements. The concentration was therefore reduced to 6 mg/L and the standard was further diluted in-syringe four times. To yield a similar concentration of chlorhexidine in tested samples, each sample was diluted one thousand times outside of the system and similarly as the standard solutions, four times in-syringe.

For method evaluation, a calibration was done with standards of concentrations up to 3 mg/L. When applied to real samples it was found that the method measured lower concentrations of chlorhexidine in the samples than was stated. The recovery was different ranging from 8.4 to 84.8 %. There is a possibility that this could be caused by interference of other components of mouthwash. In addition, chlorhexidine is a di-cationic compound and it is contained in mouthwash in a form of salt chlorhexidine digluconate. Both of these features could also affect the recovery.

7. Shrnutí

V práci byla popsána metoda založena na disperzní kapalinové mikroextrakci automatizované technikou Lab-In-Syringe. Všechny parametry byly optimalizovány pro stanovení obsahu chlorhexidinu v ústní vodě. Jako organické extrakční rozpouštědlo bylo zvoleno 250 µL čistého oktanolu a 50 µL činidla methyloranž bylo použito pro vytvoření extrahovatelného iontového páru s chlorhexidinem.

V první části práce byly studovány parametry pouze pro samotnou extrakci. Cílem optimalizace bylo zlepšit účinnost a zároveň snížit produkci odpadu a redukovat čas potřebný pro měření. Extrakční čas a rychlost míchání byly sledovány souběžně, z důvodu předpokládaného vzájemného ovlivnění. Při rychlosti míchání 1470 ot/min byla zjištěna vyšší účinnost než při rychlosti 930 ot/min. Extrakční čas byl nastaven na 30 s, jelikož jeho další navyšování nevedlo ke zvýšení absorbance.

Ve druhé části byla k extrakci jako další krok přidána zpětná extrakce do 500 µL kyseliny chlorovodíkové o koncentraci 0,125 mg/L s cílem zlepšit selektivitu metody. Čas pro zpětnou extrakci byl nastaven na 30 s. Časy pro separaci fází pro extrakci i zpětnou extrakci byly nastaveny na 40 s. Tento čas byl vybrán na základě pozorování fázové separace.

Koncentrace standardního roztoku chlorhexidinu, který byl použit k optimalizaci, byla 20 mg/L. Během měření bylo zjištěno, že naměřená absorbance pro tuto koncentraci byla příliš vysoká, což snižuje přesnost měření. Proto pro další měření byla koncentrace snížena na 6 mg/L a každý standard byl navíc čtyřikrát naředěn uvnitř pístového čerpadla. K dosažení podobné koncentrace chlorhexidinu v testovaných vzorcích byl každý vzorek tisíckrát zředěn a podobně jako standardy, také navíc čtyřikrát naředěn uvnitř pístového čerpadla.

Pro vyhodnocení metody byla provedena kalibrace. Testováním skutečných vzorků bylo zjištěno, že ve vzorcích byly naměřeny nižší koncentrace chlorhexidinu, než bylo deklarováno. Výtěžnost byla různá a pohybovala se v rozmezí od 8,4 do 84,8 %. Nízká výtěžnost mohla být způsobena interferencí ostatních složek ústní vody. Skutečnosti, že chlorhexidin je dvojmocný kation a v ústní vodě se vyskytuje ve formě diglukonátu, mohly také výrazně ovlivnit výtěžnost měření.

