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Direktor: Univ.-Prof. Dr. med. Daniel Teupser

Configuration, optimization and evaluation of a novel instrumental platform for automated SPE-LC-MS/MS analysis of drugs in whole blood

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For my dearest family and friends

„… All things work together for good to them that love God …“

(Romans)

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Table of Contents

Abbreviations

AC	Affinity Chromatography
ACN	Acetonitrile
API	Atmospheric Pressure Ionization
APPI	Atmospheric Pressure Photoionization
bar	Unit of Pressure: 1 bar = $1*10^{-5}$ Pa = 1 N/m^2
Cal	Calibration
CDB	Cell Disintegrated Blood
cf.	Compare
CI	Confidence Interval
CID	Collision-Induced-Dissociation
CRM	Charge Residue Model
CyA	Cyclosporine A
CyD	Cyclosporine D
DBE	Dried Blood Extract
DBS	Dried Blood Spot
DMSO	Dimethyl Sulfoxide
dp	Particle Diameter
EDTA	Ethylene Diamine Tetraacetic Acid
eg	Exempli gratia
ESI	Electrospray Ionisation
EtOH	Ethanol
FDA	Federal Drug Administration of the United States
g	Gramm
Hct	Hematocrit
HLB	Hydrophilic Lipophilic Balance
HPLC	High Performance Liquid Chromatography

H_2O	Water
ICH	**I**nternational **C**onference on **H**armonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
ID	Inner Diameter
i.e.	Id est
IEM	Ion Evaporation Mechanism
IEX	Ion Exchange
IS	Internal Standard
LC	Liquid Chromatography
LOD	Limit of Detection
LLOQ	Lower Limit of Quantification
M	Molarity (Unit for Molar Concentration: 1 M = 1 mol/L)
mAU	Milli Absorption Unit
MeOH	Methanol
mg	Milli Gramm
min	Minute
mL	Milli Liter
mm	Milli Meter
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometer
m/z	Mass-to-charge ratio
µL	Micro Liter
µm	Micro Meter
NaCl	Sodium Chloride
nm	Nano Meter (Unit of Length: 10^{-9} m)
PCI	Post-Column Infusion
PFP	Pentafluorophenyl
RAM	Restricted Access Material
RP	Reversed Phase

rpm	Rotations per Minute
RSD	Relative Standard Deviation
SEC	Size Exclusion Chromatography
S/N	Signal-to-Noise Ratio
SPE	Solid Phase Extraction
SRM	Single/selected Reaction Monitoring
SV	Switching Valve
TAS	Total Analysis System
TDM	Therapeutic Drug Monitoring
TFC	Turbulent Flow Chromatography
t_A	Breakthrough time of Analyte
t_M	Elution time of Matrix
t_T	Transfer time of Analyte
UV	Ultra Violet
Vis	Visible
WB	Whole Blood

1 Introduction

1.1 Preparation of whole blood for clinical-chemical LC-MS/MS analysis: State-of-the-art

The combination of High Performance Liquid Chromatography with selective and sensitive Tandem Mass Spectrometry (HPLC-MS/MS) has become the technique of choice for the analysis of low-molecular weight compounds in biological fluids in clinical-chemical and pharmaceutical laboratories. However, complex body fluids, such as whole blood, have to be pretreated prior to LC-MS/MS analysis in order to deplete the protein matrix and interfering sample components.

Sample preparation often represents the most laborious and time-consuming step in an analytical protocol. It significantly affects the quality, throughput and costs of the analysis. Thus, an appropriate sample preparation is the key factor for an accurate, reliable and efficient analytical procedure.

Pretreatment of whole blood involves at least two steps. First, the protein matrix has to be depleted, if not removed completely. Second, the resulting preprocessed blood sample has to be further cleaned by Solid Phase Extraction (SPE).

There are different ways to deplete the protein matrix of an anticoagulated blood sample [1].

Membrane filtration of anticoagulated whole blood (cf. Figure 1, A): Depending on the pore diameter, i.e. the molecular weight cut-off, either the blood cells or a fraction of plasma proteins is retained by the filter and removed. The remaining plasma proteins in the filtrate are depleted by precipitation followed by centrifugation.

Hemolysis of anticoagulated whole blood (cf. Figure 1, A): After dilution of a blood sample with distilled water (blood/water, 1/20, v/v) or the addition of a lysis-buffer, the erythrocytes depleted from hemoglobin, i.e. the “ghosts”, are removed by centrifugation. The protein matrix of the resulting hemolysate is depleted by precipitation followed by centrifugation.

Preparation of secondary specimens, i.e. plasma or serum (cf. Figure 1, A): After centrifugation of an anticoagulated / clotted sample of whole blood, the resulting plasma / serum aliquots are precipitated and centrifuged again.

Precipitation of whole blood (cf. Figure 1, A): The protein matrix is denatured by addition of a precipitating agent and removed by centrifugation.

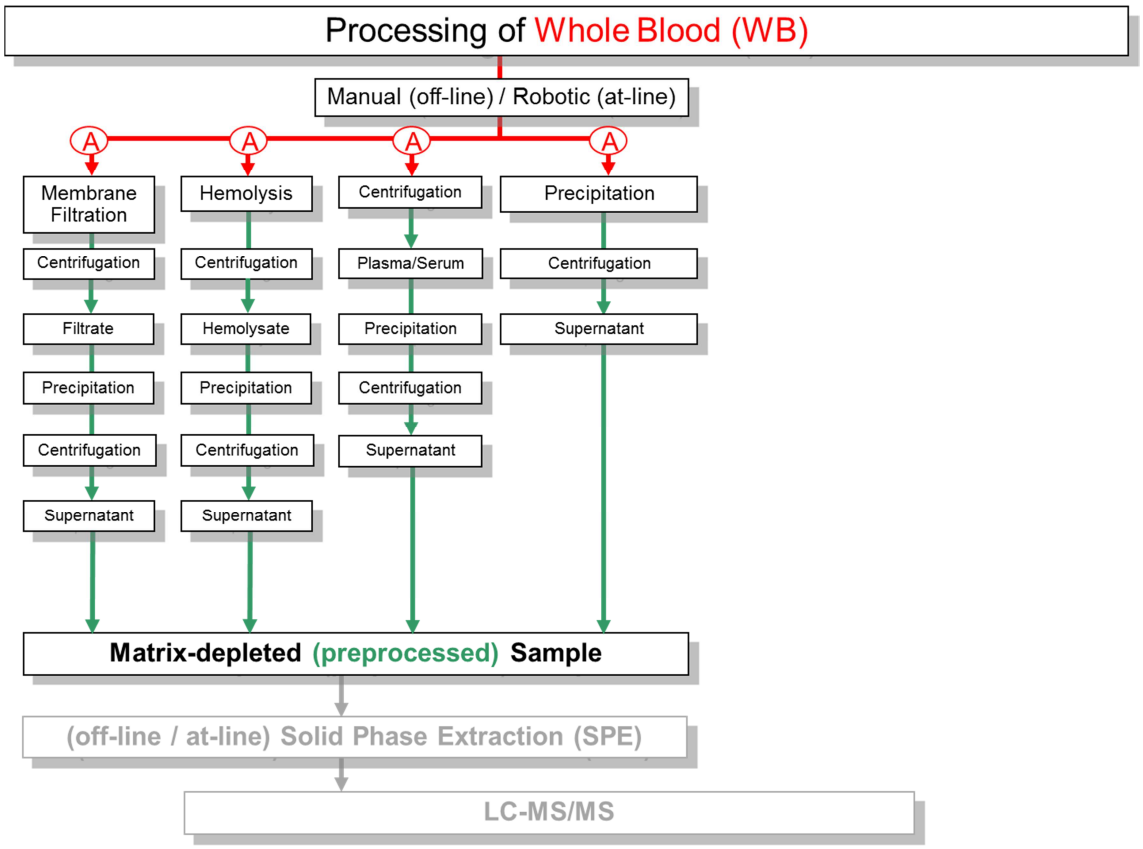


Figure 1: Operational procedures for preparation of whole blood: Protein precipitation.

In all four procedures listed in Figure 1, precipitation of the protein matrix takes place. Protein precipitation is a simple and commonly used technique to deplete the protein matrix of a body fluid and to disrupt protein-drug binding [2]. The relative efficiency of different precipitants has been extensively investigated by Blanchard [3]. For whole blood, a mixture of MeOH / $ZnSO_4$ 0.4 M (80/20, v/v) is often used [4-7], as this mixture results in a tight pellet after centrifugation. An appropriate Internal Standard (IS) is preferably dissolved in and added with the precipitating agent. The technique of protein precipitation has some essential disadvantages. The potential co-precipitation of analyte(s) impairs the recovery, and the incomplete removal of matrix

proteins leads to matrix effects during ESI-MS/MS detection [8,9].

Another methodological approach, which is currently the most intensively investigated technique in the pharmaceutical industry, is the Dried Blood Spot (DBS, cf. Figure 2, B) technique.

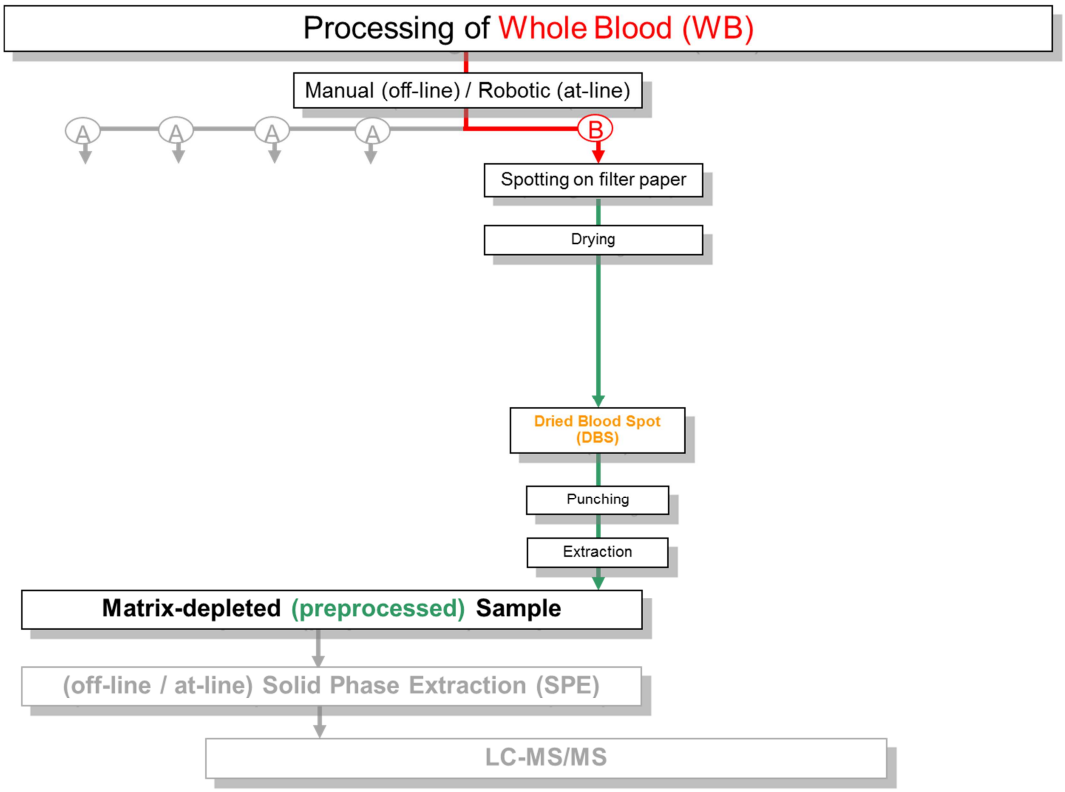


Figure 2: Operational procedures for preparation of whole blood: Dried Blood Spot (DBS).

A defined amount, e.g. 30 µL of native whole blood is carefully pipetted and spotted onto a special marked filter paper/card without touching the card with the filter tip. After drying at room temperature for at least three hours, such a Dried Blood Spot (DBS) is punched, if necessary repeatedly. The target analyte(s) are extracted from the corresponding disk(s) by vortexing in the presence of an organic solvent for 60 min. Finally, only an aliquot of the supernatant is subjected to further clean-up. Thus the sample is diluted before the final analysis. Furthermore, the addition of the Internal Standard (IS) takes place late, at the extraction step (Figure 3).

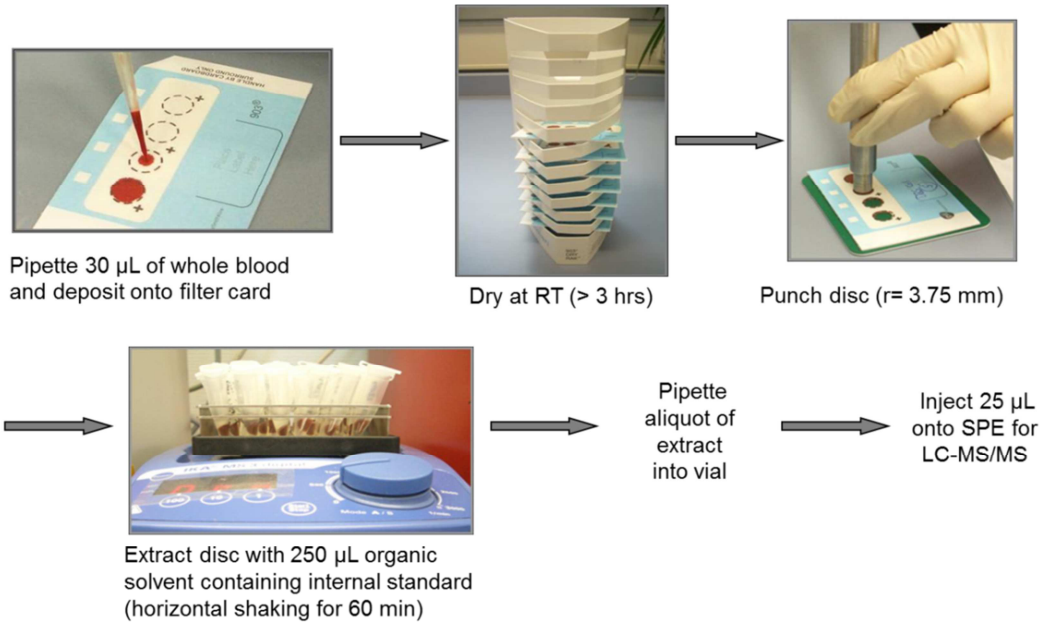


Figure 3: Preparation of Dried Blood Spot (DBS).

Recently, an alternative procedure with respect to DBS, the preparation of Dried Blood Extract (DBE), has been developed in the Laboratory of BioSeparation, Institute of Laboratory Medicine, Medical Center of the University of Munich (cf. Figure 4, C and Figure 5) [10]. A sample of whole blood is added to a precipitating agent (organic solvent with dissolved Internal Standard), contained in a special filtration tube which prevents dripping of the agent. Upon low-speed centrifugation, the resulting filtrate is directly collected into a HPLC vial and simultaneously evaporated to dryness by applying a vacuum. After removing the filtration tube, the DBE can be shipped in a closed HPLC vial and then simply reconstituted by addition of a solvent prior to SPE-LC-MS/MS analysis.

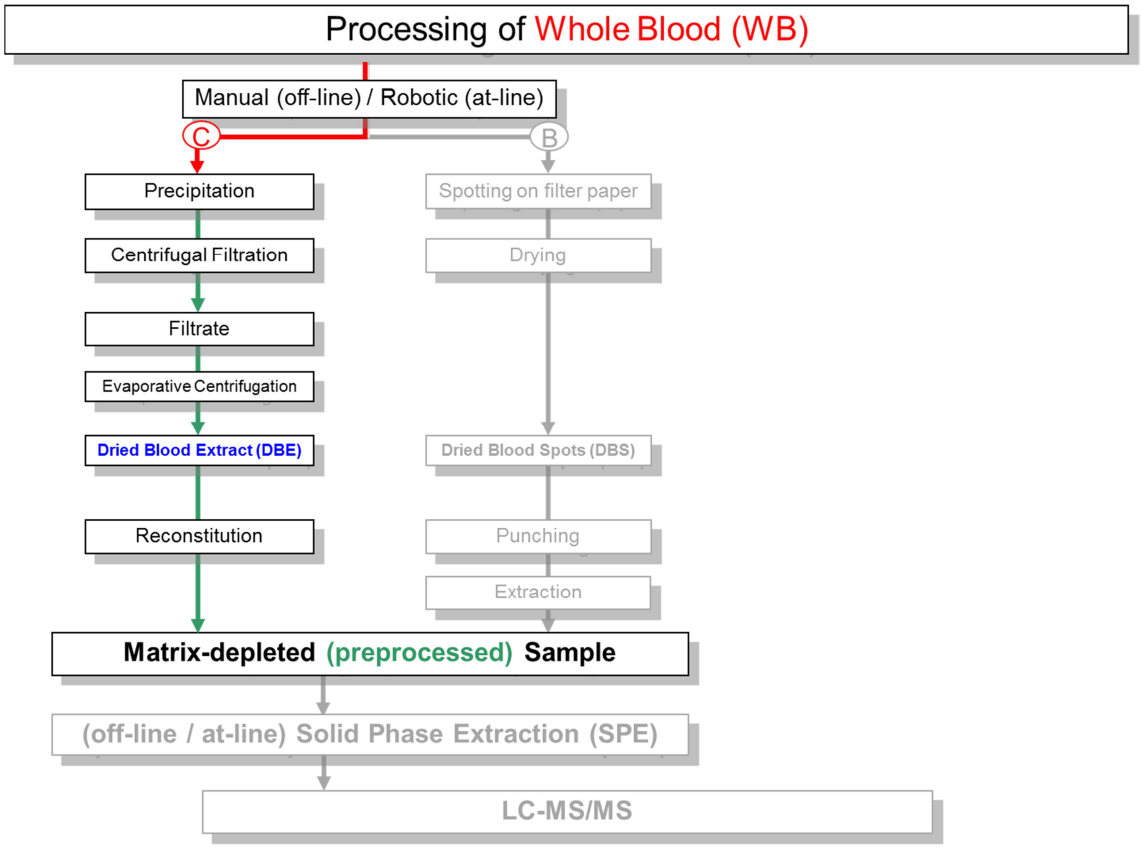


Figure 4: Operational procedures for preparation of whole blood: Dried Blood Extract (DBE).

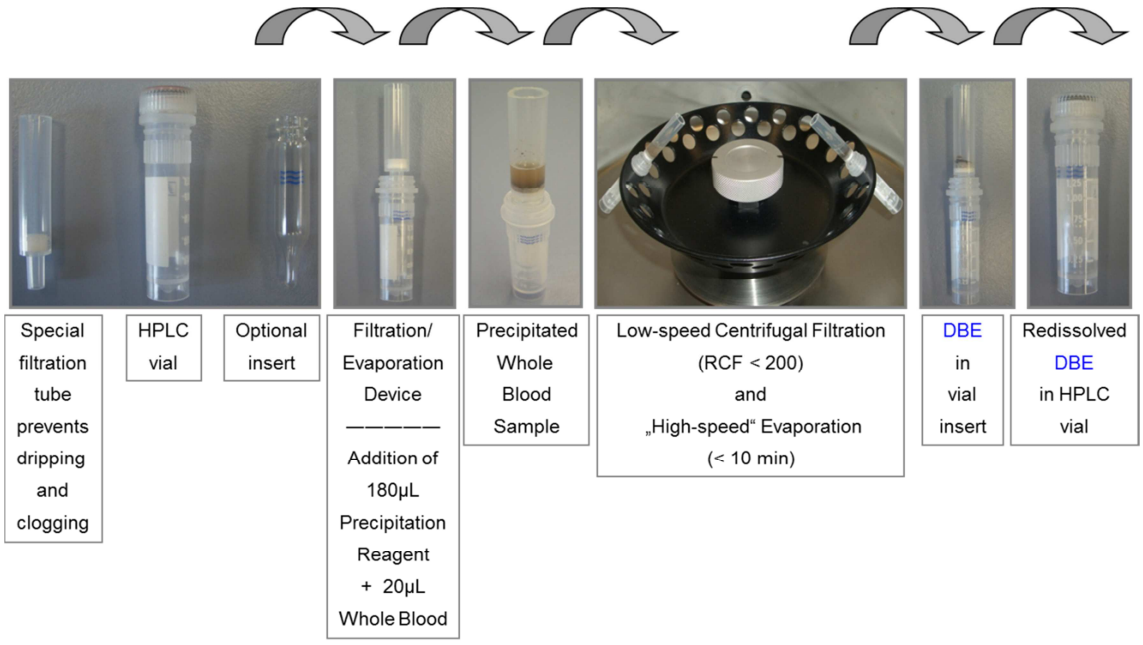


Figure 5: Preparation of Dried Blood Extract (DBE).

However, all the methods described so far can only be partly automated. This makes the preparation of whole blood sample time consuming, error-prone and cost-intensive.

One step towards total automation has been the introduction of tailor-made SPE packing materials and the application of on-line SPE-LC column switching [11,12]. These special SPE columns allow a repetitive injection and fractionation of native body fluids such as plasma, serum and urine.

The fractionation into low- and high-molecular weight compounds is based on Size Exclusion Chromatography (SEC) or Turbulent Flow Chromatography (TFC). The small molecules, i.e. target analytes, are retained on the stationary phase of the SPE column, whereas the high-molecular weight components, i.e. proteins, carbohydrates and nucleic acids are eluted to waste.

SPE columns packed with Restricted Access Materials, for example, rely on two-dimensional chromatography, i.e. SEC in combination with Reversed Phase (RP) -, Ion Exchange (IEX) - or Affinity Chromatography (AC). SPE columns packed with conventional porous materials, such as alkyl-bonded phases, and operated at a high linear flow velocity rely on Turbulent Flow Chromatography (TFC). Due to the very short residence time in the SPE column (size 50 x 0.5 mm ID), macromolecular sample components cannot diffuse into the pores and thus are eluted in the void volume to waste [13]. Recently, the analytical platform RapidFire was launched by Agilent. It consists of a switching valve and a SPE cartridge. The latter is not coupled to a LC-column but directly to a MS/MS detector [14].

The described integrated sample preparation principles, however, cannot be applied for whole blood samples, because the blood cells clog the capillaries, sieves and SPE columns (cf. Figure 6, D).