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Appendix

Appendix 1 *Method used in experiments without back-extraction*

'Define HardwareSettings
Hardware Settings Wavelength 1 (nm) 420 *'Yellow form in organic phase*
Hardware Settings Wavelength 2 (nm) 465 *'Yellow form in water*
Hardware Settings Wavelength 3 (nm) 508 *'Red form in water*
Hardware Settings Wavelength 4 (nm) 585 *'Reference*

Hardware Settings Detectors to Average 5
Hardware Settings Samples to Average 5
Hardware Settings Integration Time (msec) 10
Hardware Settings Scan Rate (Hz) 20
Hardware Settings Use Wavelength 4 as Reference

'Define variables
Variable Define New MeasureSample
Variable Define New VolCleaning
Variable Define New OptionTube
Variable Define New OptionBlank

/
Positions of the selection valve:
1. Detection cell, 2. Extraction solvent, 3. Buffer, 4. MO, 5. Standard/Sample, 6. Isopropanol,7. Water, 9. Air/Waste
**/*

OptionTube = 0
OptionBlank = 0

Syringe Pump2 Command (?) J0R
'Close syringe piston channel
Valve port 2

'Empty syringe
Syringe Pump2 Command (?) O9R
Syringe Pump2 Flowrate (microliter/sec) 250
Syringe Pump2 Empty
Syringe Pump2 Delay Until Done

'Cleaning of tube - *optional*
If OptionTube = 1
Syringe Pump2 Command (?) O2R
Syringe Pump2 Aspirate (microliter) 300
Syringe Pump2 Delay Until Done

Syringe Pump2 Command (?) O9R
Syringe Pump2 Empty
Syringe Pump2 Delay Until Done
End If

If OptionBlank = 1
'Cleaning of syringe with isopropanol
VolCleaning = 500
Insert File C:\Users\Obsluha\Documents\FIA3000 data měření\P Rehakova\ProcedureCleanSyringeWithAuxSolvent.fia

'Cleaning of syringe with water and measurement of blank - *optional*
Loop Start (#) 1
Syringe Pump2 Command (?) O9R
Syringe Pump2 Aspirate (microliter) 500
Syringe Pump2 Delay Until Done
Syringe Pump2 Command (?) J7R
Syringe Pump2 Command (?) O7R
Syringe Pump2 Aspirate (microliter) 1000
Syringe Pump2 Delay Until Done
Delay (sec) 3
Syringe Pump2 Command (?) J0R
Syringe Pump2 Command (?) O1R
Syringe Pump2 Flowrate (microliter/sec) 100
Syringe Pump2 Empty
Delay (sec) 5
Spectrometer Reference Scan
Syringe Pump2 Flowrate (microliter/sec) 200
Syringe Pump2 Delay Until Done
Loop End
End If

Loop Start (#) 1 *'Different conditions*
MeasureSample = 0
Loop Start (#) 2 *'Different solutions (water/standard)*
Loop Start (#) 3 *'Repetitions of measurement*

'Cleaning of syringe with isopropanol
VolCleaning = 500
Insert File C:\Users\Obsluha\Documents\FIA3000 data měření\P Rehakova\ProcedureCleanSyringeWithAuxSolvent.fia
'Cleaning of syringe with water
VolCleaning = 1000
Insert File C:\Users\Obsluha\Documents\FIA3000 data měření\P Rehakova\ProcedureCleanSyringeWithWater.fia
Insert File C:\Users\Obsluha\Documents\FIA3000 data měření\P Rehakova\ProcedureCleanSyringeWithWater.fia

'Aspiration of solutions
Syringe Pump2 Flowrate (microliter/sec) 100
Syringe Pump2 Pump Command (?) O3R *'Buffer*
'Syringe Pump2 Pump Aspirate (microliter) 200
Syringe Pump2 Delay Until Done
Delay (sec) 1

Syringe Pump2 Pump Command (?) O2R *' Extraction Solvent*
Syringe Pump2 Pump Aspirate (microliter) 250
Syringe Pump2 Delay Until Done
Delay (sec) 2

Syringe Pump2 Command (?) O4R *'MO*
Syringe Pump2 Aspirate (microliter) 50
Syringe Pump2 Delay Until Done
Delay (sec) 1