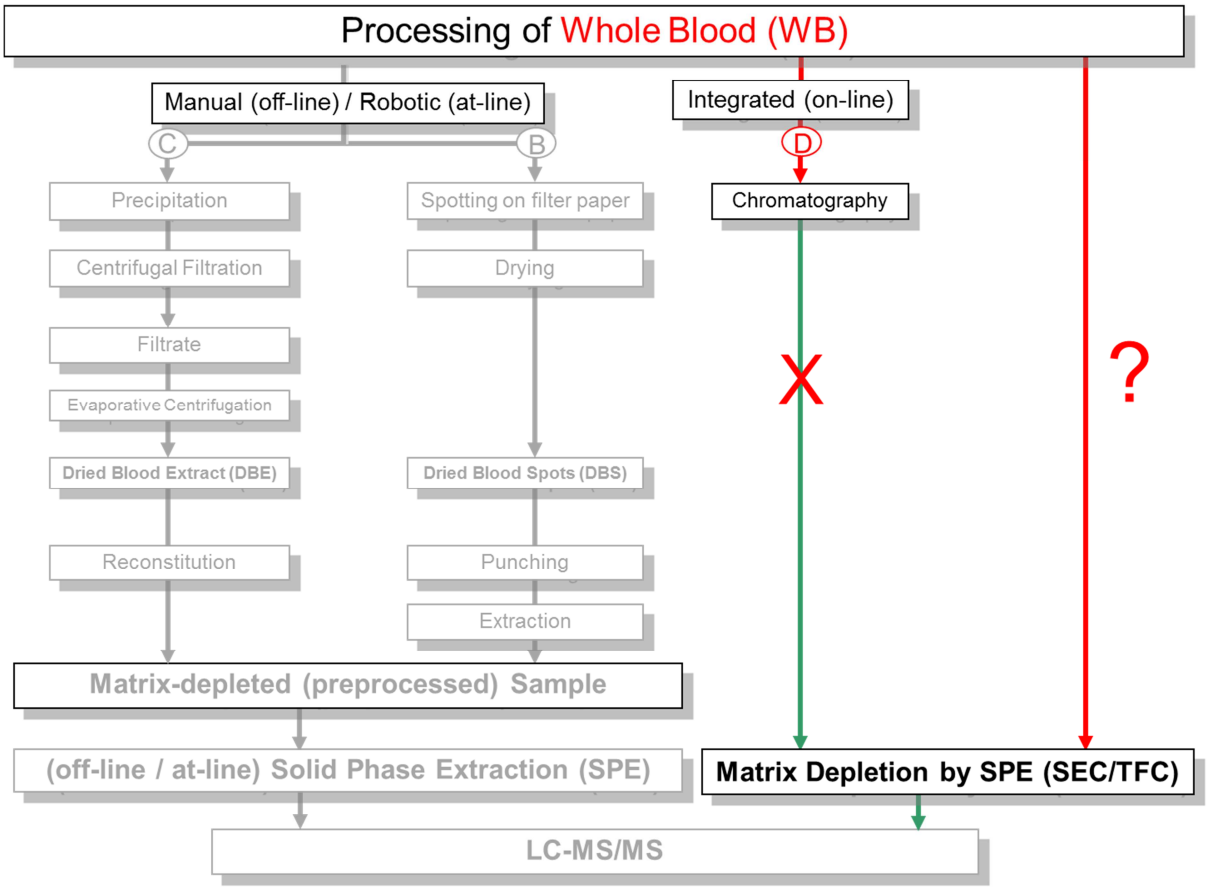


Figure 6: Operational procedures for preparation of whole blood: Chromatographic clean-up.

In order to enable on-line SPE of an anticoagulated blood sample, two special, embedded treatment procedures have been developed in the Laboratory of BioSeparation, Institute of Laboratory Medicine, Medical Center of the University of Munich [15-17]. Whole blood is treated in-line either by heat-shock treatment for 13 seconds at 75 ℃ or by snap-freezing with liquid ni trogen followed by slow thawing. In both cases, whole blood is converted into so-called Cell-Disintegrated Blood (CDB). The disintegration of blood cells generates subcellular particles which have an average diameter of 1 µm. These particles do not sediment on standing and do not clog a chromatographic system (cf. Figure 7).

Thus, anticoagulated whole blood can be directly processed, converted in-line to CDB, treated on-line by SPE and finally analyzed by LC-MS/MS in a fully automated way (cf. Figure 8, E).

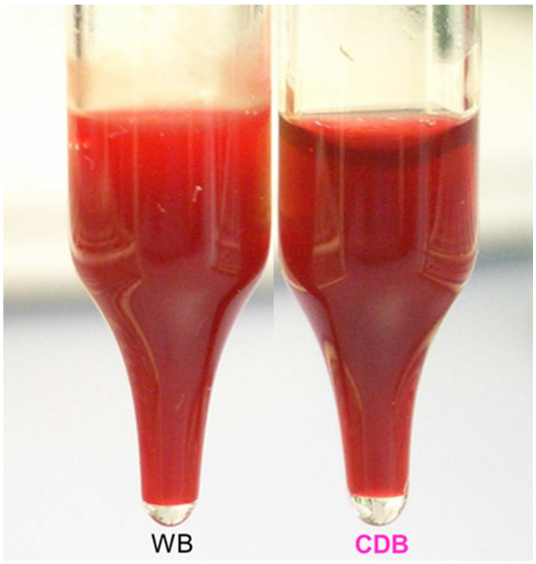


Figure 7: Conversion of anticoagulated whole blood (WB) into cell-disintegrated blood (CDB)

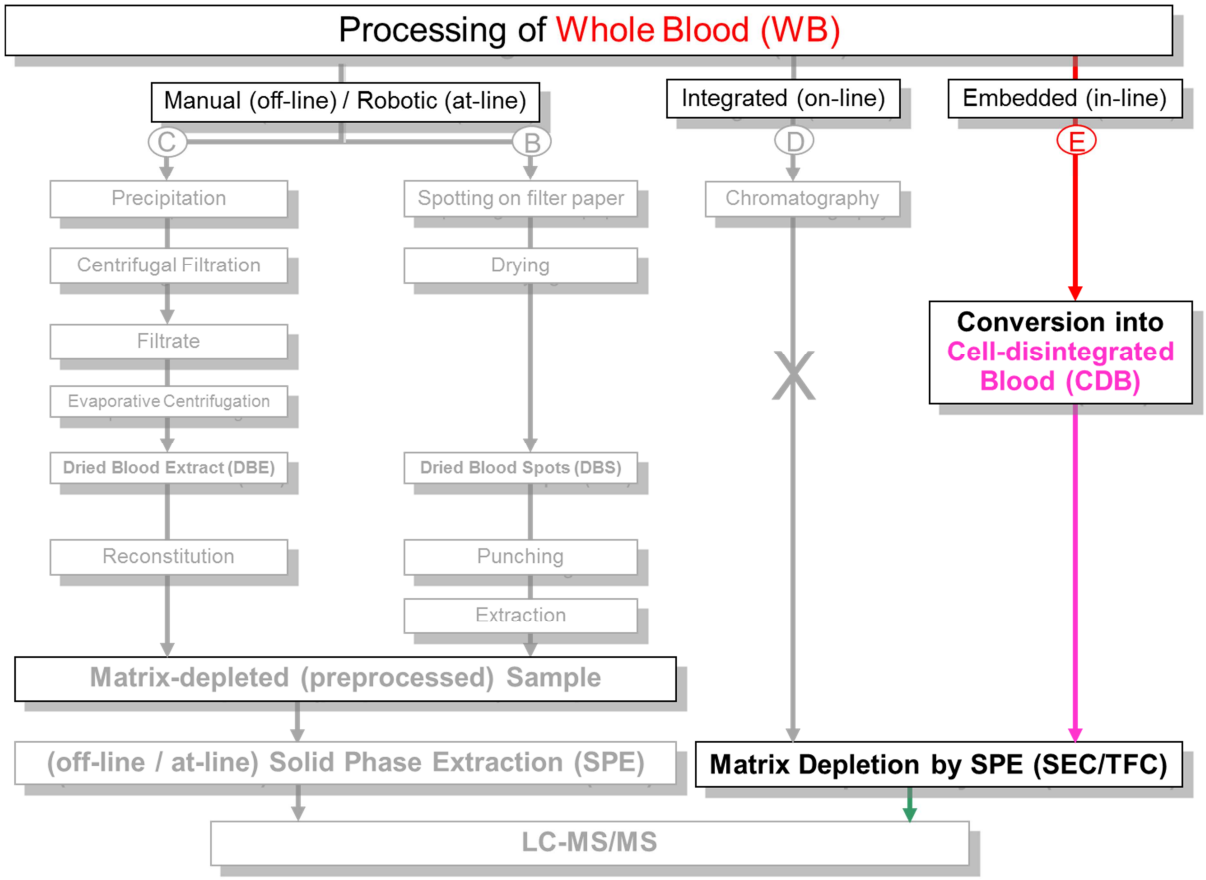


Figure 8: Operational procedures for preparation of whole blood: In-line processing of whole blood and conversion into Cell-Disintegrated Blood (CDB).

1.2 Aims of the thesis

The major aims of the thesis were to set-up, program, optimize and evaluate an instrumental platform for fully automated SPE-LC-MS/MS analysis of a model analyte in whole blood.

The immunosuppressant Cyclosporine A was chosen as the model analyte, as this drug predominantly binds to erythrocytes.

For the sampling and mixing of anticoagulated whole blood, two novel liquid-handling modules and a mixing unit had to be integrated in a XYZ-autosampler, programmed and optimized with regard to their mode of operation.

For in-line processing of whole blood, i.e. its conversion into Cell-Disintegrated Blood (CDB), home-made modules for heat-shock treatment and cryogenic treatment had to be installed, programmed and optimized with regard to each treatment procedure.

The embedded, in-line sample processing modules then had to be hyphenated with a SPE-unit which had to be operated at high flow rates (Turbulent Flow Chromatography).

Furthermore, in order to set-up a robust analysis system, different in-line filters had to be tested and optimized towards a maximum of analysis cycles.

In the next step, the filter and SPE-unit had to be coupled on-line via switching valves to a LC-MS/MS system. In addition, the different wash steps of the filter, columns and capillaries had to be optimized to ensure a routine, unattended operation.

Another goal was to proof the applicability of commercial calibrators and quality control samples for the analysis platform. Furthermore, the overall analysis procedure had to be validated with respect to linearity, range, sensitivity, accuracy, precision and recovery of the model analyte.

Finally, a method comparison with the routine, semi-automated procedure of the Institute of Laboratory Medicine, University of Munich, was performed and interpreted.

2 Theoretical part

2.1 Whole blood

Whole blood is the main transport medium in the human body, and thus the most important biological fluid for clinical-chemical analysis.

Whole blood represents a two-compartment-system, which is composed of liquid blood plasma and blood cells (erythrocytes, leukocytes, thrombocytes and subtypes) as shown in Figure 9.

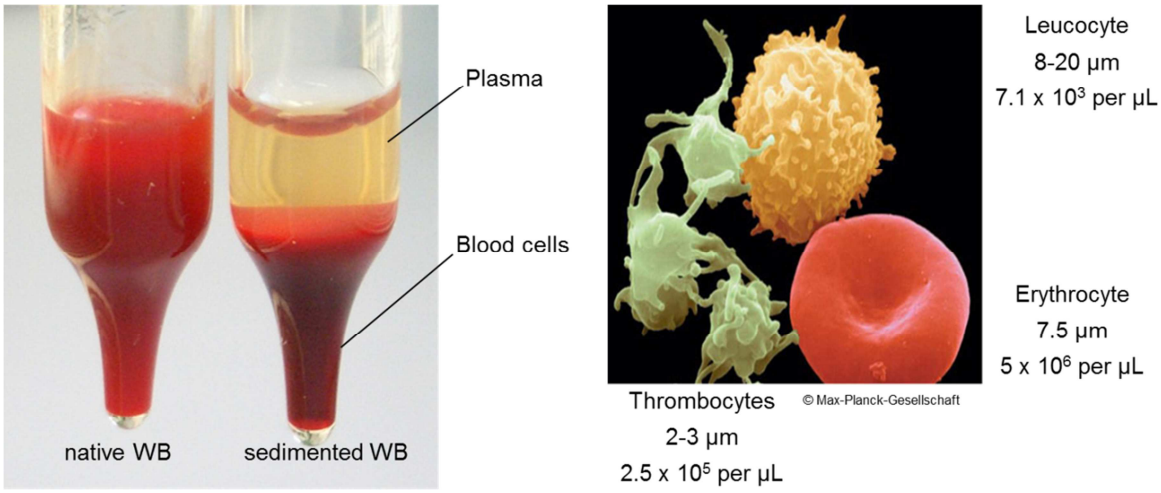


Figure 9: Components of whole blood [18]

About 45 % of whole blood is represented by blood cells, whereby erythrocytes are the most abundant cells with more than 95 % of total blood cells.

Mature erythrocytes are oval, biconcave disks with a size of about 7 µm. They lack a cell nucleus and have a fixed period of existence before their components are recycled [19]. Erythrocytes are very flexible and can be deformed easily when flowing through capillaries [20]. The reddish color of erythrocytes is due to hemoglobin, which makes up about 97% of the dry content of erythrocytes [21].

Leucocytes are divided according to their morphological and functional characteristics into monocytes, granulocytes and lymphocytes. Leucocytes have a nucleus and are variously shaped. The number of leucocytes in whole blood is

normally 6000 / µL. They play a central role in the immune system.

Thrombocytes are small, irregularly shaped, enucleated cell fragments, which are 1-3 µm in diameter [22]. The number of thrombocytes in whole blood is between 200.000 – 300.000 / µL. One of the primary functions of thrombocytes is to facilitate blood clotting.

The hematocrit is defined as the volume percentage of erythrocytes in whole blood. The hematocrit varies depending on sex and environmental conditions, and is normally about 45%.

The liquid component of blood is blood plasma, which is straw-colored. It is prepared by centrifugation of anticoagulated blood. Blood plasma consists of more than 90 % water, 7-8 % proteins, and other dissolved constituent parts, which are fats, enzymes, lipids, hormones, minerals, metabolites and waste products. The composition of plasma is shown in Figure 10. Plasma proteins possess a large surface area with numerous hydrophilic and lipophilic binding sites for endogenous compounds and drugs [23]. Albumin is the most abundant of the plasma proteins.

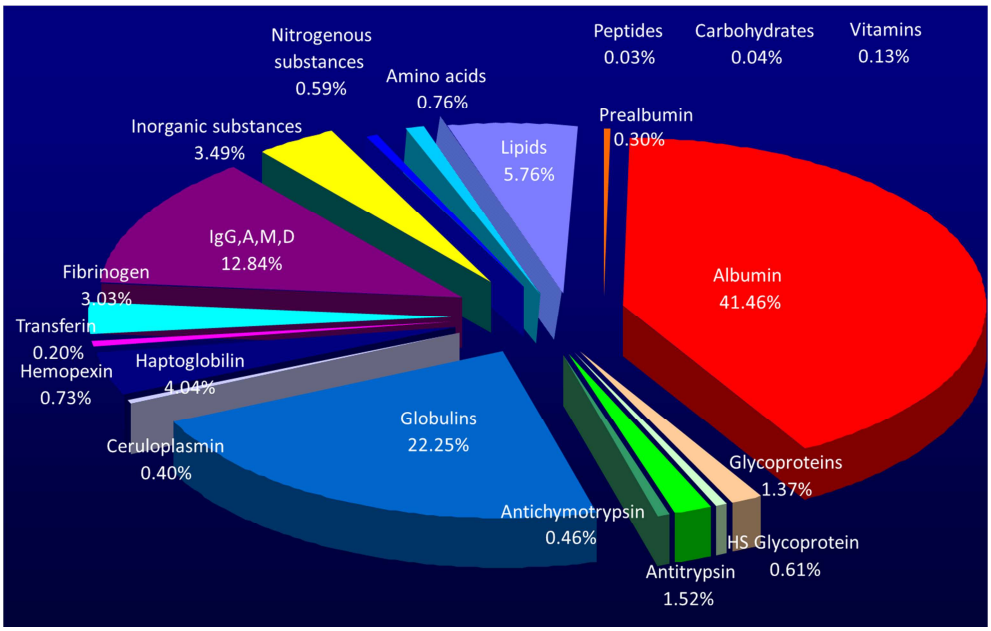


Figure 10: Composition of human blood plasma (according to Geigy Scientific tables) [24].

2.2 On-line Solid Phase Extraction (SPE)

The pretreated samples of whole blood - both the matrix-depleted sample produced by established technologies and the matrix containing CDB sample – have to be further processed by solid phase extraction (SPE) before an aliquot can be injected onto a LC-MS/MS analysis platform.

2.2.1 Solid Phase extraction

Due to its simplicity and versatility, SPE is widely used as a clean-up technique in instrumental bioanalysis. SPE physically extracts an analyte from a liquid matrix by partitioning the solutes between the solid phase (sorbent) of the SPE column and the liquid phase in order to isolate and pre-concentrate the analyte(s) [25,26]. The analyte(s) of interest is then eluted from the sorbent while interferences either pass straight through the sorbent bed or remain adsorbed to it.

The SPE procedure usually consists of four distinct and consecutive steps [25,27]:

Column conditioning / equilibration: The sorbent bed must be conditioned with an organic solvent of appropriate polarity to promote good surface contact between the two phases before the extraction step. This step also includes the equilibration of the sorbent bed with a mobile phase that is as similar to the sample as possible, in terms of polarity, ionic strength and pH.

Adsorption: The liquid sample is percolated through the SPE column at a suitable flow rate by application of a gentle pressure or vacuum, which causes the depletion of the matrix and the simultaneous enrichment of the analyte(s). The optimal flow rate depends on the particle size, the column dimensions and the packing characteristics of the sorbent.

Column washing: The intention of this step is to remove the undesired compounds (interference material) from the sorbent as completely as possible without eluting the analyte(s).

Elution: The adsorbed analyte(s) are eluted from the sorbent using a suitable solvent or a mixture of solvents and returned into a liquid phase suitable for analytical measurements.

2.2.2 On-line SPE-LC via column switching

In on-line SPE, a SPE column is connected to an analytical column via an electrically or pneumatically driven six-port valve. The retained analyte(s) is directly eluted from the SPE column onto the LC system via a column switching-valve [28].

The general procedure for column switching consists of three steps. Firstly, the pretreated / untreated sample is injected onto the SPE column and fractionated. The target analyte is extracted from the sample matrix and retained on the sorbent. Concurrently, the interfering sample constituents are flushed into waste. Secondly, after rotation of the valve, the retained analyte is eluted on-line onto a series-connected HPLC column. Thirdly, the analytical separation takes place, and at the same time, the SPE column is reconditioned [11,29].

In this work, the valve switching time parameters (t_A, t_M and t_T) were determined according to Majors et al. [29]. The operational procedure and steps to determine valve switching times are documented in 3.2.3.1.

The hyphenation of SPE with a LC-column via a switching valve is essential for automation [25,27]. Automation of SPE for sample preparation is important for many reasons. First, it means release from tedious and intensive manual labor and therefore a higher sample throughput. The costs involved are also reduced through fewer manpower requirements and decreased analysis times. In addition, it reduces the exposure of operators to hazardous solvents and infectious samples. Furthermore, it improves the analytical quality due to enhanced reproducibility, avoidance of human error and the possibility of multiple step elution for cleanup of complex samples [26]. Possible disadvantages of such an automation could be systematic errors, “carry-over” and matrix-effects due to insufficient fractionation [26,30]. These drawbacks can be overcome by optimized wash steps and / or multidimensional SPE.

2.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

After sample clean-up, there is still a need for chromatographic separation of the analytes and residual matrix components prior to detection. This is especially true when analyzing complex biological samples, such as whole blood. In this work, the pretreated sample is separated by High Performance Liquid Chromatography (HPLC) prior to the detection by a tandem mass spectrometer (MS/MS).

A tandem mass spectrometer is capable of ionizing and fragmenting many classes of compounds eluting from a LC column. The three principal components of a mass spectrometer are: an ion source for formation of gas phase ions which are positively or negatively charged, a mass analyzer for separating ions according to their m/z ratios and an ion detector for detection of the selected ions.

The enormous growth of LC-MS/MS is due to the development of atmospheric pressure ionization (API) techniques, which enabled LC to be easily and reliably interfaced with MS [31,32]. LC-MS/MS offers selective and sensitive detection of target analytes, and has played an increasingly important role in clinical laboratories during the last 10-15 years [31].

2.3.1 Ionization techniques

The API techniques are generally referred to as soft ionization techniques, since the predominant ion detected is the quasi-molecular ion rather than other ions resulting from fragmentation of the molecule. The three commonly used ionization sources are atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and the recently introduced atmospheric pressure photoionization (APPI) [33-36].

The suitability of these ionization techniques for the analysis of different compounds is often classified by their applicability to analytes with different polarities and molecular weights, as shown in Figure 11.

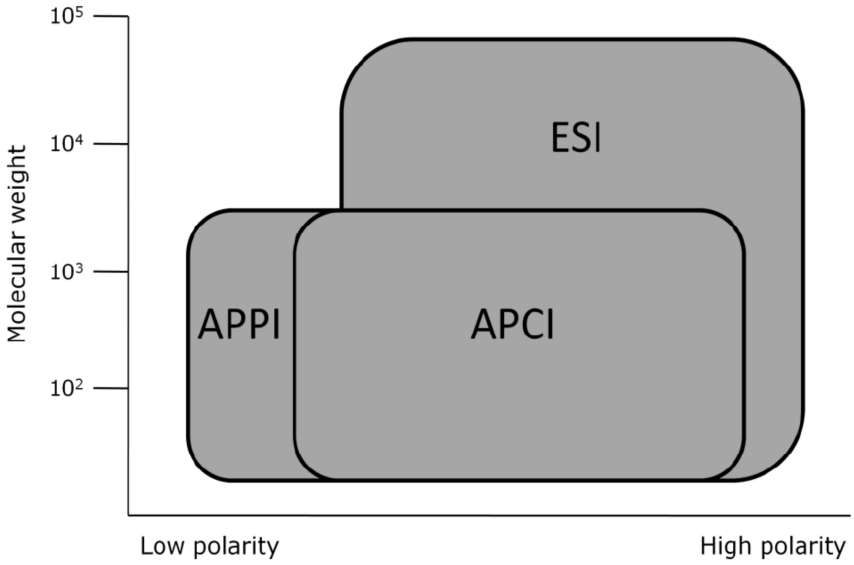


Figure 11: Application range of ESI, APCI and APPI ionization techniques for analytes with different polarity and molecular weight [37].

It is clear that ESI works best over a broader range of different analytes. Furthermore, analytes with a high polarity and a high molecular weight can only be analysed by ESI. In this work, ESI was applied for the detection of the target analyte, i.e. the immunosuppressant Cyclosporine A.

The effluent from the LC column enters the ESI source region through the spray needle at atmospheric pressure. It is evaporated in a fine mist of droplets facilitated by a flow of a neutral gas (e.g. nitrogen) and a high temperature. An electrical field is generated by applying a potential difference between the needle and the entrance (orifice) of the mass analyzer. The applied electric field leads to the formation of a Taylor cone at the needle tip [38]. As the droplet decreases in size by evaporation, the electrical charge density at the droplet surface increases up to a critical point, known as the Rayleigh stability limit [39]. At this point, the electrostatic repulsion between like charges on its surface exceeds the surface tension, and the droplet clusters divide into small droplets by Coulombic explosion (Figure 12) [40-42]. This process is repeated several times until the sample ions are ejected into the gas phase by field desorption. The mechanism of production of the gas phase ions is not yet fully understood. According to two proposed theories, the charge residue model (CRM) and the ion evaporation mechanism (IEM), the analytes in ESI are ionized in solution. The formation of adducts with buffer ions is a common phenomenon in ESI.

However, it is possible to increase or decrease the amount of adducts formed by altering the mobile phase solvent composition [43,44].

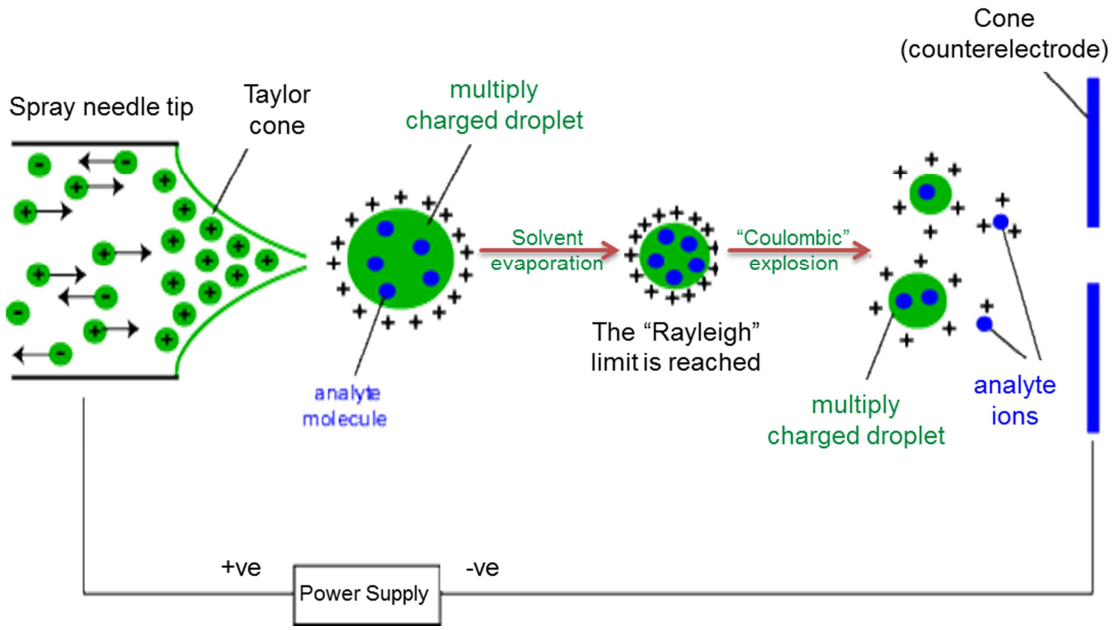


Figure 12: A simplified scheme of ion formation in the electrospray ionization (ESI) process, operated in positive ion mode [45].

The number of charges retained by an analyte is affected by different factors, such as the composition and pH of the electrosprayed solvent, droplet size, liquid surface tension, solvent volatility, surface charge and ion solvation strength. For small molecules (< 1000 Da), ESI typically generates singly charged ions; for medium sized molecules (1000-2000 Da), ESI generates single or double charged ions; and for large molecules (> 2000 Da), the ESI process typically gives rise to a series of multiply-charged species. Among the common MS techniques, ESI is unique in that multiply-charged gas phase ions may be formed, and is suitable for detection of large proteins and other biomolecules. A drawback of ESI is its sensitivity to the sample matrix [46,47] and dependency on the flow rate.

2.3.2 Coupling of LC to ESI-MS

As mentioned previously, the development of atmospheric pressure ionization (API) techniques enabled LC to be easily and reliably interfaced with MS [31,32]. Generally, the ion formation in ESI mode is most efficient at low flow rate, around 5-10 µL/min [48]. However, the flow rate can be enhanced up to 1 mL/min through the addition of heating capabilities and improvements in the use of drying and nebulizing gases [49,50]. A split of the flow to 100-200 µL/min is often recommended [51].

The choice of solvents used as LC mobile phase affects the formation of ions in solution. The composition of the mobile phase must be compatible with the ESI-MS detection system and allow chromatographic separation. Sometimes a compromise between the sensitivity and the retention may be necessary. The separation efficiency of the column is important, even if the MS detector is selective and can separate compounds by their mass-to-charge ratios (m/z). Co-eluting peaks may give rise to severe suppression of the analyte response. A retention time long enough to separate the analyte from interferences, especially early eluting polar matrix interferences, is often advantageous. Buffers and pH modifier in the mobile phase should be volatile to avoid contamination or plugging of the sample orifice. Operating the mass spectrometer with non-volatile buffers, such as phosphate and borate, is technically possible, but requires periodic cleaning of the ion source to remove the salt deposits. It is recommended to replace nonvolatile buffers with the following volatile buffers: formic acid, acetic acid, ammoniac, triethylamine, carbonates, ammonium formate, ammonium acetate and ammonium carbonate. The concentration of buffer must be kept as low as possible [52]. Ion-pairing agents and surfactants should be avoided or added in a very low concentration to avoid ion-suppression. Furthermore, ESI does not tolerate high salt concentrations or non-polar solvents. The trifluoroacetic acid also largely decreases the analyte response [52].

2.3.3 Tandem Mass Spectrometry

After ionization, the analyte ions present in the gas phase are directed into a mass detection system, which consists mainly of a mass analyzer and a detector.

In the mass analyzer, the ions are separated according to their m/z ratios. Depending on the information required from the ionized analytes, there are several types of mass analyzers: quadrupole, ion trap, linear ion trap, orbitrap, magnetic sectors, Fourier transform ion cyclotron resonance and time-of-flight [53,54]. Hyphenation of different mass analyzers is a trend to increase the versatility and allow multiple experiments to be performed [52]. The hybrid instruments, such as triple-quadrupole, quadrupole-time-of-flight or quadrupole-ion trap, allow one to obtain a mass spectrum resulting from the decomposition of an ion selected in the first analyzer [52]. It is possible to fragment over several generations (MS^n), where n refers to the number of generations of ions being analyzed.

In the most common tandem mass spectrometry (MS/MS, or MS^2), a precursor ion is isolated in the first analyzer, and then undergoes a fragmentation to yield product ions, which are analyzed in the second analyzer (Figure 13). The fragmentation of the precursor ion can be achieved by collisional activation via collisions of selected ion with neutral gas molecules (collision-induced dissociation, CID). MS/MS can be performed either in space by the coupling of two physically distinct analyzers (e.g. triple-quadrupole), or in time by performing an appropriate sequence of events in an ion storage device (ion-traps).

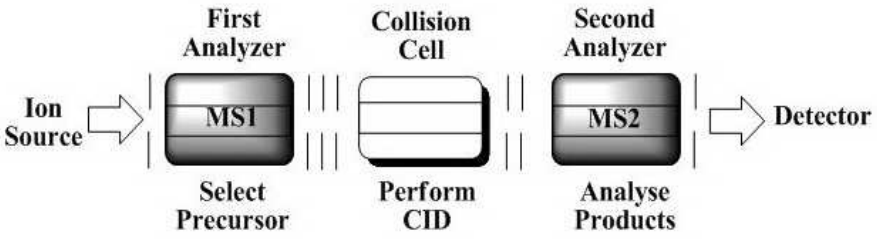


Figure 13: Principle of tandem mass spectrometry [55].

The four most common MS/MS scan modes are shown in Figure 14.

A) *Product ion scanning (daughter scanning):* A precursor ion (or parent ion) of a chosen m/z ratio is selected by MS1 at a certain time, and undergoes CID in the collision cell. The resultant product ions (daughter ions) are determined by MS2. This process can be repeated for different precursors.

B) *Precursor ion scanning (parent scanning):* MS2 is set to transmit a specific / defined fragment ion to the detector. MS1 is scanned to detect all the precursor ions that produce this fragment.

C) *Neutral loss scanning:* Both analyzers, MS1 and MS2, are scanned in a synchronized manner, but with a defined mass offset between the two. For a selected neutral fragment, all the fragmentations leading to the loss of that neutral are detected.

D) *Multiple ion / reaction monitoring (MRM):* It consists of a series of single / selected reaction monitoring (SRM), in which MS1 is focused on one selected precursor and MS2 on one specific fragment characteristic for that precursor. The absence of scanning in both analyzers allows for focusing on the precursor and fragment ions over longer times and increases the sensitivity. Typically, MRM is used for detection of analytes with known fragmentation properties in complex samples.

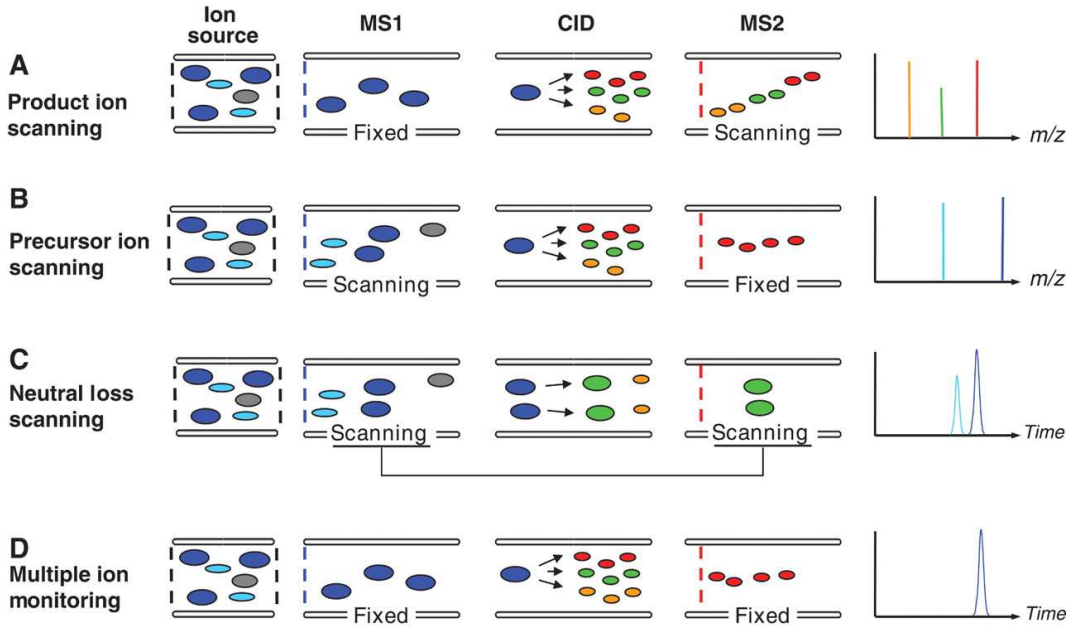


Figure 14: Multiple scan modes routinely used in tandem mass spectrometry (MS/MS) [56].

2.3.4 Matrix effects

As noted earlier in 2.3.1, a drawback of ESI-MS is the occurrence of matrix effects. Matrix effects were originally discussed by Kerbale and Tang, who showed that the response of organic bases decreased when increasing the concentrations of other organic bases [41].

Matrix effects are the alteration of ionization efficiency by the co-eluting compounds and can lead to a significant increase or decrease in the response of an analyte in a matrix sample compared to the same concentration in a neat sample [57,58].

The mechanism by which matrix effects occur is still unclear, but is thought to be as a result of competition between the interfering matrix components and the analyte during the ionization process for access to the droplet surface and subsequent emission into the gas phase.

Generally, matrix effects vary from sample to sample and from analyte to analyte [58-64]. They also depend on the sample preparation procedure, the mobile phase, the chromatographic separation and the ionization mode [65]. These compounds are known to cause matrix effects: anticoagulants, dosing vehicles, salts, fatty acids, triglycerides, phospholipids and constituents of sampling material (e.g. polymers) [66-69].

It must be kept in mind that the enhancement in the response of the analyte may sometimes come from some other compound with the same m/z of both the parent and the product ions [70]. This kind of increase in signal intensity differs from the ionization enhancement by matrix effects.

Matrix effects can cause significant errors in the sensitivity, accuracy and precision of an analytical procedure [71]. Thus, according to the FDA guidelines [72,73], the evaluation of matrix effects is required as part of a quantitative method development.

Although how to evaluate and eliminate the presence of matrix effects is not stated in the guidelines, there are two most commonly used methods to study matrix effects.

A) Post extraction supplementation technique

The post extraction supplementation technique has been developed by Matuszewski

et al. for quantitative measurement of matrix effects [60]. A spiked neat sample is analyzed with LC-ESI-MS/MS and gives the MS response of the peak area $A_{neat\ sample}$. A spiked matrix sample at the same concentration gives the MS response of peak area $A_{matrix\ sample}$. The matrix effect can be calculated with the formula according to Niessen et al. and Taylor [57,58]:

$$\text{Matrix Effects (\%)} = \left(\frac{\text{Area of analyte in matrix sample}}{\text{Area of analyte in neat sample}} - 1\right) \text{x } 100\% \qquad \textbf{(Equation 1)}$$

A value of 0 indicates no matrix effects, a negative value indicates ion-suppression and a positive value indicates ion-enhancement due to coeluting sample compounds.

In addition, matrix effects can be calculated as a relative difference of slops of two calibration curves constructed from the spiked neat sample and the spiked matrix sample at the corresponding concentrations [74]. In this application, the intercepts of both calibration curves should be negligible. This indicates that the matrix effects are independent of the concentration of the analyte.

B) Post-column infusion

A spiked neat solution is constantly infused into the LC eluent via a T-union inserted between the LC column and the MS detector. The MS response of this solution is monitored while injecting a blank matrix sample for SPE-LC-MS/MS analysis. The constant MS response (baseline) of the spiked neat solution varies if compounds elute from the LC column which suppress or enhance the ionization process [75].

The matrix effects can be decreased by injecting less sample or a diluted sample. However, this is accompanied with less detection sensitivity [76]. Another measure to reduce matrix effects is to minimize the number of coeluting compounds. This can be accomplished by more effective extraction/clean-up techniques (e.g. SPE extraction) or improving chromatographic resolution of the analyte peak from the peaks of interfering matrix compounds [77-84].

The matrix effects can only rarely be fully eliminated during method development. Thus, the matrix effects are practically unavoidable in ESI-MS and need to be compensated for to improve the quality of the analytical results. The use of an Internal Standard (IS) is recommended, as the extraction- and ionization efficiency are expected to be identical with regard to the analyte and the IS [52,85].

The IS method is based on a comparison of the intensity of the signal corresponding to the analyte with one of the IS. This comparison allows the elimination of various error sources. As the analyte and the IS undergo the same losses in the extraction steps and the same errors in the ionization step, their ratio remains unchanged [52]. It is recommended to add the IS as early as possible in the analysis procedure. This allows the IS to be treated exactly the same as the analyte and leads to the maximum precision of the analysis method.

The physical and chemical properties of the IS should be as close as possible to those of the analyte [52,86]. There are three categories of IS: structural analogues labeled with stable isotopes, structural homologues and compounds from the same chemical family. In this work, two different Internal Standards are used for the quantitation of Cyclosporine A: the isotope labeled deuterated Cyclosporine A (D12-Cyclosporine A) and the structural homologue Cyclosporine D.

2.4 Target analytes: Immunosuppressants

Immunosuppressants are powerful drugs that affect the immune system. They are used to prevent the body from organ rejection in post-transplantation patients and slow down the immune response in autoimmune disease therapy [87].

Immunosuppressants can be classified based on their primary sites of action. The most commonly used immunosuppressants include cyclosporine, tacrolimus, sirolimus, everolimus, glucocorticoids, azathiopurine and mycophenolate mofetil [88,89].

Due to the availability of appropriate immunosuppressive regimens, the outcome of post-transplatation patient care has been significantly improved over the last decades [89].

However, all of these immunosuppressants have variable pharmacokinetic profiles, relative narrow therapeutic ranges, and show toxic side effects [90-94]. The required dosage of immunosuppressants also varies in different individuals and ethnicities. In transplant recipients, both supratherapeutic and subtherapeutic drug concentration can have devastating effects. Thus, individualizing the drug therapy of patient is recommended to obtain the optimum balance between the therapeutic efficiency and the risk of experiencing toxic side-effects. This has been achieved by therapeutic drug monitoring (TDM) during drug treatment [95].

Therapeutic drug monitoring (TDM) is the measurement of drug levels in the blood at timed intervals in order to maintain a blood level within the narrow therapeutic range by adjusting the individual dosage. Immunosuppressants are mainly bound to erythrocytes [96-103]. 58% of Cyclosporine A, for example, is present in erythrocytes at a concentration of 500 µg/L at 20 ℃ [104]. Thus TDM of immunosuppressants has to be performed in whole blood. There are two categories of available analytical techniques for TDM of immunosuppressants: immunoassay and LC based methods [89]. During last 10-15 years, LC-MS/MS has gained increasing popularity in clinical laboratories due to its high selectivity and sensitivity, as mentioned previously in 2.3. At present, the most common method for pretreatment of whole blood samples prior to SPE-LC-MS/MS analysis of immunosuppressants is protein precipitation [105,106]. However, by applying the analysis platform described in this work, the

blood level of immunosuppressants can be directly determined from whole blood without any manual pretreatment.

In this work, the immunosuppressant Cyclosporine A is used as the model analyte. For determination of the blood level of Cyclosporine A, two different Internal Standards are used: D12-Cyclosporine A and Cyclosporine D. Figure 15 shows the structural form of Cyclosporine A and its Internal Standards.

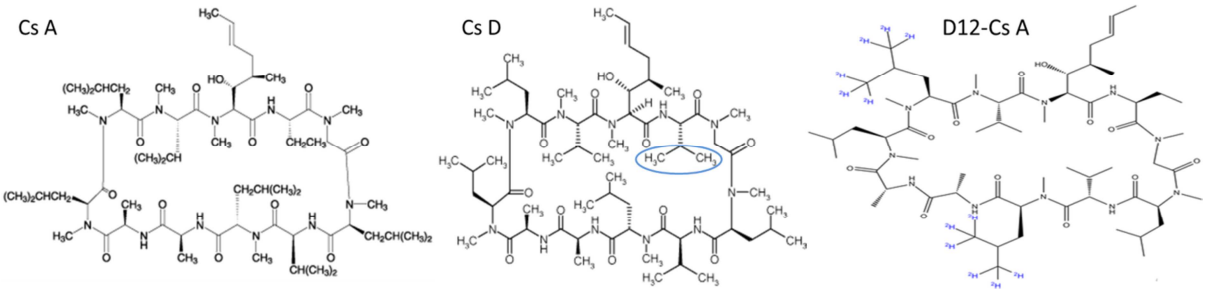


Figure 15: Structural formula of Cyclosporine A, Cyclosporine D and D12-Cyclosporine A.

3 Experimental Part

3.1 Set-up of a fully automated analysis platform

Towards a total automation of LC-MS/MS analysis of whole blood samples a dedicated instrumental platform has been developed and is described in the following. This platform enables direct injection, in-line processing and on-line SPE-LC-MS/MS analysis of drugs and endogenous, low-molecular weight compounds present in whole blood samples.

The fully automated analysis platform consists of an autosampler, an on-line SPE unit, several HPLC pumps, a UV-VIS detector and a tandem mass spectrometer (Figure 16).

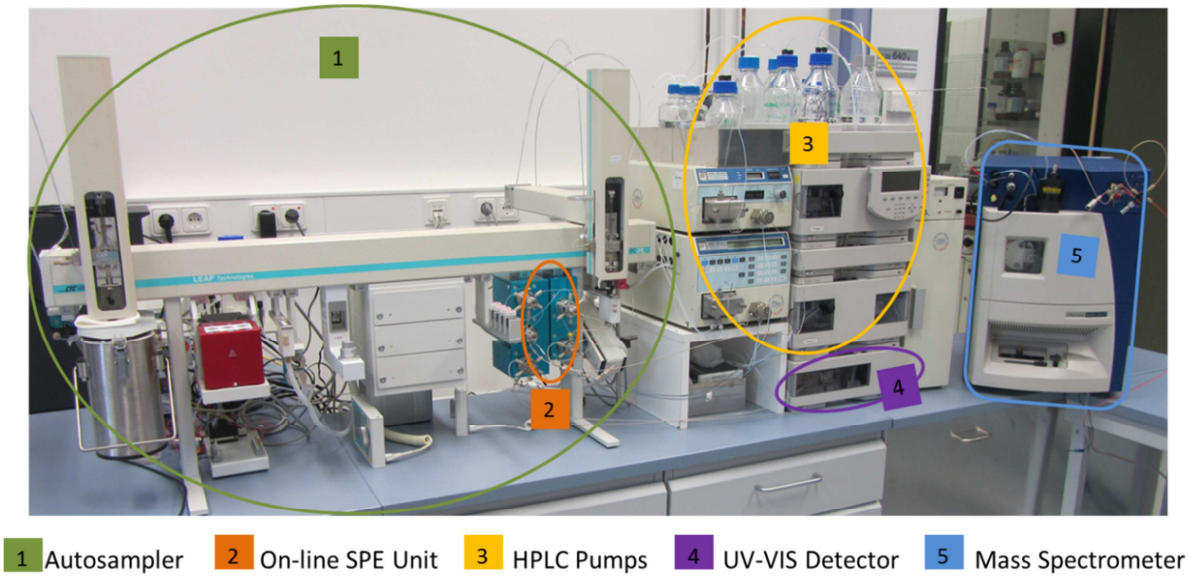


Figure 16: Instrumental set-up of the fully automated analysis platform.

This platform represents a total analysis system (TAS). As shown in Figure 17, it consists of the following units: sample handling/injection unit, processing unit, clean-up unit, separation unit, detection unit and data managing unit.