Syringe Pump2 Command (?) [J7R *'Activate stirring*
Syringe Pump2 Command (?) O7R *'Water*
Syringe Pump2 Delay Until Done
If MeasureSample = 1
Syringe Pump2 Command (?) O5R *'Standard*
End If

Syringe Pump2 Flowrate (microliter/sec) 250
Syringe Pump2 Aspirate (microliter) 250
Syringe Pump2 Delay Until Done
Delay (sec) 1

Syringe Pump2 Command (?) O7R *'Water*
Syringe Pump2 Aspirate (microliter) 750
Syringe Pump2 Delay Until Done
Delay (sec) 1

'Extraction and phase separation
Delay (sec) 30
Syringe Pump2 Command (?) J0R *'Deactivate stirring*
Delay (sec) 40

'Measurement
Syringe Pump2 Pump Command (?) O1R
Syringe Pump2 Flowrate (microliter/sec) 45
Syringe Pump2 Dispense (microliter) 630
Spectrometer Absorbance Scanning
Delay (sec) 4
Analyte New Sample
Analyte Name S
Delay (sec) 10
Spectrometer Stop Scanning
Syringe Pump2 Pump Command (?) O9R
Syringe Pump2 Flowrate (microliter/sec) 250
Syringe Pump2 Empty
Syringe Pump2 Delay Until Done

Loop End
MeasureSample = 1
Loop End
Loop End

Appendix 2 *Method used in experiments including back-extraction*

'Define HardwareSettings
Hardware Settings Wavelength 1 (nm) 420 *'Yellow form in organic phase*
Hardware Settings Wavelength 2 (nm) 465 *'Yellow form in water*
Hardware Settings Wavelength 3 (nm) 508 *'Red form in water*
Hardware Settings Wavelength 4 (nm) 585 *'Reference*

Hardware Settings Detectors to Average 5
Hardware Settings Samples to Average 5
Hardware Settings Integration Time (msec) 10
Hardware Settings Scan Rate (Hz) 20
Hardware Settings Use Wavelength 4 as Reference

'Define variables
Variable Define New MeasureSample
Variable Define New VolCleaning
Variable Define New OptionTube
Variable Define New OptionBlank

/
Positions of the selection valve:
1. Detection cell, 2. Extraction solvent, 3. HCl, 4. MO, 5. Standard/Sample, 6. Isopropanol,7. Water, 9. Air/Waste
**/*

OptionTube = 0
OptionBlank = 0

Syringe Pump2 Command (?) J0R

'Close syringe piston channel
Valve port 2

'Empty syringe
Syringe Pump2 Command (?) O9R
Syringe Pump2 Flowrate (microliter/sec) 250
Syringe Pump2 Empty
Syringe Pump2 Delay Until Done

'Cleaning of tube - *optional*
If OptionTube = 1
Syringe Pump2 Command (?) O2R
Syringe Pump2 Aspirate (microliter) 300
Syringe Pump2 Delay Until Done

Syringe Pump2 Command (?) O9R
Syringe Pump2 Empty
Syringe Pump2 Delay Until Done
End If

If OptionBlank = 1
'Cleaning of syringe with isopropanol
VolCleaning = 500
Insert File C:\Users\Obsluha\Documents\FIA3000 data měření\P Rehakova\ProcedureCleanSyringeWithAuxSolvent.fia

'Cleaning of syringe with water and measurement of blank *- optional*
Loop Start (#) 1
Syringe Pump2 Command (?) O9R
Syringe Pump2 Aspirate (microliter) 500
Syringe Pump2 Delay Until Done
Syringe Pump2 Command (?) J7R
Syringe Pump2 Command (?) O7R
Syringe Pump2 Aspirate (microliter) 1000
Syringe Pump2 Delay Until Done
Delay (sec) 3
Syringe Pump2 Command (?) J0R
Syringe Pump2 Command (?) O1R
Syringe Pump2 Flowrate (microliter/sec) 100
Syringe Pump2 Empty
Delay (sec) 5
Spectrometer Reference Scan
Syringe Pump2 Flowrate (microliter/sec) 200
Syringe Pump2 Delay Until Done
Loop End
End If