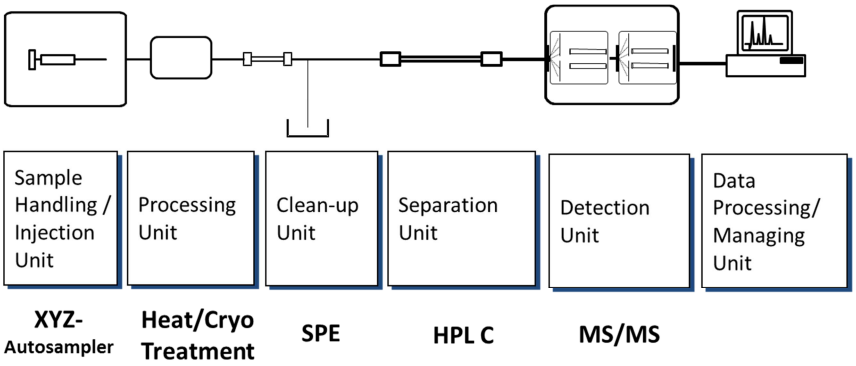


Figure 17: Total analysis system for direct and automated determination of drugs in whole blood.

The sample handling/injection unit is a XYZ - autosampler, which has been configured in cooperation with LEAP Technologies (Carrboro, NC, USA). The software Chronos was modified and adjusted for controlling and timing of this dedicated autosampler in cooperation with Axel Semrau (Sprockhövel, Germany).

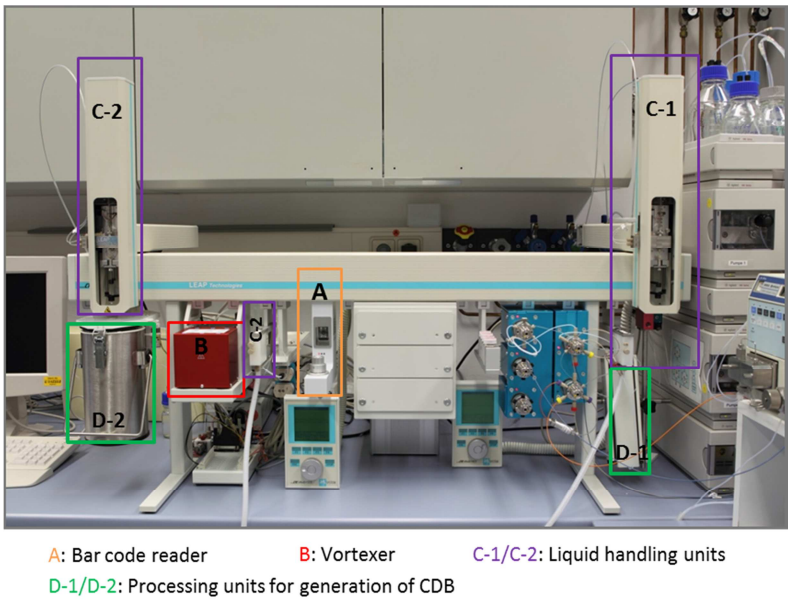


Figure 18: Modules of XYZ – Autosampler.

As shown in Figure 18 the autosampler is composed of a bar code reader, a heatable vortexer, two liquid handling units and two processing units, which independently convert anticoagulated whole blood into the novel blood matrix, i.e. cell-disintegrated blood (CDB).

3.1.1 Liquid Handling/Injection Unit: C-1 module

This unit is composed of a barcode reader for automatic identification of samples, a vortexer for sample mixing, and a DLW liquid handling unit for sampling and injection. The DLW liquid handling unit consists of a DLW injection adapter (Figure 19) with syringe (100 µL), a solenoid/actuator valve, two inlets for DLW active micro pumps, a sample holding loop (100 µL), a spring loaded needle guide and replaceable injection needle, a DLW wash station, a wash solvent pump station with two active micro pumps for DLW wash step and a self-priming PTFE micro pump for external active wash of the injection needle.

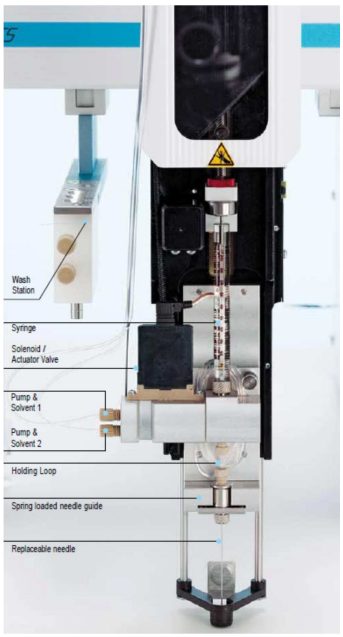


Figure 19: Components of DLW injection module.

For operation of the C-1 module the Chronos software was programmed accordingly. A sedimented sample of anticoagulated blood stored in the sample hotel at 4 ℃, is transferred to the vortexer for mixing. The homogenized whole blood sample is identified by a barcode reader (if required) and placed back into the sample hotel. A defined amount of an air segmented blood sample is aspirated by the syringe needle and stored into the holding loop. After sampling, the injection needle is washed from outside using the active wash option. This is followed by aspiration of a defined volume of IS stored in the sample hotel, in case an in-line addition of IS is required.

Finally, all segmented fluids present in the injection needle and holding loop, respectively, are injected into the sample loop (100 µL) via the injection valve. By this the in-line processing and on-line SPE step are initiated. Simultaneously, the liquid handling unit is moved to the DLW wash station and the whole sample path is washed from the rear with up to two solvents.

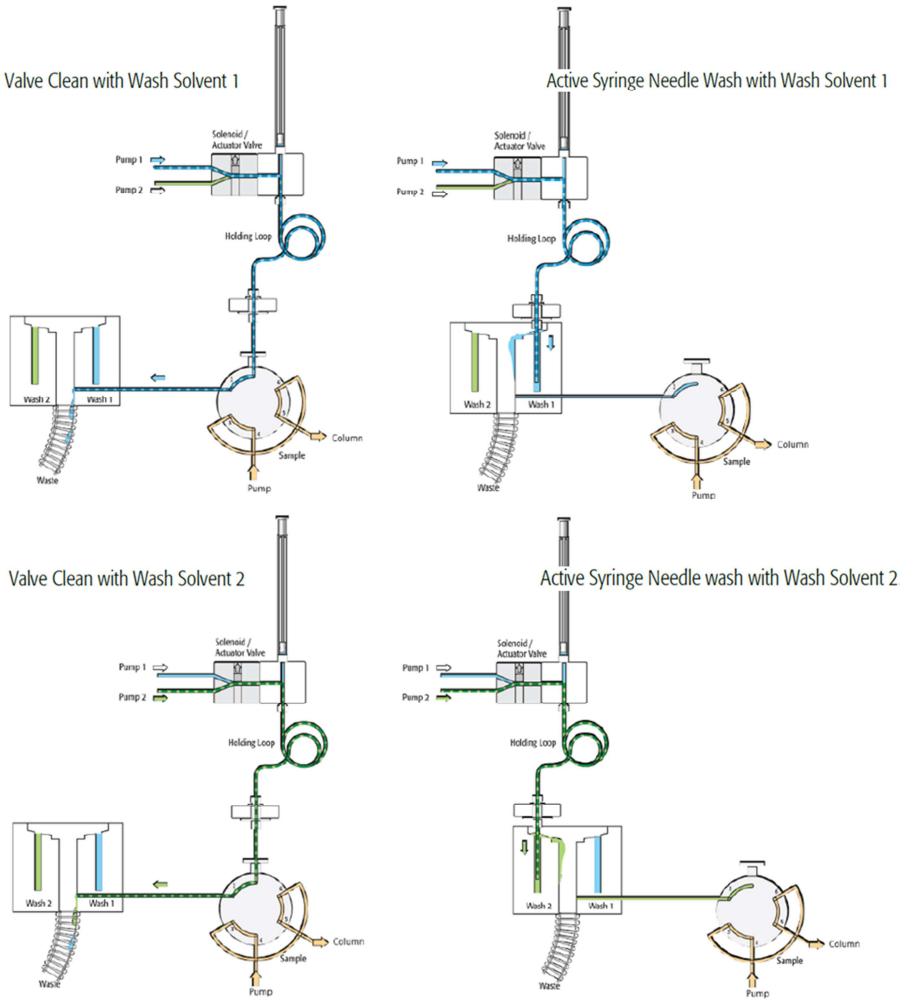


Figure 20: DLW wash / injection module and principle.

The principle of the DLW wash module is displayed in Figure 20. The two active micro pumps at the wash solvent pump station deliver a defined amount of wash solvent to the injection adapter. The solenoid/actuator valve functions in a precise manner, in starting/stopping the solvent delivery system and solvent switching. As a consequence all parts which have been in contact with the sample, i.e. the highly viscous blood samples, are completely washed from both inside and outside.

As mentioned before, the injection needle has to be washed externally after sampling, in order to get rid of contamination of residual sample components. However this wash step cannot be implemented into the DLW wash module. Therefore, a special active wash module (Figure 21) has been developed by combining a self-priming PTFE micro pump with the DLW wash station (C-2). The IN port of the micro pump was connected to the wash solvent bottle, and the OUT port was connected to the wash 1 station (Figure 21) of the DLW wash module (C-2). After sampling the injection needle is inserted to wash 1 station, and the micro pump is activated. The wash solvents flow from the bottom of wash 1 station and clean the injection needle externally in backflush direction.

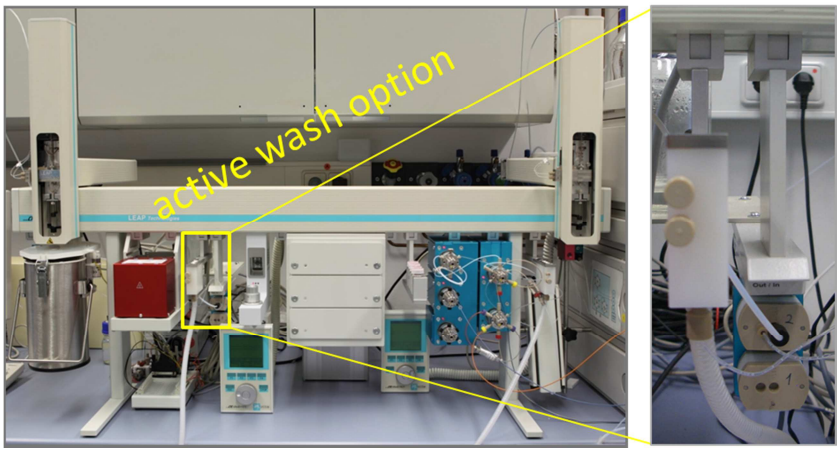


Figure 21: Active wash module.

3.1.2 Liquid Handling / Injection Unit: C-2 Module

The operation of the C-2 module was programmed with the Chronos software. A sedimented sample of anticoagulated blood stored in the sample hotel at 4 ℃, is transferred to the vortexer for mixing. The homogenized whole blood sample is identified by the barcode reader (if required) and placed back into the sample hotel. A defined volume (e.g. 40 µL) of an air segmented blood sample is aspirated into the special stainless steel processing needle. This needle has an inner diameter of 1.6 mm and a volume of 116 µL. After sampling, the processing needle is washed

externally using the active wash option. Then the processing needle is inserted into the standpipe for snap-freezing, as described in 3.1.3. After removal of the processing needle from the standpipe, the snap-frozen sample is allowed to melt at room temperature. Then the processed blood sample (cryo CDB) is deposited into a second vial placed in the sample hotel. The processing needle is moved to the DLW wash station, and the whole sample path is washed with water and organic solvent. Finally, the inner lumen of the processing needle is dried with nitrogen.

3.1.3 Processing Units

There are two embedded processing units in this platform for the two independent procedures to convert anticoagulated whole blood into CDB.

One of the processing units is for heat-shock treatment of anticoagulated whole blood. As shown in Figure 22 A, a heated stainless-steel HPLC capillary (300 x 0.5 mm ID) is placed in a column oven and used as processing device. The whole blood sample is injected into and pumped through this heated capillary under defined conditions.

A.  B. 

Figure 22: A. Device for heat-shock treatment; B. Device for cryogenic treatment.

The other processing unit is for cryogenic treatment of anticoagulated whole blood. As shown in Figure 22 B, a stainless-steel syringe-needle is used as processing device. The blood sample is aspirated and snap-frozen by contacting the syringe needle with liquid nitrogen followed by slow thawing. Liquid nitrogen is contained in a stainless-steel thermo-flask (Thermolyne® Brand Thermo-Flask®), which is sealed

with a special cover board.

In the center of the cover board a standpipe is located, which serves as a sleeve for the processing needle. The sleeve can be filled with liquid nitrogen through a Teflon tube, one end of which is located at the bottom of the thermo-flask. At the moment the autosampler places the processing needle into the standpipe, a valve closes the overpressure line. By this liquid nitrogen is forced through the Teflon tube into the standpipe and snap-freezes the blood sample contained in the processing needle.

This valve-triggered freezing process was established together with LEAP Technologies (Carrboro, NC, USA). For more technical information see S. Dai patent application (WO 2010/065145A1).

3.1.4 Clean-up unit

After conversion of whole blood into CDB this fluid contains all matrix components of whole blood. Thus CDB has to be pretreated by solid phase extraction (SPE) prior to LC-separation.

For the clean-up of CDB a SPE column coupled with an in-line filter was installed. Different SPE columns and filters were tested during method development.

The SPE column and the filter were connected to different 6-port switching valves of the on-line SPE unit. Thus, the SPE column and the filter were separately washed and reconditioned after every sample. In this way, the lifetime of both SPE column and filter was extended effectively. This not only reduces costs, but also improves robustness of the overall analysis procedure.

3.1.5 Separation unit

After fractionation of CDB the extracted analytes were transferred on-line from the SPE column to an analytical column and separated there. The preferred analytical column for the separation of the model compounds immunosuppressants was a pentafluorophenyl (PFP) column (50 x 2.1 mm ID, dp 5 µm). The model analyte Cyclosporine A is very strongly bound to the SPE material investigated. Thus only an eluent with a high amount of organic solution can desorb the target analyte from the SPE column. However, under this condition no retention and thus no separation of the analyte(s) on the analytical column can be achieved. This problem was solved by a so-called in-line dilution step.

For that purpose a T union was placed in front of the analytical column (Figure 35). This configuration allows the addition of an aqueous mobile phase via the T union to the mobile phase eluting from the SPE-column. By adjusting appropriate flow-rates any composition of the mobile phase being optimal for the retention/separation of the target analyte(s) can be achieved.

3.1.6 Detection unit

For the detection of drugs and endogenous, low-molecular weight compounds two detectors were integrated in the platform.

First, a UV-VIS detector was used for on-line monitoring the elution profiles of matrix components. In SPE-based sample clean-up, the UV-VIS detector was used to monitor the protein matrix at 280 nm and the hemoglobin fraction at 428 nm.

Second, a tandem mass spectrometer was used for selective and sensitive detection of the low-molecular weight model compound, i.e. the immunosuppressant Cyclosporine A.

3.2 Development of a SPE-LC-MS/MS method for direct analysis of Cyclosporine A in whole blood via heat-shock treatment

The development of an on-line SPE-LC-MS/MS method for direct analysis of drugs and endogenous, low-molecular weight compounds in blood samples includes implementation and optimization of the SPE based sample clean-up step, the LC based separation step and the MS/MS detection. In addition, the operational parameters for the sample handling/injection unit and as well as the processing unit (heat-shock treatment) of whole blood had to be adopted and optimized, respectively.

3.2.1 Optimization and standardization of sample handling/injection

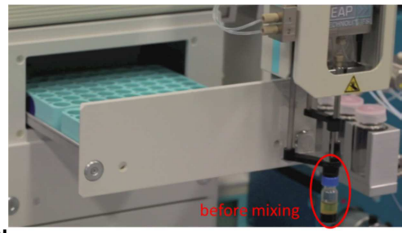
The standardization of handling/injection of whole blood samples involved the following operational steps:

1) Mixing of sedimented blood sample
2) Addition of Internal Standard (IS) if required
3) Optimization of sample segmentation by air and liquids
4) Optimization of syringe speed for aspiration and dispensing
5) Optimization of both in-between and after injection wash of injection needle

3.2.1.1 Sample mixing

Whole blood represents a heterogeneous, i.e. a two-phase system composed of blood plasma and blood cells. Thus, blood cells sediment on standing.

A sedimented whole blood sample present in a HPLC vial and located in the sample hotel is placed into the vortex as shown in Figure 23 A, and shaken (Figure 23 B) to yield a homogenized sample ready for injection as shown in Figure 23 C.

A. 

B. 

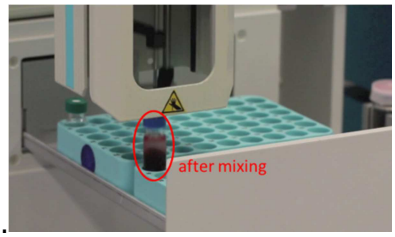
C. 

Figure 23: Sequence of mixing a sedimented sample of whole blood

3.2.1.2 Addition of Internal Standard (IS)

The Internal Standard (IS) for the analysis of the model analyte Cyclosporine A is Cyclosporine D or D12-Cyclosporine A. These compounds are very hydrophobic and thus only soluble in organic solvents. In this work, dimethyl sulfoxide (DMSO) is used for preparation of the Internal Standard. The addition of DMSO to a sample of whole blood even improves the conversion of whole blood sample into CDB [16]. As DMSO becomes solid when stored in the sample hotel at 4 ℃, mixtures of DMSO and sodium chloride solution were investigated.

It turned out, that a mixture of DMSO/NaCl (80/20 v/v) provided the best result with regard to solubility at 4 ℃.

3.2.1.3. Sample Segmentation

So far, a whole blood sample has been air segmented before injection, in order to avoid the lysis of the blood cells when in contact with mobile phase [15]. Yet, during in-line heat-shock treatment, an additional temperature dependent convection of the

blood sample within the heated capillary caused a mixing of blood cells with the mobile phase. This dilution leads to a modified hematocrit. Thus, the heating time which is dependent on hematocrit, cannot be adjusted properly in order to disintegrate the blood cells in a highly efficient and reproducible way. In addition, the dilution with the mobile phase causes unwanted hemolysis.

Thus, much more efficient sample segmentation has been developed by adding additional plugs of sodium chloride solution. In addition, volume and position of those plugs were optimized.

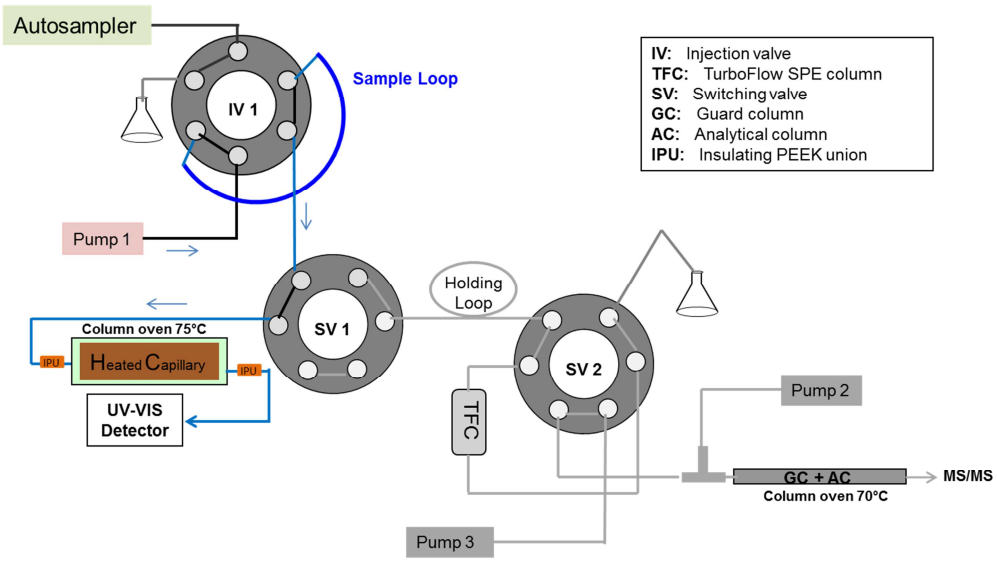


Figure 24: Instrumental set-up for optimization of sample segmentation

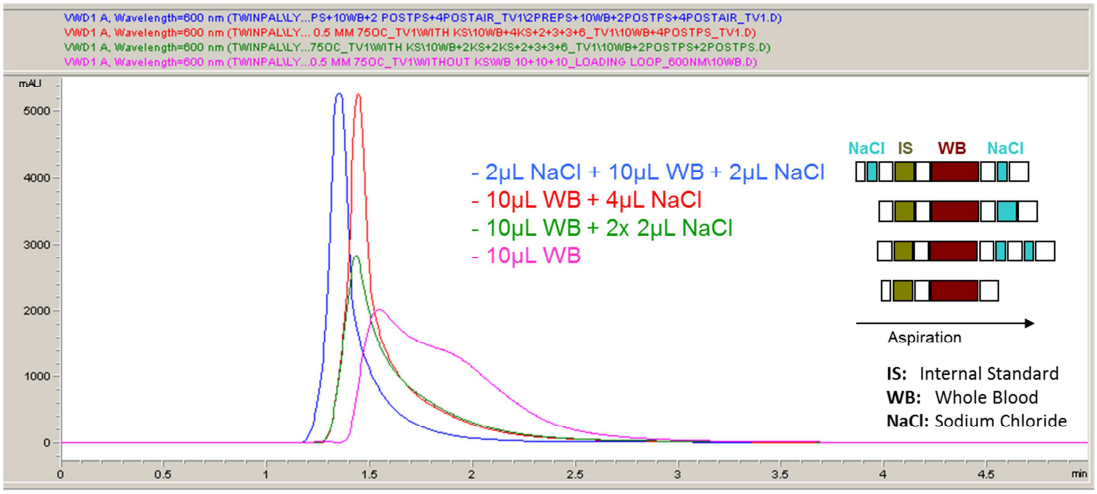
The system configuration for optimization of the sample segmentation is shown in Figure 24. The dilution of the processed blood sample, i.e. CDB was directly monitored with a UV-VIS detector. The wavelength was set to 600 nm, the absorption minimum of hemoglobin, because the detection of hemoglobin in CDB samples at 428 nm (maximum absorption of hemoglobin) exceeded the linear measuring range.

As shown in Figure 25, addition of a post-sample sodium chloride solution plug considerably reduced the dilution of the blood sample, and thus its elution time. This is reflected in a sharper dispersion profile, i.e. peak compression of the hemoglobin fraction (Figure 25: compare the red and green chromatograms to the pink one).

The sample segmentation was further optimized by introducing a pre-sample sodium chloride solution plug (the blue chromatogram in Figure 25). By this measure the

peak maximum shifted forwards and the overall elution time of the CDB matrix was further reduced from 3 minutes to 2 minutes in total.

It is clear, pre- and post-sample sodium chloride solution plug coupled to air segment efficiently protected whole blood sample from dilution by aqueous mobile phase and lysing of blood cells before in-line processing.



Processing	Sample : 10 µL of whole blood Heat-Shock treatment (75°C) and conversion into CDB Mobile Phase : H_2O/ACN (95/5, v/v) ; Flow-rate : 200 µL/min ; 5 min
Detection	UV-VIS Detector, 600nm

Figure 25: Optimization of sample segmentation: Elution profiles of a differently segmented blood sample.

3.2.1.4 Optimization of syringe speed for aspiration and dispensing

A major problem to be solved during the development of the described instrumental platform was the dosing and injection of a sample of whole blood.

For conventional applications (i.e. diluted and/or precleaned samples), the speed of the injection syringe is set to 100 µL/s. However, a sample of whole blood is very viscous and thus contaminates the injection line very easily. This leads to significant carry over effects. In this work it was found that sampling of whole blood at a syringe speed of 2 µL/s in combination with the optimized aspiration and segmentation sequence completely reduced carry over effects.

3.2.1.5 Optimization of in-between and after injection wash of injection needle

As mentioned above (3.1.1), the injection needle should be washed from outside by active wash after sampling of blood. In this step, the needle penetration at wash 1 station for active wash was optimized. It should be deep enough, so that the outer surface of the needle, which has been in contact with blood, can be completely cleaned by wash solvent. On the other hand, there should be some space between the bottom of wash 1 station and the tip of the injection needle. This prevents that the wash solvent is pumped into the lumen of the needle.

For wash of the whole sample path using DLW wash function (3.1.1), the components of the wash solvents had to be optimized. Residual blood is removed by rinsing with an aqueous solvent. Remaining analyte(s) on the surface of the injection path is washed away with an organic solvent. It turned out, that the optimal combination for the DLW wash is: H_2O/ACN (95/5, v/v, wash 1) for the aqueous needle rinse and a mixture of organic solvents (wash 2) for the organic needle rinse. The ACN is present in wash 1 to prevent microbial growth in the reservoir.

It is known that adding an organic solvent to a biological sample causes protein precipitation. Therefore the organic solvent should never come in contact with the blood sample. Otherwise the injection line would get clogged. So after the sample injection, the syringe and injector was washed first with wash 1, then with wash 2, and again with wash 1 to remove organic solvent.

3.2.2 In-line processing of blood samples by heat-shock treatment

The in-line processing of anticoagulated whole blood by heat-shock treatment and the corresponding processing parameters (t_{min}, t_{max}, t_{heat}) were described by Milojković [16]. The same processing parameters determined by Milojković were used here. For a mixture of whole blood and IS in 2.5 vol% DMSO, the optimal heating time is 13 seconds at 75 ℃. The timing of the sample and mobile phase, respectively, can be achieved by adjusting the flow rate. The flow rate is calculated

by dividing the volume (59 µL) of the heated capillary (300 x 0.5 mm ID) by the heating time (13 seconds), yielding 270 µL/min.

Besides temperature and heating time, the processing procedure is predominantly affected by dilution of blood sample and lysis of blood cells by the aqueous mobile phase (see also 3.2.1.3). In order to minimize these effects, the blood sample has to be segmented accordingly (see 3.2.1.3). In addition, the dead volume between the injection port and the heated capillary has to be as small as possible. This could be achieved by connecting the heated capillary directly to the injection valve, as shown in Figure 26. Instead of placing the heated capillary in a column oven (Figure 22 A, Figure 24), a heated sleeve (250 x 1.587 mm ID, AgileSLEEVE Plus Capillary Heater) was used to heat the stainless-steel HPLC capillary.

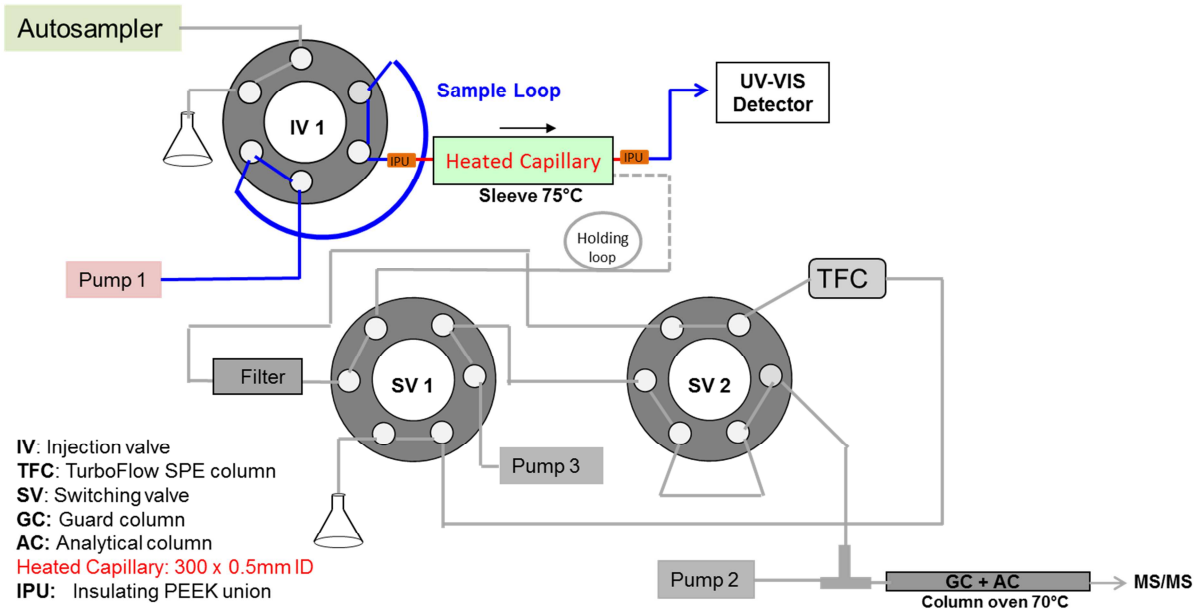


Figure 26: Instrumental set-up for monitoring the heat-shock treatment process of whole blood.

The duration of the total blood-processing procedure was monitored by connecting the outlet of the heated capillary to a UV-VIS detector. 20 µL of whole blood were injected into the sample loop and pumped through the heated capillary to the UV-VIS detector with a flow rate of 270 µL/min and a mobile phase composed of H_2O/ACN (95/5, v/v).

As shown in Figure 27, the overall duration for in-line processing of 20 µL whole blood was 45 seconds.

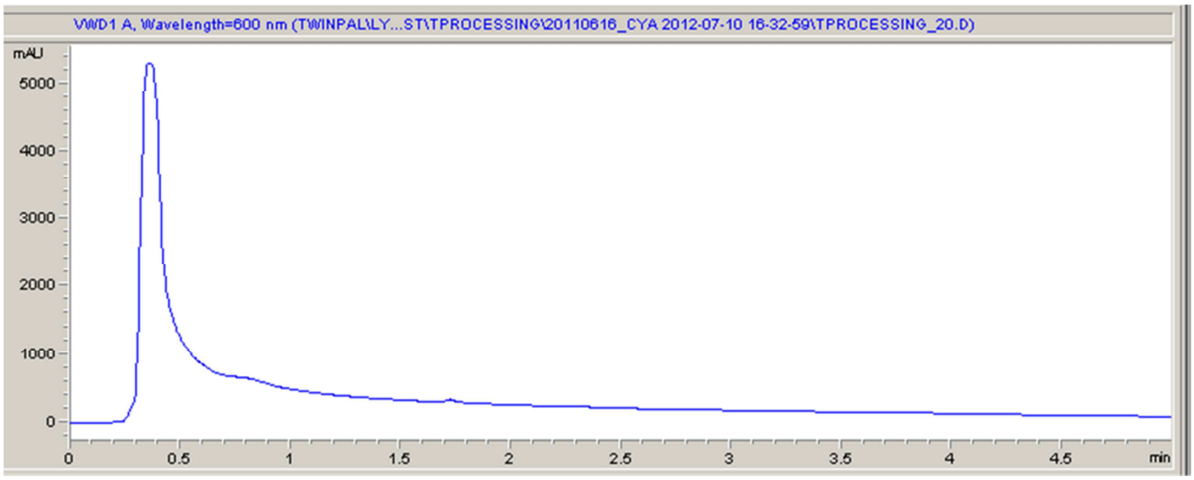


Figure 27: Monitoring of the overall heat-shock treatment process of whole blood via a UV-VIS detector: Elution profile of a segmented and processed blood sample.

Comparison of Figure 27 and Figure 25 shows, that reduction of the dead volume between the injection port and the heated capillary shifted the peak maximum forwards. In addition, the peak-dispersion was significantly diminished, as seen in the reduction of the half-peak-width from 10 seconds to 7 seconds.

3.2.3 Development of an on-line SPE method for clean-up of a cell-disintegrated blood (CDB) sample

For on-line solid phase extraction of processed whole blood (i.e. CDB), attentions should be taken in order to prevent clogging of capillaries and column sieves by particles / precipitate. First, the inner diameter of capillaries between sample inlet and SPE-column as well as between SPE-column and valve-port “waste” should be 0.5 or 0.8 mm. Furthermore, a replaceable in-line filter should be inserted between sample inlet and SPE column.

The most essential part of the in-line filter is a special three-layered stainless steel sieve, the smallest pore diameter of which is 1µm. This sieve is positioned in the housing, which is free of dead volume, and sealed by a Teflon seal together with a

conical pressure screw (Figure 28). As only one sieve is used in this in-line filter, it is also called (in-line) single sieve filter. Because of the small pore diameter of the sieve, all the components and agglomerates formed during the in-line heat-shock treatment of blood samples and being larger than 1 µm are withheld by the in-line filter. This prevents the clogging of the SPE column and even the analytical column.

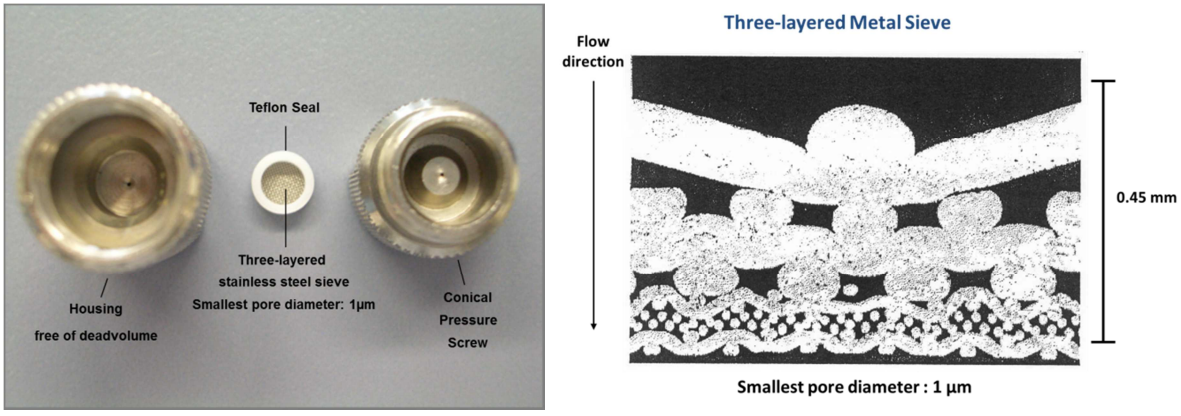


Figure 28: Parts of single sieve filter.

Clean-up of the processed blood sample, i.e. cell-disintegrated blood (CDB) was performed on-line using a TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) coupled to an in-line single sieve filter. The first step to set-up an on-line SPE system was to determine the valve switching times (3.2.3.1). After that, the wash process for the single sieve filter and the SPE column was optimized separately (3.2.3.2).

3.2.3.1 On-line SPE: Determination of valve switching times

The operational procedure and steps to determine valve switching times are described in the next paragraphs.

Step 1: Determine time for complete depletion of sample matrix = t_M

After in-line processing of whole blood, the cell-disintegrated blood matrix is fractionated into analyte and sample matrix. The target analyte is retained on the

stationary phase of the SPE column, and the residual sample matrix is flushed to waste.

When depleting a protein matrix, care should be taken to ensure that the loading mobile phase does not possess denaturing properties. With regard to the amount of organic modifier, it is recommended to use less than 15 vol% methanol, or less than 10 vol% acetonitrile or less than 5 vol% isopropanol. For displacement of protein bound analytes, such as immunosuppressant, the addition of 2 to 5 vol% of ACN and 2 to 10 vol% of MeOH is recommended. In this work, a mixture of 95/5 H_2O/ACN has been used as mobile phase for fractionation.

In order to determine the parameter t_M, the TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) was directly coupled to UV-VIS detector (Figure 29). Then the elution profile of the CDB matrix was monitored at 428 nm, the absorption maximum of hemoglobin.

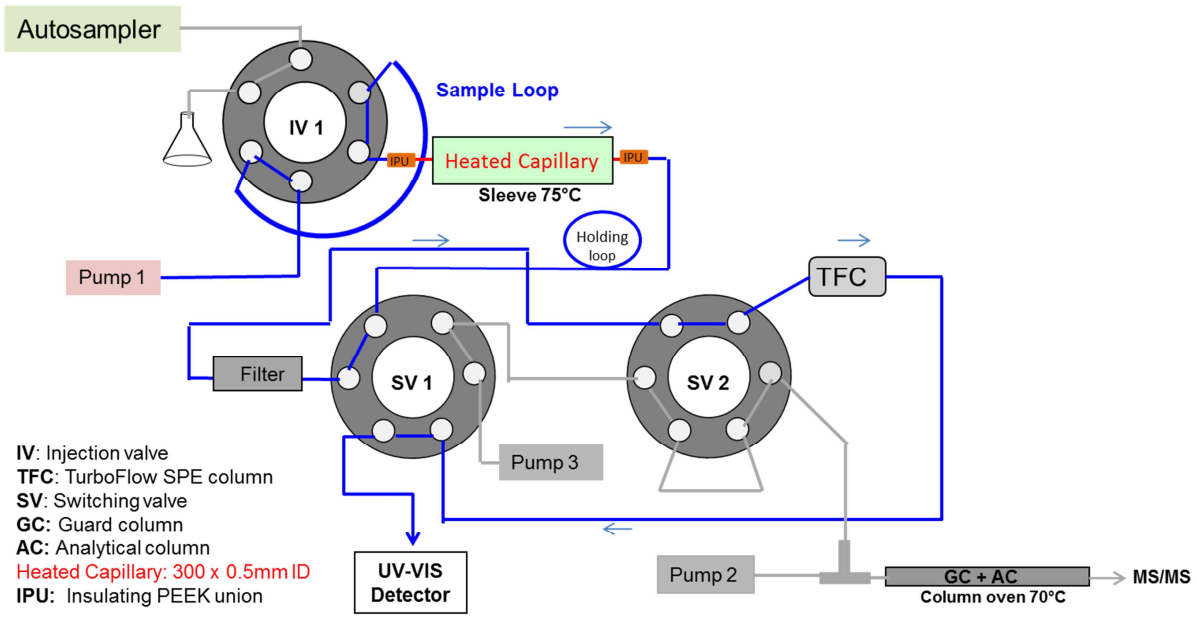
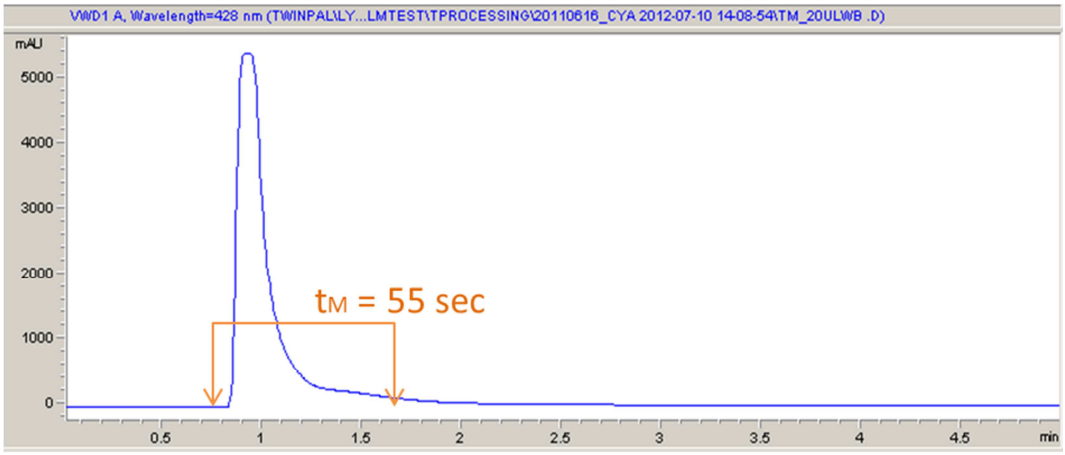


Figure 29: On-line SPE: Instrumental set-up for the determination of the SPE-parameters tM and tA.

The TurboFlow SPE column (50 x 0.5 mm ID) requires a high flow rate for sample fractionation. Such a high flow rate (2 mL/min) and linear flow velocity (10.2 m/min), respectively, is not compatible with the ones (270 µL/min, linear velocity: 1.38 m/min) applied for the heat-shock treatment. The solution was, to store the processed sample, i.e. CDB in a holding loop (254 x 1.0 mm ID, 200 µL holding volume). This

technical “trick” allows pumping the CDB sample through the TurboFLow SPE column at the required flow rate of 2 mL/min. The elution profile of the CDB matrix was monitored at 428 nm, i.e. the absorption maximum of hemoglobin. A representative elution profile of a CDB matrix is shown in Figure 30. The matrix elution time t_M for 20 µL of processed whole blood (CDB) on the TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) amounts to 55 seconds at a flow rate of 2 mL/min.



Sample	20 µL of whole blood and CDB, respectively
SPE column	TurboFlow Cyclone-P (50 x 0.5 mm ID)
Mobile Phase	H_2O / ACN (95/5, v/v) , 270 µL/min for 45 seconds; followed by 2 mL/min for 4,25min
Detection	UV-VIS Detector, 428 nm

Figure 30: On-line SPE: Monitoring of elution profile of CDB matrix and determination of SPE-parameter t_M.

Step 2: Determine breakthrough time (t_A) of analyte

For the determination of the parameter value tA the same instrumental set-up as for tM (Figure 29) is used. Instead of a UV-VIS detector, the tandem mass spectrometer is used to monitor the breakthrough profile of the analyte Cyclosporine A on the TurboFlow SPE column.

A standard solution of the analyte is injected onto the SPE column. The SPE column is operated at a high flow rate of 2 mL/min. After fractionation for 12 minutes with H_2O/ACN (95/5, v/v) at 2 mL/min, the extracted analyte is desorbed with 100 % MeOH (see also step 4) at the same flow rate for 4 minutes. The resulting elution profile of the analyte is shown in Figure 31. No signal is detected for the first 12

minutes (no breakthrough). After switching to 100 % MeOH, the analyte is immediately desorbed. Thus, the breakthrough time of analyte t_A exceeds 12 minutes at a flow rate of 2 mL/min.

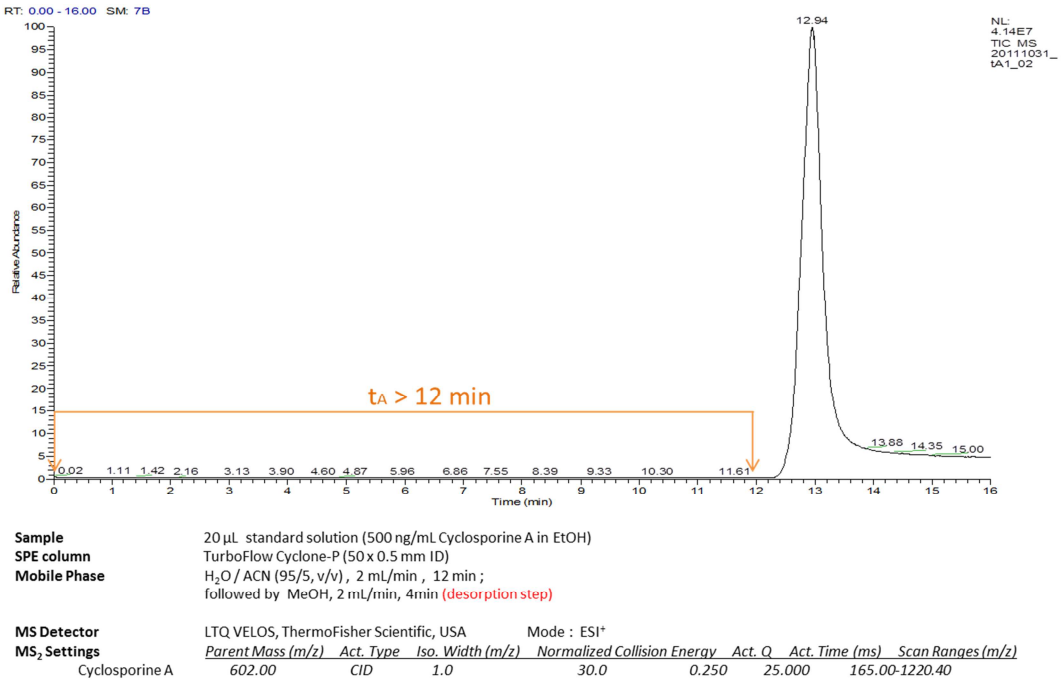


Figure 31: On-line SPE: Monitoring of breakthrough of model analyte CyA and determination of SPE-parameter t_A.

Step 3: Calculate the first switching time for both switching-valves

The first switching time (t_{V1}) for both switching-valves terminates the fractionation step. Simultaneously, the SPE-column is coupled with the analytical column. The parameter t_{v1} is calculated as follows:

$t_{v1} = t_{processing\ step} + t_M + x$ **(Equation 2)**

$= 45\ s + 55\ s + 10s = 110\ s$

x (= 10 s) is the add-on time for security / robustness reasons.

Step 4: Determine desorption / transfer time (t_T) of analyte(s) from SPE column

The instrumental set-up for the determination of t_T is displayed in Figure 32.