Loop Start (#) 1 *'Different conditions*
MeasureSample = 0
Loop Start (#) 2 *'Different solutions (water/sample)*
Loop Start (#) 3 *'Repetitions of measurement*

'Cleaning of syringe with isopropanol
VolCleaning = 500
Insert File C:\Users\Obsluha\Documents\FIA3000 data měřeni\P Rehakova\ProcedureCleanSyringeWithAuxSolvent.fia

'Cleaning of syringe with water
VolCleaning = 1000
Insert File C:\Users\Obsluha\Documents\FIA3000 data měřeni\P Rehakova\ProcedureCleanSyringeWithWater.fia
Insert File C:\Users\Obsluha\Documents\FIA3000 data měřeni\P Rehakova\ProcedureCleanSyringeWithWater.fia

'Aspiration of solutions
Syringe Pump2 Pump Command (?) O2R *'Extraction solvent*
Syringe Pump2 Pump Aspirate (microliter) 250
Syringe Pump2 Delay Until Done
Delay (sec) 2

Syringe Pump2 Command (?) O4R *'MO*
Syringe Pump2 Aspirate (microliter) 50
Syringe Pump2 Delay Until Done
Delay (sec) 1

Syringe Pump2 Command (?) J7R *'Activate stirring*
Syringe Pump2 Command (?) O7R *'Water*
Syringe Pump2 Delay Until Done
If MeasureSample = 1
Syringe Pump2 Command (?) O5R *'Standard*
End If

Syringe Pump2 Flowrate (microliter/sec) 250
Syringe Pump2 Aspirate (microliter) 250
Syringe Pump2 Delay Until Done
Delay (sec) 1

Syringe Pump2 Command (?) O7R *'Water*
Syringe Pump2 Aspirate (microliter) 750
Syringe Pump2 Delay Until Done
Delay (sec) 1

'Extraction and phase separation
Delay (sec) 30
Syringe Pump2 Command (?) J0R *'Deactivate stirring*
Delay (sec) 40

'Water discharged to the waste
Syringe Pump2 Command (?) O9R *'Water to waste*
Syringe Pump2 Dispense (microliter) 950
Syringe Pump2 Delay Until Done
Delay (sec) 1

'Washing of the sample
Syringe Pump2 Command (?) O7R *'Water*
Syringe Pump2 Aspirate (microliter) 1500
Syringe Pump2 Delay Until Done
Syringe Pump2 Command (?) J4R
Delay (sec) 1
Syringe Pump2 Command (?) J0R
Syringe Pump2 Command (?) O9R *'Water to waste*
Syringe Pump2 Dispense (microliter) 1500
Syringe Pump2 Delay Until Done
Delay (sec) 1

'Aspiration of HCl
Syringe Pump2 Aspirate (microliter) 150
Syringe Pump2 Command (?) O3R *'HCl*
Syringe Pump2 Aspirate (microliter) 500
Syringe Pump2 Delay Until Done
Delay (sec) 1

'Back-extraction and phase separation
Syringe Pump2 Command (?) J7R
Delay (sec) 30
Syringe Pump2 Command (?) J0R
Delay (sec) 40

'Measurement
Syringe Pump2 Pump Command (?) O1R
Syringe Pump2 Flowrate (microliter/sec) 45
Syringe Pump2 Dispense (microliter) 630
Spectrometer Absorbance Scanning
Delay (sec) 4
Analyte New Sample
Analyte Name S
Delay (sec) 10
Spectrometer Stop Scanning
Syringe Pump2 Pump Command (?) O9R
Syringe Pump2 Flowrate (microliter/sec) 250
Syringe Pump2 Empty
Syringe Pump2 Delay Until Done

Loop End
MeasureSample = 1
Loop End
Loop End