First, a standard solution of the analyte is injected, and pumped at a flow rate of 270

µL/min through the heated capillary (75 °C) with the mobile phase used for the fractionation step H2O/ACN (95/5, v/v) and “parked” in the holding loop (see Figure 32 A). After 45 seconds, the flow rate is enhanced to 2 mL/min for 65 seconds in order to operate the TurboFlow SPE column under optimal conditions for extraction of the target analyte.

Switching the valve after 110 seconds couples the SPE column with the mass spectrometer. The analyte is back-flushed and transferred from the SPE column to the MS detector. The transfer time is defined as complete elution of the analyte from the SPE column.

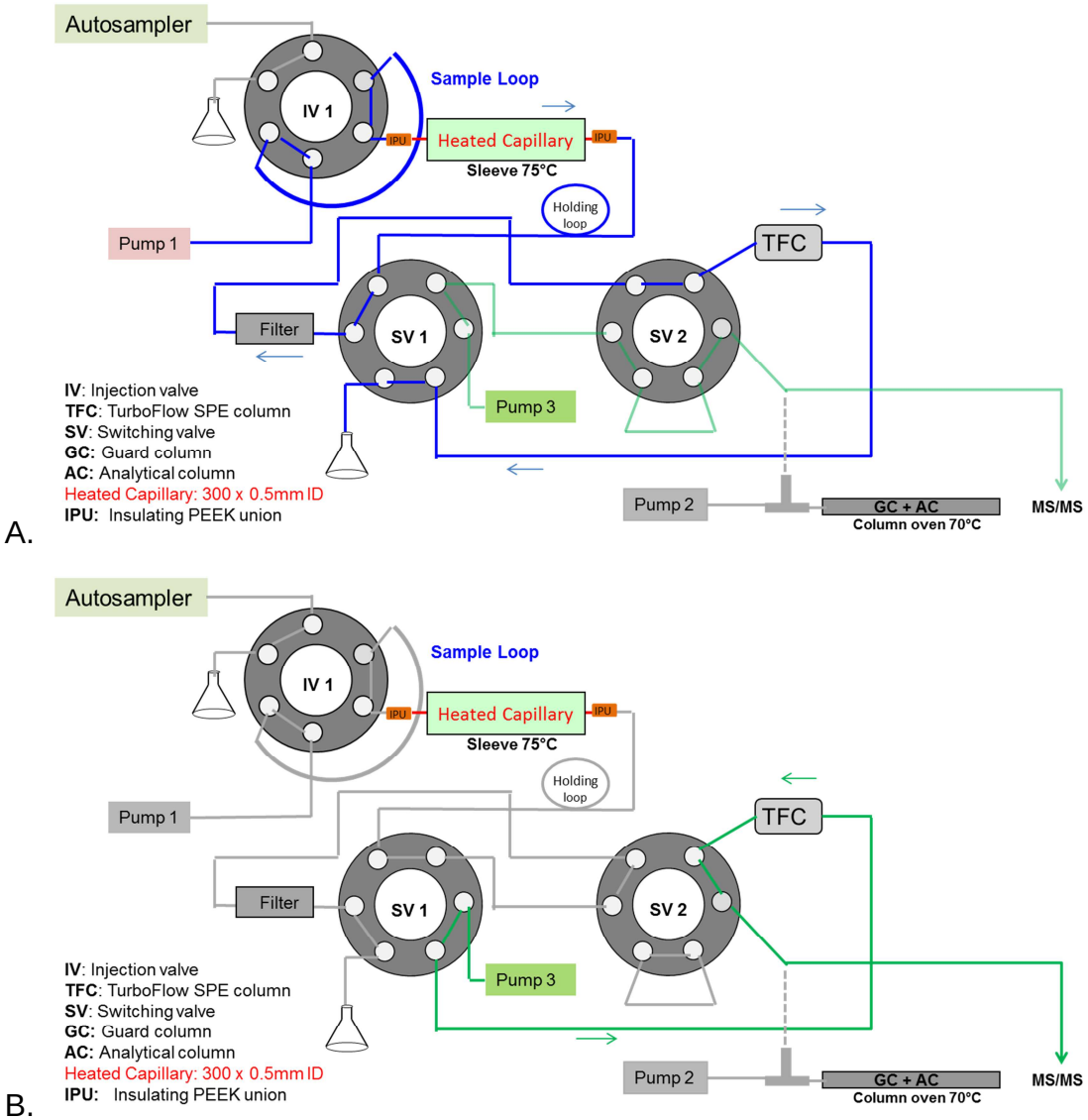


Figure 32: Instrumental set-up for the determination of the SPE-parameter t_T.

A) Extraction of target analyte on TurboFlow SPE column; B) Desorption of target analyte from TurboFlow SPE column and transfer to analytical column

To determine the optimal amount of organic modifier for the elution step, a series of isocratic elutions with varying organic concentrations is run. A standard solution of Cyclosporine A is directly injected onto the TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) and then eluted by a mobile phase containing different amounts of Methanol. In order to assure quantitative elution, i.e. transfer to a series-connected analytical column, an additional desorption step with 100% MeOH is applied. The resulting elution profiles are shown in Figure 33. As demonstrated in chromatograms C and D, memory effects occur when eluting the target analyte with less than 80% of MeOH.

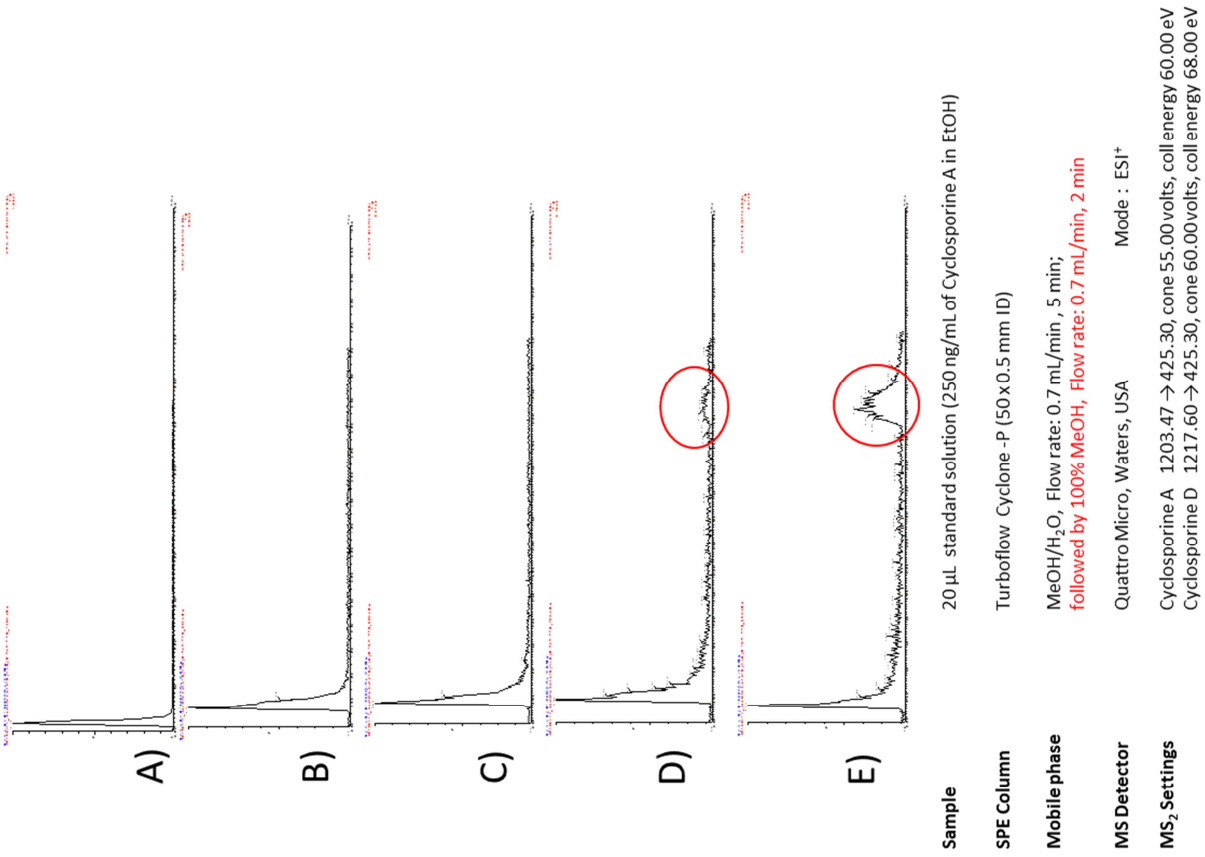
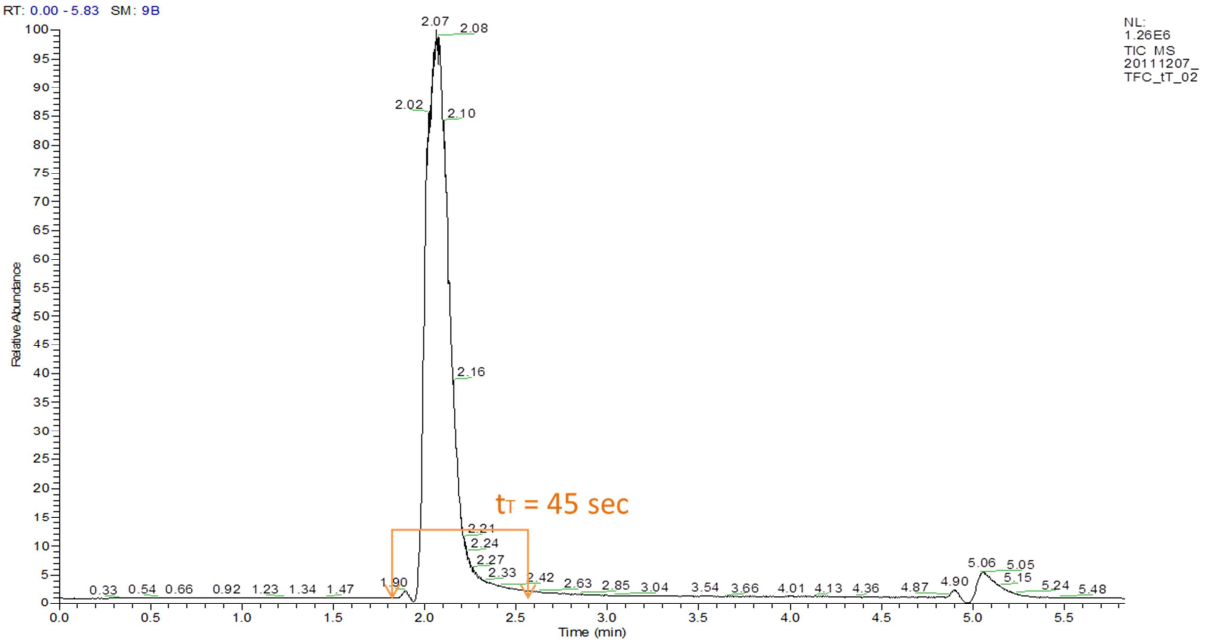


Figure 33: On-line SPE: Elution profiles of Cyclosporine A from the SPE column under varying isocratic conditions.

A) MeOH/H_2O, 100/0, v/v; B) MeOH/H_2O, 90/10, v/v; C) MeOH/H_2O, 80/20, v/v; D) MeOH/H_2O, 70/30, v/v; E) MeOH/H_2O, 60/40, v/v

As a result, a mobile phase composed of 100 % MeOH was chosen for the elution and transfer step, respectively.

As shown in Figure 34, the total elution/transfer time of analyte CyA from the TurboFlow SPE column to the analytical column was 45 seconds at 200 µL/min (see 3.2.4).



Sample	20 µL standard solution (500 ng/mL Cyclosporine A in EtOH)
Processing	Heat-shock treatment (13 sec at 75°C), Mobile Phase: H_2O/ACN (95/5, v/v); Flow-rate: 270 µL/min; 45 sec
Fractionation	SPE column: Turboflow Cyclone P (50 x 0.5 mm ID)
	Mobile Phase: H_2O/ACN (95/5, v/v); Flow-rate: 2 mL/min; 65 sec
Transfer	Mobile Phase: MeOH; Flow-rate: 200 µL/min; 4 min

MS Detector LTQ VELOS, ThermoFisher Scientific, USA Mode: ESI^+

MS_2 Settings

	Parent Mass (m/z)	*Act. Type*	*Iso. Width (m/z)*	*Normalized Collision Energy*	*Act. Q*	*Act. Time (ms)*	*Scan Ranges (m/z)*
Cyclosporine A	*602.00*	*CID*	*1.0*	*30.0*	*0.250*	*25.000*	*165.00-1220.40*

Figure 34: On-line SPE: Monitoring of desorption and transfer of model analyte CyA from the SPE column and determination of SPE-parameter t_T.

Step 5: Calculate the second switching time for both switching-valves

The second switching time (t_{V2}) for both switching-valves terminates the transfer step. Simultaneously, the analytical column is decoupled with the SPE-column. The parameter t_{v2} is calculated as follows:

$t_{v2} = t_{v1} + t_T + y$ **(Equation 3)**

$= 110 \text{ s} + 45 \text{ s} + 15\text{s} = 170 \text{ s}$

y (= 15 s) is the add-on time for security / robustness reasons.

3.2.3.2 Optimization of washing step for in-line filter and SPE column

As mentioned above, the smallest pore diameter of the three-layered stainless steel sieve in the in-line filter is 1µm. Thus, all components and agglomerates formed during the in-line heat-shock treatment of blood samples and being larger than 1 µm, are withheld by the in-line single sieve filter. This prevents the clogging of the SPE column and even the analytical column. However, these residues can cause significant carry over effects. This problem is solved by back-flushing the in-line single sieve filter during the transfer step. First, an aqueous mobile phase (H_2O/ACN, 95/5, v/v) is used to remove the residues from the filter at a flow rate of 2 mL/min for 30 seconds. Secondly, any remaining analyte(s) on the surface of the filter is washed away using a mixture of Acetonitrile /Isopropanol/Acetone (45/45/10, v/v/v) at a flow rate of 2 mL/min for 30 seconds.

After transfer of analyte from the TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) to the analytical column, the SPE column is disconnected from the analytical column and series-connected with the in-line single sieve filter (see Figure 41). A mixture of organic solvent is used to clean the in-line single sieve filter and TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) at a flow rate of 2 mL/min for 185 seconds (see 3.2.6).

3.2.4 Optimization of the analytical separation of the model analyte(s)

In this work, a pentafluorophenyl (PFP) analytical HPLC column (Hypersil GOLD PFP, 50 x 2.1 mm ID, dp 5 µm) with a guard column (Hypersil GOLD PFP, 10 x 2.1 mm ID, dp 5µm) both operated at 70℃ in a column oven was used for the separation of the model analyte(s). The separation of the analyte(s) is influenced by the mobile phase used for the transfer step of the analyte(s) from the SPE column to the analytical column. Therefore optimal transfer and separation conditions for the model analyte(s) had to be investigated. Finally, the wash process for the analytical column was optimized.

3.2.4.1 Optimization of transfer conditions

This goal was achieved by a so-called in-line dilution step. As shown in Figure 35 a mixing T union was installed in front of the analytical column and was connected to pump 2 and the TurboFlow SPE column via switching valve 2 (SV 2). This instrumental set-up allows the addition of an aqueous mobile phase by pump 2 to the eluate of the SPE column before it reaches the top of the analytical column. In this way the amount of organic modifier can be decreased to such an extent that the analyte(s) are enriched at the top of the analytical column (peak compression or focusing) prior to their separation.

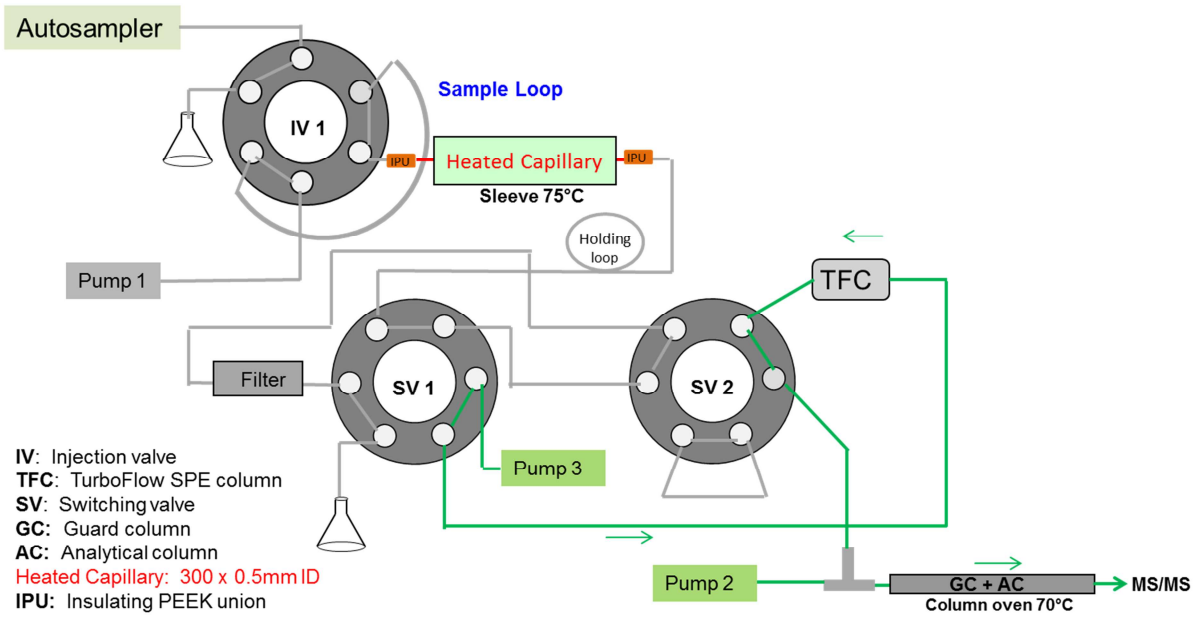
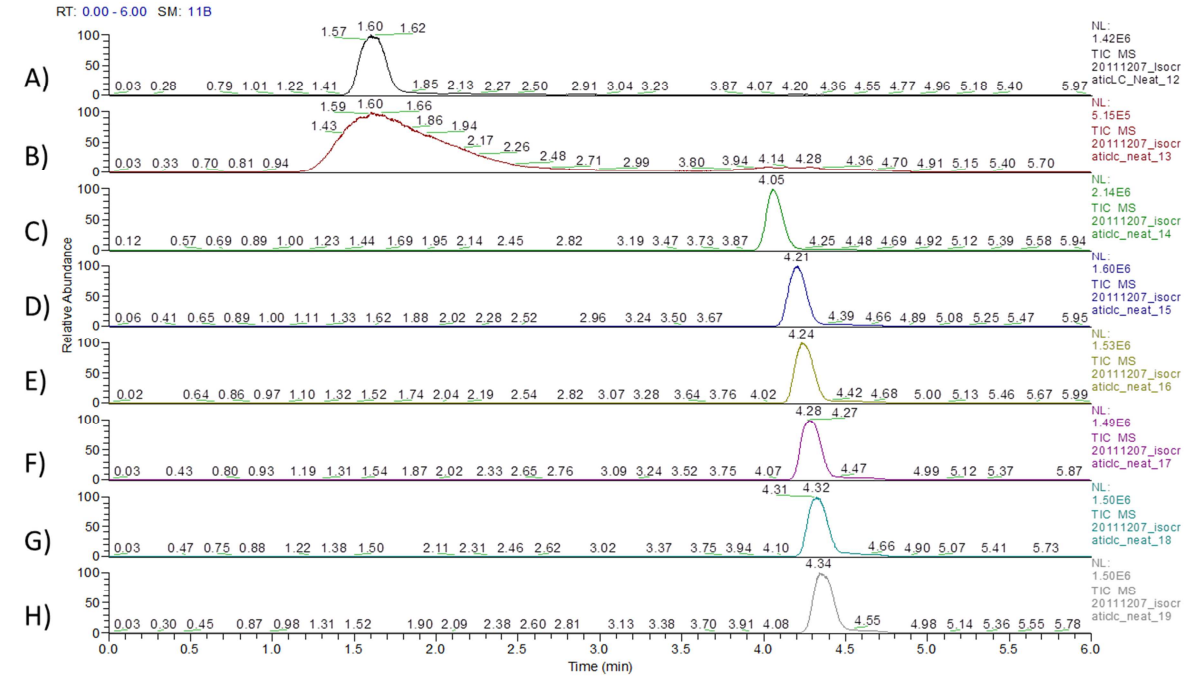


Figure 35: Instumental set-up for in-line dilution of the eluate from the SPE column.

In order to find out the optimal composition, i.e. the amount of organic modifier of the mobile phase for the separation of the model analyte(s), a series of experiments were carried out (Figure 36).



Sample	10 µL standard solution (500ng/mL of Cyclosporine A in EtOH)
Analytical Column	Hypersil GOLD PFP (50 x 2.1mm ID), dp 5 µm; encased in a 70°C column oven
Elution mobile phase	MeOH / H_2O, Flow rate : 0.7 mL/min , 2.5 min followed by 100% MeOH, Flow rate : 0.7 mL/min, 3.5 min
MS Detector	LTQ VELOS, ThermoFisher Scientific, USA Mode : ESI+

MS_2 Settings

	Parent Mass (m/z)	*Act. Type*	*Iso. Width (m/z)*	*Normalized Collision Energy*	*Act. Q*	*Act. Time (ms)*	*Scan Ranges (m/z)*
Cyclosporine A	*602.00*	*CID*	*1.0*	*30.0*	*0.250*	*25.000*	*165.00-1220.4*

Figure 36: Separation profiles of Cyclosporine A from the analytical column using decreasing amounts of organic modifier.

A) MeOH/H_2O, 90/10, v/v; B) MeOH/H_2O, 80/20, v/v; C) MeOH/H_2O,70/30, v/v; D) MeOH/H_2O, 60/40, v/v; E) MeOH/H_2O, 50/50, v/v; F) MeOH/H_2O, 40/60, v/v; G) MeOH/H_2O, 30/70, v/v; H) MeOH/H_2O, 20/80, v/v

For this purpose, a standard solution of Cyclosporine A was directly injected onto the analytical column (Hypersil GOLD PFP, 50 x 2.1 mm ID, dp 5 µm) and eluted by a mobile phase containing different amounts of MeOH. In order to assure quantitative elution of the analyte(s) from the analytical column, an additional desorption step with 100% MeOH has been applied. The resulting chromatograms are shown in Figure 36. No retention of the model analyte Cyclosporine A on the analytical column is achieved when eluting with more than 80% of MeOH, as demonstrated in chromatogram A and B.

As a result, a mobile phase composed of MeOH/H_2O (30/70, v/v) was chosen as the optimal mobile phase. This decision was made with regard to other immunosuppressants, which are less hydrophobic than Cyclosporine A and thus can be eluted from the analytical even with less than 80% MeOH.

The in-line dilution needed for the transfer step can be calculated using equation 4. Pump 3 is the eluting pump used for the transfer step and pump 2 is the diluting pump:

$$\%\text{MeOH after T union} = \frac{\text{flow of pump3 x \%MeOH of pump3} + \text{flow of pump2 x \%MeOH of pump2}}{\text{total flow rate after T union}} \quad \textbf{(Equation 4)}$$

The total flow rate entering the analytical column (Hypersil GOLD PFP, 50 x 2.1 mm ID, dp 5 µm) after the mixing T union was set to 0.7 mL/min. In order to ensure an optimal timing for the transfer step, the flow rate of pump 3 should be as high as possible. This was achieved by setting the mobile phase of pump 2 during the transfer step to 100% water. As the "%MeOH of pump 2" is 0% and "%MeOH of pump 3" is 100%, the flow rate of pump 3 during the transfer step is calculated according to equation 3, and amounts to 0.2 mL/min.

3.2.4.2 Optimization of the separation step on the analytical column

In order to achieve an optimal separation and timing, a gradient elution was applied. It turned out, that the optimal conditions for the starting mobile phase is MeOH/H_2O (70/30, v/v). This is followed by a linear gradient of MeOH from 70% to 95% within 60 seconds. Finally, isocratic conditions for additional 60 seconds completed the separation step.

3.2.4.3 Optimization of the washing step for the analytical column

After separation of the analyte(s) on the analytical column, the analytical column was washed for 120 seconds at a flow rate of 0.7 mL/min to reduce carry over effects. A mixture of Acetonitrile/Isopropanol/Acetone (45/45/10, v/v/v) turned out to be the most effective solvent.

3.2.5 MS/MS detection of the analyte(s)

A home-made flow splitter was used to reduce the flow (0.7 mL/min) of the mobile phase eluting from the analytical column to a flow of 0.175 mL/min entering the MS ion-source. The splitting of the flow was adjusted to 0.175 mL/min by the length of the tubings (0.13 mm ID) fixed to the flow splitter. In this work, the length of the tubing to the detector was three times of the one to waste.

The optimal MS parameters for detection of Cyclosporine A and the Internal Standards (IS, Cyclosporine D or D12-Cyclosporine A) were tuned at the reduced flow rate of 0.175 mL/min. This was achieved by connecting the solvent flow from the syringe pump and the LC pump 2 by a T-union with the ion source. The syringe pump infused a standard solution of CyA and IS in EtOH at a flow rate of 25 µL/min. The LC pump added a mixture of MeOH/H_2O (95/5, v/v) at a flow rate of 150 µL/min.

In this work, the MS detection was carried out in the ESI^+ mode using two different mass spectrometers: LTQ VELOS (ThermoFisher Scientific, USA) and Micromass Quattro Micro (Waters, USA).

LTQ VELOS Mass Spectrometer:

After programming the mass spectrometer for ESI mode, the scan parameters were set up for a full-scan, single-stage mass analysis. Then the source temperature and gas flow were manually adjusted to establish a stable spray of the ESI calibration solution infused into the ion-source. The stable spray ensures that the mass analyzer is transmitting a sufficient amount of ions to the mass detector. Therefore, the mass spectra of the single charged ions in the calibration solution were monitored. Stable signals which vary by less than about 15% from scan to scan, indicate that the MS is operating properly. The crucial parameters (see below) of the MS were optimized by automatic tuning using the ESI calibration solution. It is recommended to calibrate the mass spectrometer every one to three months of operation for optimum performance over the entire mass range of the detector [107].

The most important parameters that affect signal quality in the ESI mode are: electrospray voltage, heated capillary temperature (voltage), S-lens RF (radio frequency) level, sheath gas flow rate, auxiliary gas flow rate and sweep gas flow

rate. The settings for these parameters depend on the solvent flow rate and target analyte composition. As the analytical sample was run in high-flow ESI mode using a flow rate of 0.175 mL/min in this work, a further tuning with the model analyte(s) in the ESI mode is required. The automatic tuning procedure adjusts the voltages applied to the ion optics until the ion transmission of the model analyte(s) is maximized.

As a result, the following MS operating parameters were used for detection and quantitation of CyA and D12-CyA (Figure 37):

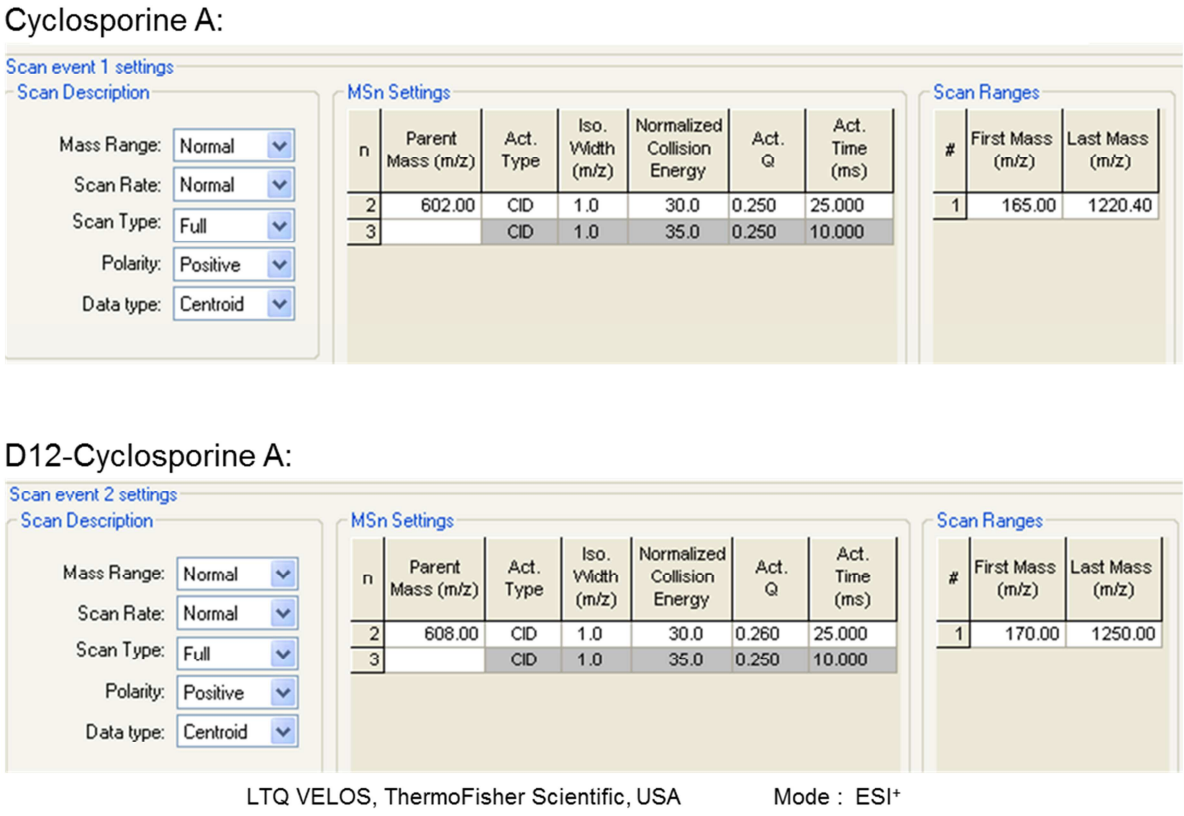


Figure 37: MS operating parameters for detection of CyA and D12-CyA using LTQ VELOS in ESI^+ mode.

Micromass Quattro Micro Mass Spectrometer:

In order to obtain a high mass accuracy, the MS was tuned and calibrated with phosphoric acid in the ESI mode. The tuning parameters in the “ES+Source” and “Analyser” menus were adjusted to optimize peak shape and intensity.

Using a full AutoTune with the model analyte(s) in the ESI mode the voltages applied

to the ion optics were further optimized.

As a result, the following MS operating parameters were used for detection and quantitation of CyA and CyD (Figure 38).

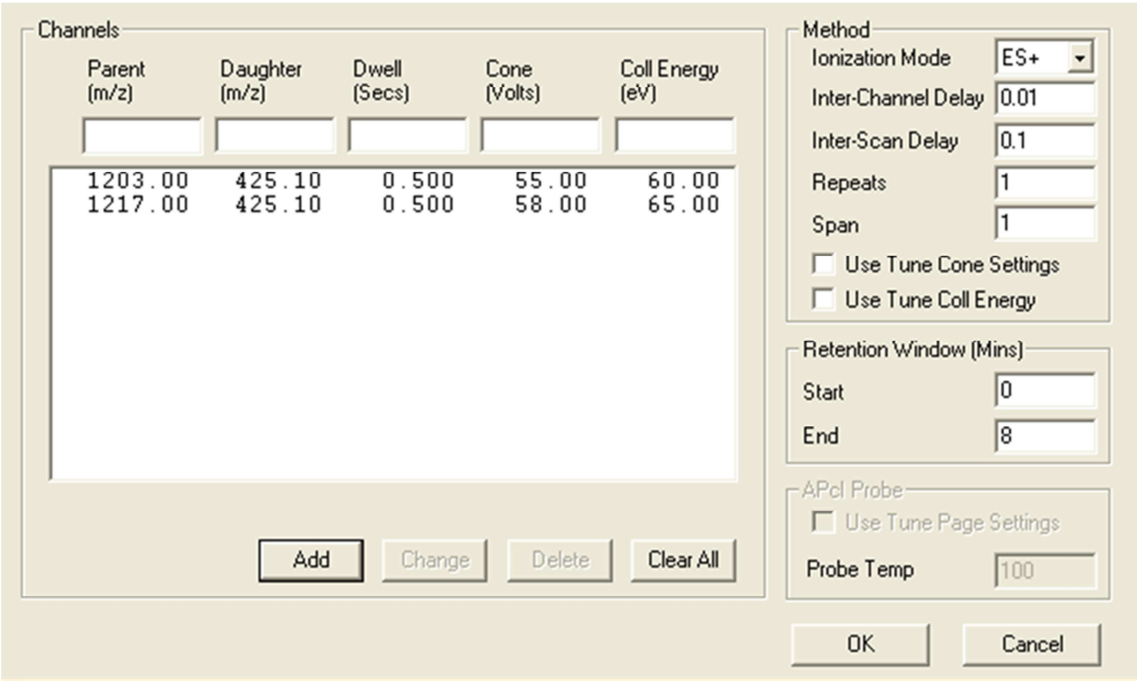
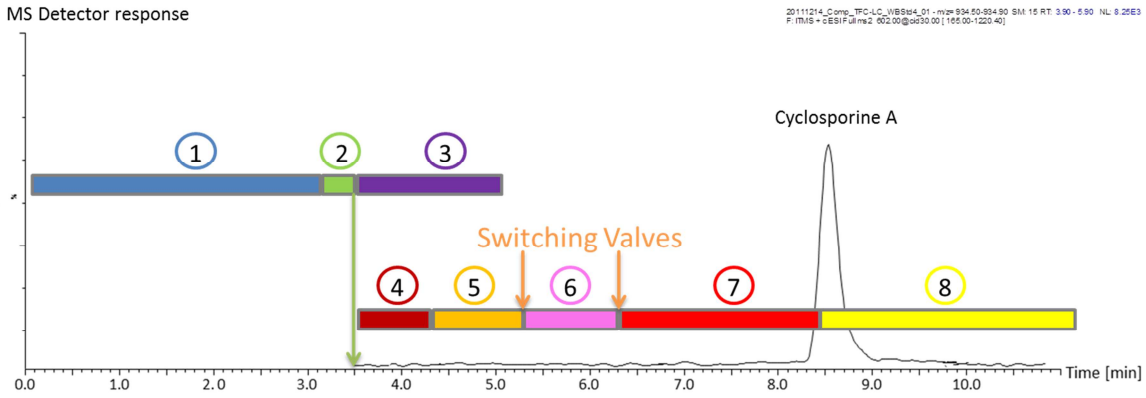


Figure 38: MS operating parameters for detection of CyA and CyD using Quatro Micro in ESI$^+$ mode.

3.2.6 Overall analysis cycle

After the optimization and standardization of the sample handling/injection, the in-line processing and the detection parameters for on-line SPE-LC-MS/MS analysis of the model analyte Cyclosporine A in whole blood, the overall analysis cycle was composed. The overall analysis cycle for the direct determination of Cyclosporine A in an anticoagulated whole blood sample (20 µL) lasts 11.2 minutes, as shown in the representative chromatogram (Figure 39). Individual steps of the overall analysis cycle are color coded.



1. Sample handling: mixing and segmentation of a sedimented whole blood sample (20 µL). Option: In-line addition of Internal Standard (IS)
2. Injection of the segmented whole blood sample
3. Wash of the injection needle after injection
4. In-line processing (heat-shock treatment) of whole blood sample and generation of Cell-Disintegrated Blood (CDB)
5. Fractionation of CDB on TurboFlow SPE column
6. Transfer of analyte(s) from TurboFlow SPE column to analytical column and focusing of the analyte(s) at the top of the analytical column
7. Separation of the analyte(s) on the analytical column
8. Rinsing/reconditioning of filter and columns

Figure 39: Overall analysis cycle for the analysis of Cyclosporine A in a whole blood sample.

The first three steps are related to sample handling and injection (see also 3.2.1) and are performed by the autosampler. First, a sedimented whole blood sample is remixed by the vortexer. Then the homogenized blood sample is aspirated following

the optimized segmentation sequence described in 3.2.1.3. After this step, the injection needle is washed externally to remove remaining contaminations. Then the segmented blood sample is injected into the sample loop at a speed of 2 µL/min (Step 2). After the injection (3.5 min), the injection needle is washed using the DLW wash function (Step 3). In parallel, the whole blood sample is processed in-line and cleaned up by on-line SPE (Step 4-5).

For the analysis of Cyclosporine A in whole blood the following filter and columns are used:

Filter: In-line single sieve Filter (free of dead volume, consists of a special three-layered stainless steel sieve with the smallest pore diameter of 1 µm, a Teflon seal, a housing and a conical pressure screw)

SPE-column: TurboFlow Cyclone-P (50 x 0.5mm ID)

LC-column: Hypersil Gold PFP guard column + Hypersil Gold PFP column (10 x 2.1mm ID+ 50 x 2.1mm ID, dp 5 µm)

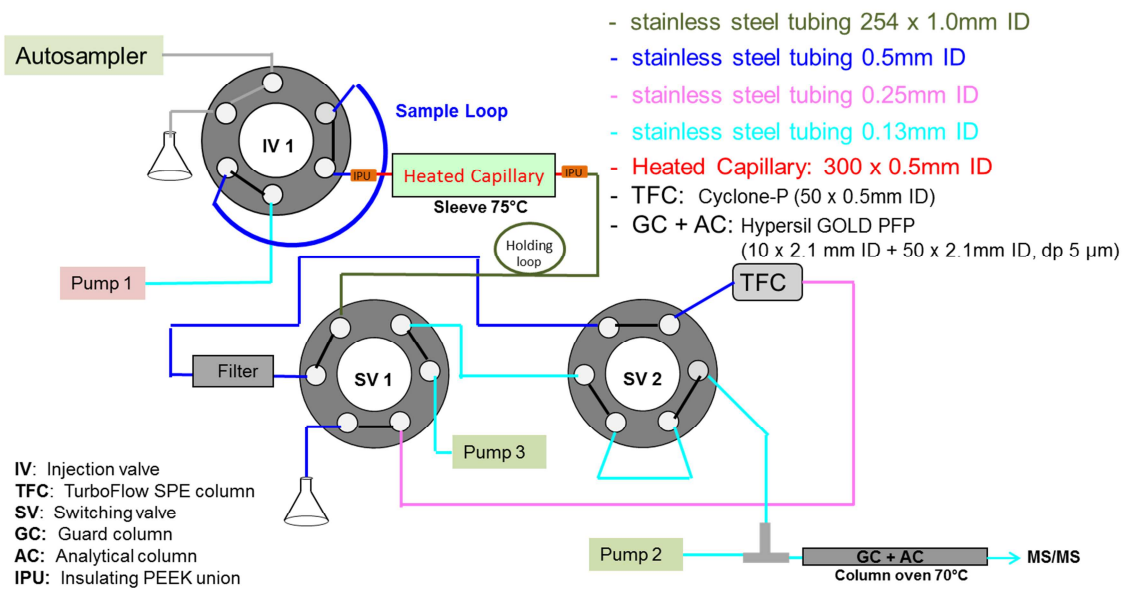
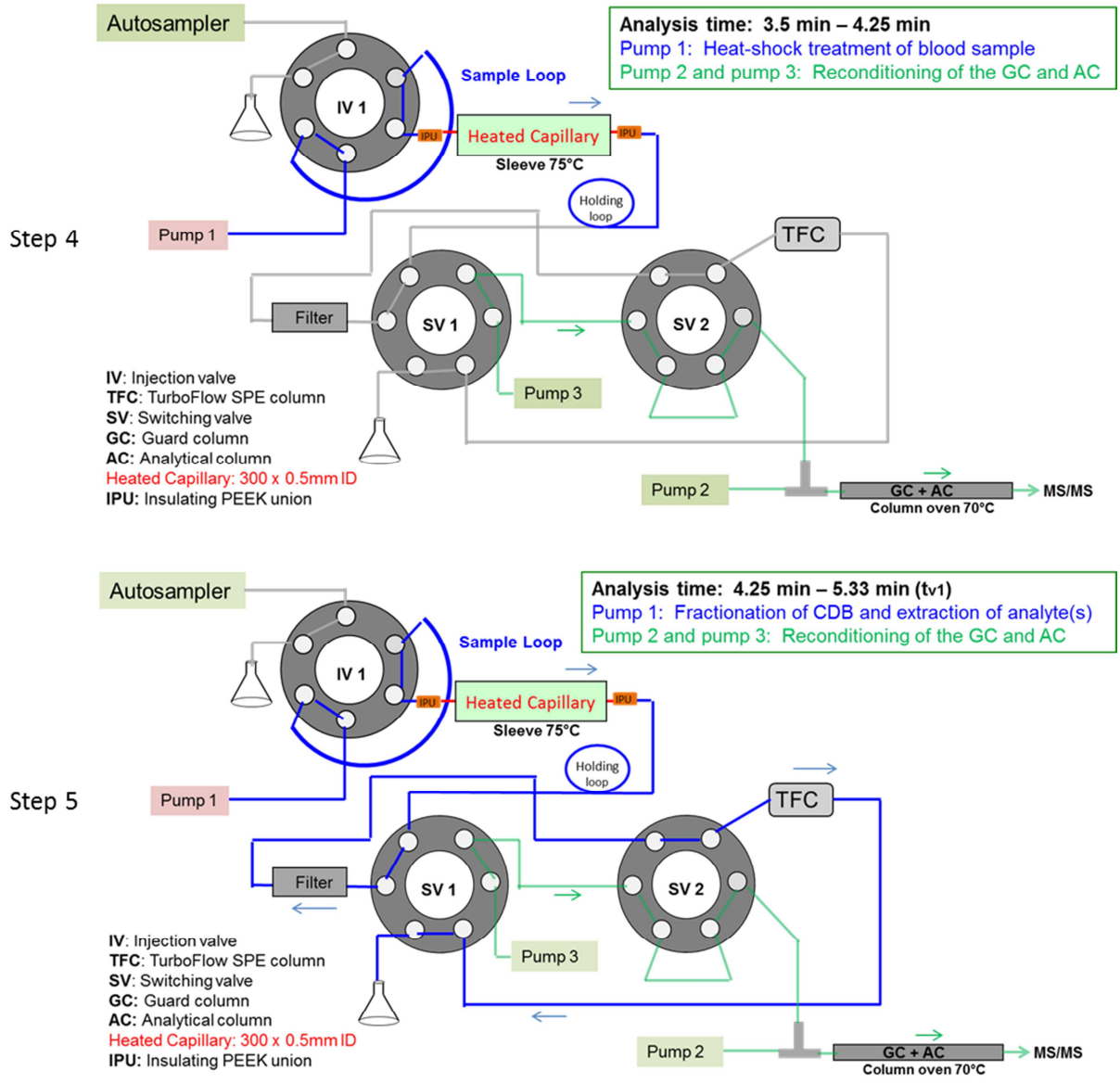


Figure 40: System configuration for in-line processing of a whole blood sample and on-line SPE-LC-MS/MS analysis of the processed sample (CDB): Analysis cycle steps 4-8.

The system configuration for in-line processing of a whole blood sample followed by on-line SPE-LC-MS/MS analysis of the processed sample (CDB) is shown in

Figure 40. The detailed instrumental set-up for the analysis steps 4 – 8 is displayed in Figure 41.

The mobile phases used in each steps of the overall analysis cycle are listed in Table 1. The software Chronos allows a time optimization of sequential analysis. The second analysis starts during the separation step of the analyte(s). Thus the duration for the second and following analysis cycle amounts to 7.7 minutes each (Step 4-8), instead of 11.2 minutes for the first analysis.



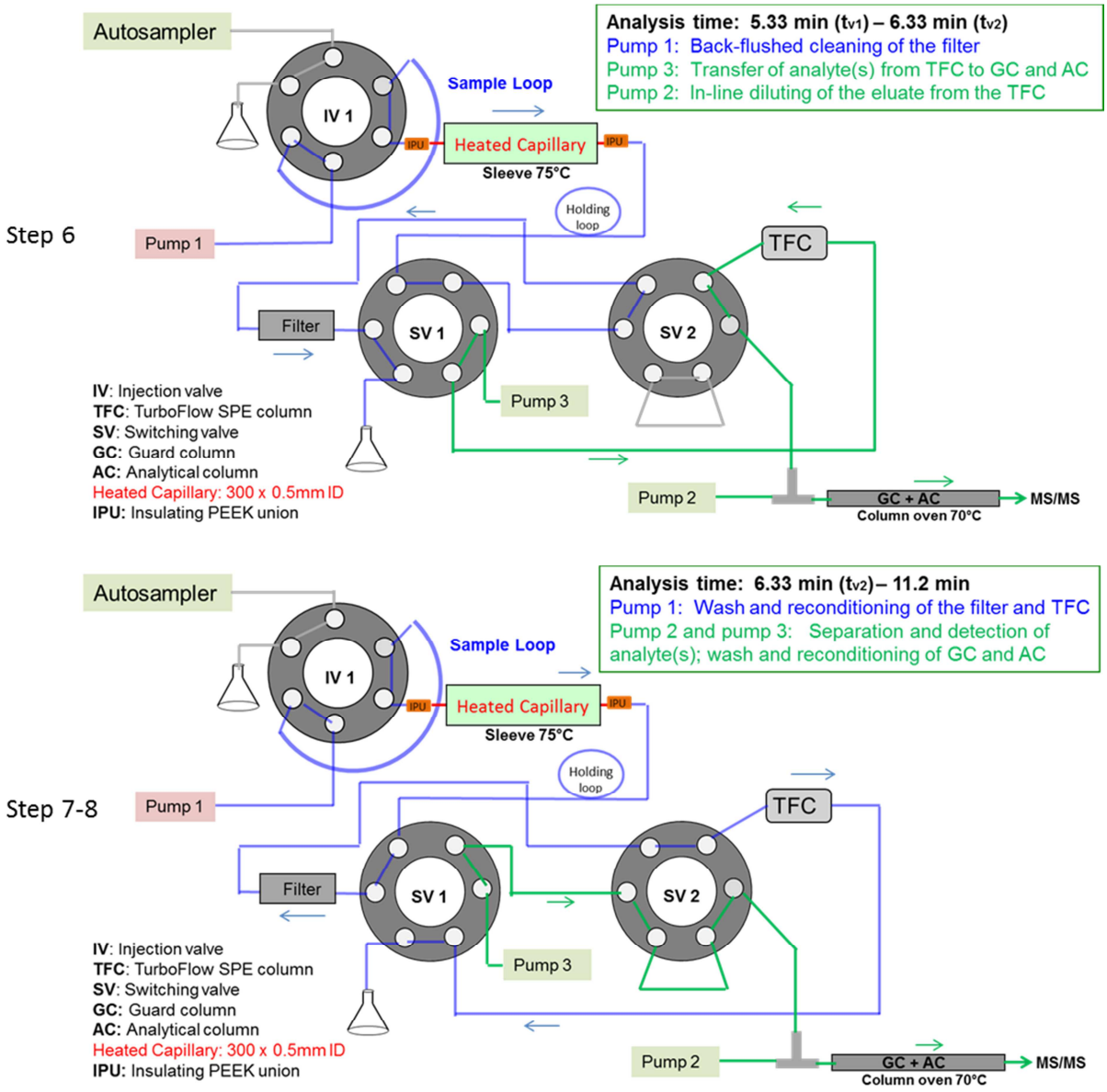


Figure 41: System configuration for in-line processing of a whole blood (heat-shock treatment) and on-line SPE-LC-MS/MS analysis of the processed sample (CDB): Analysis cycle steps 4-7.

A	Analysis Time [min]	Pump 1		
		Mobile Phase	Flow rate [mL/min]	Function
Step 1	0 - 3	H_2O/ACN (95/5, v/v)	1.0	Reconditioning of the SPE column
Step 2	3 – 3.5			
Step 4	3.5 – 4.25	H_2O/ACN (95/5, v/v)	0.27	In-line processing (heat-shock teratment) of whole blood
Step 5	4.25 – 5.33	H_2O/ACN (95/5, v/v)	2.0	Fractionation of processed whole blood (CDB) and SPE extraction of analyte(s)
Step 6	5.33 – 5.83	H_2O/ACN (95/5, v/v)	2.0	Back-flush of single sieve filter with aqueous mobile phase
	5.83 – 6.33	A mixture of organic solvents	2.0	Back-flush of single sieve filter with organic mobile phase
Step 7	6.33 – 6.42	A mixture of organic solvents	2.0	Wash single sieve filter and SPE column with organic mobile phase
	6.42 – 7.42	A mixture of organic solvents	2.0	Clean single sieve filter and SPE column with organic mobile phase
	7.42 – 8.42	A mixture of organic solvents	2.0	Clean single sieve filter and SPE column with organic mobile phase
	8.42 – 9.42	A mixture of organic solvents	2.0	Clean single sieve filter and SPE column with organic mobile phase
	9.42 – 10.4	H_2O/ACN (95/5, v/v)	2.0	Reconditioning of SPE column
	10.4 – 11.2	H_2O/ACN (95/5, v/v)	2.0	Reconditioning of SPE column

B	Analysis Time [min]	Pump 2		
		Mobile Phase	Flow rate [mL/min]	Function
Step 1	0 - 3	H2O	0.5	Reconditioning of analytical column
Step 2	3 – 3.5			
Step 4	3.5 – 4.25	H2O	0.5	Reconditioning of analytical column
Step 5	4.25 – 5.33	H2O	0.5	Reconditioning of analytical column
Step 6	5.33 – 5.83	H2O	0.5	In-line diluting of eluate from SPE column. Focusing of analyte(s) at top of analytical column
	5.83 – 6.33			
Step 7	6.33 – 6.42	H2O/MeOH, (43/57, v/v)	0.5	Start of separation
	6.42 – 7.42	H2O/MeOH, (7/93, v/v)	0.5	Separation of analyte(s) using linear gradient elution within 1 min
	7.42 – 8.42	H2O/MeOH, (7/93, v/v)	0.5	Separation of analyte(s)
	8.42 – 9.42	A mixture of organic solvents	0.5	Wash analytical column with organic mobile phase
	9.42 – 10.4	A mixture of organic solvents	0.5	Wash analytical column with organic mobile phase
	10.4 – 11.2	H2O	0.5	Reconditioning of analytical column

	Analysis Time [min]		Pump 3		
			Mobile Phase	Flow rate [mL/min]	Function
Step 1	0 - 3		MeOH	0.2	Reconditioning of analytical column
Step 2	3 – 3.5				
Step 4	3.5 – 4.25		MeOH	0.2	Reconditioning of analytical column
Step 5	4.25 – 5.33		MeOH	0.2	Reconditioning of analytical column
Step 6	5.33 – 5.83		MeOH	0.2	Transfer of analyte(s) from TurboFlow SPE column to analytical column
	5.83 – 6.33				
Step 7	6.33 – 6.42		MeOH	0.2	Start of separation
	6.42 – 7.42		MeOH	0.2	Separation of analyte(s)
	7.42 – 8.42		MeOH	0.2	Separation of analyte(s)
	8.42 – 9.42		MeOH	0.2	Wash analytical column with organic mobile phase
	9.42 – 10.4		MeOH	0.2	Wash analytical column with organic mobile phase
	10.4 – 11.2		MeOH	0.2	Reconditioning of analytical column

Note: Mobile phases of H_2O, ACN and MeOH contain 10 mM ammonium formate and 0.05 vol% formic acid
Blue shaded area = start of next analysis cycle
SPE = Solid Phase Extraction

Table 1: Time table of overall analysis cycle: Flow rates, compositions of mobile phases and functions of pump settings.

3.3 Evaluation of the total analysis platform (use in-line single sieve filter) for quantitation of Cyclosporine A after heat-shock treatment of whole blood

The described total analysis platform for the detection and quantitation of Cyclosporine A after heat-shock treatment of whole blood has been evaluated with respect to the following:

- Validation parameters
- Robustness

3.3.1 Method validation

The first step to evaluate the described analysis method is to validate it according to widely accepted protocols to confirm that the performance of the method meets the requirements for the intended application. Reliable analytical results are required to comply with international regulations and to ensure patient safety. The U.S.Food and Drug Administration (FDA) regulations require the validation of an analytical method before and during regular routine use [72]. There are many guidelines for validation of analytical assays such as those published by the FDA, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceutical for Human Use (ICH), and the Bundesärztekammer…etc [72,73,108]. In all these guidelines the following validation parameters are fundamental: linearity and range, sensitivity (LOD and LOQ), accuracy, precision, specificity and recovery.

3.3.1.1 Linearity and Range

The linearity of the signal response is determined by preparing a calibration curve within a given (therapeutic) range and is expressed by the regression coefficient.

In this work, the calibration curve was constructed with six different analyte concentrations. The stock solution of the model analyte Cyclosporine A (100 µg/mL) was prepared in DMSO. This solution was added to a drug free EDTA blood sample in order to obtain a 1000 ng/mL solution. This spiked blood sample was further diluted with the original blood sample to obtain the remaining calibration standards: 500, 200, 80, 40 and 10 ng/mL. 10 µL of the Internal Standard solution (10 µg/mL of D12-CyA in DMSO) was added to 490 µL of the calibration standard samples. The drug free EDTA blood sample was used as matrix blank sample.

The calibration curve was constructed from double runs. The peak area ratios of the analyte to the IS were plotted against the concentration of the analyte.

The calibration curve shown in Figure 42 proved to be linear over the range of 10 ng/mL to 1000 ng/mL. The regression coefficient for the calibration curve was 0.990.

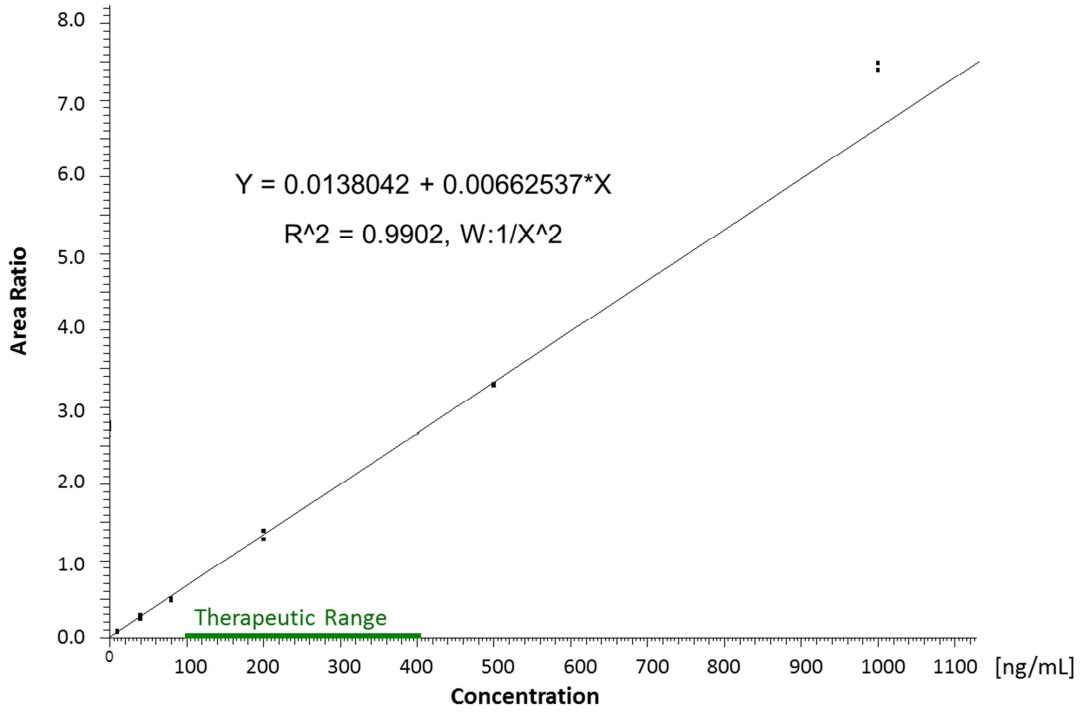


Figure 42: Calibration curve for Cyclosporine A in 20 µL of processed whole blood, i.e. CDB.

3.3.1.2 Sensitivity

The sensitivity of an analytical method is determined by measuring the limit of detection (LOD) and the lower limit of quantitation (LLOQ). The LOD is the lowest analyte concentration that can be distinguished from noise. The signal to noise ratio (S/N) of the analyte peak must be at least 3:1. The LLOQ is defined as the lowest analyte concentration that can be determined quantitatively. The analyte signal must be at least five times higher when compared to a blank sample.

The LOD values were found to be 3.3 ng/mL for CyA in 20 µL of anticoagulated whole blood sample. The LLOQ turned out to be 10 ng/mL.

3.3.1.3 Accuracy and Precision

The accuracy is a measure of the closeness of mean test results obtained by the method to the true concentration of the analyte. The accuracy is calculated using the following equation:

$$\%\text{Diff (Accuracy)} = \frac{\text{Calculated Concentration} - \text{Spiked Concentration}}{\text{Spiked Concentration}} \text{ x } 100\% \quad \textbf{(Equation 5)}$$

It is recommended to measure the accuracy using a minimum of five replicate samples at each concentration level of the quality control (QC), namely low, mid and high level.

The precision is the closeness of agreement (degree of scatter around the mean) between a series of individual measurements obtained from multiple sampling of the same sample. Precision is expressed as percentage of the relative standard deviation (%RSD) of the replicate analyses. It is recommended to measure the precision using a minimum of five replicate samples at each concentration level of the quality control (QC), namely low, mid and high level. Precision in this work was further investigated with respect to intra-day and inter-day variations. The inter-day assay was carried out using daily-prepared QC samples and calibration curve.

The intra- and inter-day accuracy and precision were determined from QC samples

prepared by spiking CyA into a whole blood sample at a concentration of 800 ng/mL and serially diluting the spiked sample with the original whole blood sample to 400 ng/mL and 25 ng/mL.

Table 2 and Table 3 document the data obtained for intra- and inter-day accuracy and precision respectively. The intra-day accuracy ranges from 0.12 to 13.6 %, and the intra-day precision ranges from 4.07 to 7.01 %RSD. The average values of all the three concentration levels from two consecutive runs on two separate days are within 15% of the expected concentration. The inter-day precision ranges from 3.8 to 9.04 %RSD.

CyA Level in blood/CDB	Spiked Concentration [ng/mL]	Calculated Concentration [ng/mL]	Accuracy [%Diff]	Precision %RSD (n=5)
Low 1	25	24.85	-0.59	
Low 2	25	24.04	-3.84	
Low 3	25	28.40	13.60	
Low 4	25	24.29	-2.83	7.01
Low 5	25	24.90	-0.37	
Middle 1	400	392.84	-1.79	
Middle 2	400	390.52	-2.37	
Middle 3	400	391.76	-2.06	
Middle 4	400	429.36	7.34	4.07
Middle 5	400	400.88	0.22	
High 1	800	897.76	12.22	
High 2	800	846.80	5.85	
High 3	800	867.60	8.45	
High 4	800	799.04	-0.12	4.23
High 5	800	860.64	7.58	

Table 2: Determination of intra-day accuracy and precision at 3 different concentration levels of Cyclosporine A in 20 µL of processed whole blood, i.e. CDB.

CyA Level in blood/CDB	Spiked Concentration [ng/mL]	Accuracy [%Diff]		Precision %RSD (n=10)
		Day 1	Day 2	
Low 1	25	-0.59	1.81	9.04
Low 2	25	-3.84	3.89	
Low 3	25	13.60	-9.68	
Low 4	25	-2.83	4.43	
Low 5	25	-0.37	22.96	
Middle 1	400	-1.79	-3.37	3.8
Middle 2	400	-2.37	-2.55	
Middle 3	400	-2.06	0.82	
Middle 4	400	7.34	6.67	
Middle 5	400	0.22	2.95	
High 1	800	12.22	2.91	9.02
High 2	800	5.85	29.90	
High 3	800	8.45	9.26	
High 4	800	-0.12	-6.53	
High 5	800	7.58	1.58	

Table 3: Determination of inter-day accuracy and precision at 3 different concentration levels of Cyclosporine A in 20 µL of processed whole blood, i.e. CDB.

3.3.1.4 Recovery

The recovery is an assessment of how efficient the extraction and the overall analysis procedure are. In this work, recovery was further investigated with respect to matrix-dependent and matrix-independent recovery.

Matrix-dependent recovery was measured by comparing the on-line SPE-LC-MS/MS analysis of a spiked blood sample with the spiked EtOH solution.

Comparison of on-line analysis with off-line analysis of a solution of the analyte dissolved in EtOH yields the matrix-independent recovery. Off-line analysis of this solution was achieved by direct injection of the sample onto the analytical column. For the calculation of the recovery the mean value of the resulting concentrations was assumed to be 100%.

These two recoveries are calculated using the following equations:

$$\text{Matrix} - \text{dependent Recovery} = \frac{\text{Measured Concentration of QC EtOH (on} - \text{line)}}{\text{Measured Concentration of QC Blood (on} - \text{line)}} \text{ x } 100\% \quad \textbf{(Equation 6)}$$

$$\text{Matrix} - \text{independent Recovery} = \frac{\text{Measured Concentration of QC EtOH (on} - \text{line)}}{\text{Measured Concentration of QC EtOH (off} - \text{line)}} \text{ x } 100\% \quad \textbf{(Equation 7)}$$

As shown in Table 4, the matrix-dependent recovery values ranges from 80.9% to 94.3% with standard deviation from 7.0% to 17.2%. In addition, the spiked neat solutions were analyzed off-line as well, and the corresponding matrix-independent recoveries are presented in Table 5.

CyA Level in WB /CDB	QC EtOH: measured concentration of on-line analysis [ng/mL]	QC WB/CDB: measured concentration of on-line ananlyis [ng/mL]	Matrix-dependent recovery
Low 1	32.765	39.998	94.3 ± 17.2 %
Low 2	36.883	35.762	
Low 3	36.555	28.502	
Low 4	34.602	28.560	
Low 5	36.592	34.435	
Middle 1	582.768	481.689	80.9 ± 7.0 %
Middle 2	591.099	467.070	
Middle 3	540.983	439.911	
Middle 4	638.279	459.394	
Middle 5	516.701	473.267	
High 1	1129.486	830.631	90.5 ± 11.2 %
High 2	1009.713	971.761	
High 3	1082.945	1011.772	
High 4	1002.491	1035.442	
High 5	989.990	868.892	

WB = whole blood, CDB = Cell-Disintegrated Blood (Processed WB), QC = Quality Control

Table 4: Matrix-dependent recovery of Cyclosporine A at 3 different concentrations in 20 µL of processed whole blood, i.e. CDB.

CyA Level in EtOH	QC EtOH: measured concentration of on-line analysis [ng/mL]	QC EtOH: measured concentration of off-line analysis [ng/mL]	Matrix-independent recovery
Low 1	32.765	34.056	99.5 ± 4.1 %
Low 2	36.883	35.983	
Low 3	36.555	35.677	
Low 4	34.602	36.856	
Low 5	36.592	35.706	
Middle 1	582.768	585.437	99.3 ± 7.6 %
Middle 2	591.099	576.759	
Middle 3	540.983	570.891	
Middle 4	638.279	580.632	
Middle 5	516.701	574.028	
High 1	1129.486	1002.067	99.5 ± 8.2 %
High 2	1009.713	997.538	
High 3	1082.945	1105.364	
High 4	1002.491	1085.962	
High 5	989.990	1062.853	

QC = Quality Control

Table 5: Matrix-independent recovery of Cyclosporine A at 3 different concentrations in 20 µL of processed whole blood, i.e. CDB.

3.3.2 Determination and improvement of the robustness of the total analysis platform

3.3.2.1 Determination of the robustness of individual subunits of the platform

In order to determine the robustness of the developed analysis method, each individual subunit of the original platform was tested with regard to its robustness. The test involved the injection needle of the sample handling/injection unit, the heated capillary of the processing unit (heat-shock treatment), the in-line filter sieve and the TurboFlow SPE column of the clean-up unit, the guard column and the analytical column of the separation unit.

For the test, the analysis cycle (see 3.2.6) of 20 µl anticoagulated whole blood sample with a HCT value of 0.45 was repeated so many times until the system back pressure exceeded its limit (550 bar). After figuring out and exchanging the part which caused the high back pressure, the analysis cycle was continued until the next high back pressure built up again.

The results of the robustness test for the individual units are documented in the following:

The injection needle was not clogged after 400 injections of each of 20 µL whole blood.

The heated stainless-steel capillary (300 x 0.5mm ID) did not develop a back pressure after 400 injections of each of 20 µL whole blood. This result compares quite well with the one reported by Berger [15].

During the repeated processing and analysis of whole blood, a layer of matrix proteins (Biofilm) is built up on the inside of all tubings, which are in contact with the biofluid, especially the heated capillary. Thus, it is recommended to clean injection needle and all tubings with BCA-reagent after 200 injections of each of 20 µL whole blood, in order to get rid of the biofilm. Otherwise, there might be a carryover or memory effect.

The sieve of the in-line single sieve builts up a back pressure of more than 550 bar after 185 injections of each 20 µL of processed whole blood, i.e. CDB. In another run, 215 injections of each of 20 µL CDB did not cause any significant raise in back pressure.

The back pressure of the TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) raised to about 400 bar after 220 injections of each of 20 µL whole blood, i.e. CDB.

The analytical column (Accucore PFP, 50 x 2.1 mm ID) developed a back pressure of more than 550 bar after 220 injections each of 20 µL of processed whole blood, i.e. CDB.

The build-up of a high back pressure by the in-line single sieve filter, by the TurboFlow SPE column and by the analytical column, point to an insufficient clean-up of the matrix. Thus, the overall analysis procedure was further optimized (see below) in order to achieve at least 500 consecutive injections without the need to exchange any hardware.

3.3.2.2 Improvement of the robustness of the total analysis platform

The robustness of the total analysis system was improved by changing operational parameters, composition of mobile phases and hardware.

Step 1: Varying the fractionation volume

The build-up of back pressure in the columns was most probably caused by matrix components remaining in the SPE column after the fractionation step. This residual matrix then is denatured during the transfer step using 100 % MeOH and clog both SPE and analytical column. In order to confirm this, 20 µL of heat-shock treated whole blood (CDB) samples were fractionated using different volumes of mobile phase (H2O/ACN, 95/5, v/v). The back pressure of the SPE column (coupled with the in-line single sieve filter) was monitored during the repeating injections as shown in Figure 43. New SPE column and in-line single sieve filter were used for each new fractionation series. A home-made test SPE column (40 x 1 mm ID) packed with Oasis HLB material (dp 10 µm) was used. This SPE column was comparable with the TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID).

From Figure 43 it is obvious that the fractionation volume for 20 µL of a CDB sample using a mobile phase of H_2O/ACN (95/5, v/v) at 2 mL/min should be at least 5 mL (169 times of dead volume of the SPE column).

The TurboFlow SPE (TFC) column has a smaller ID and thus a higher linear flow velocity at 2 mL/min. Thus the flow rate for fractionation on the Oasis HLB SPE column was enhanced to 5 mL/min (the maximum of the available HPLC pump) in order to get a linear flow velocity comparable with the TFC column.

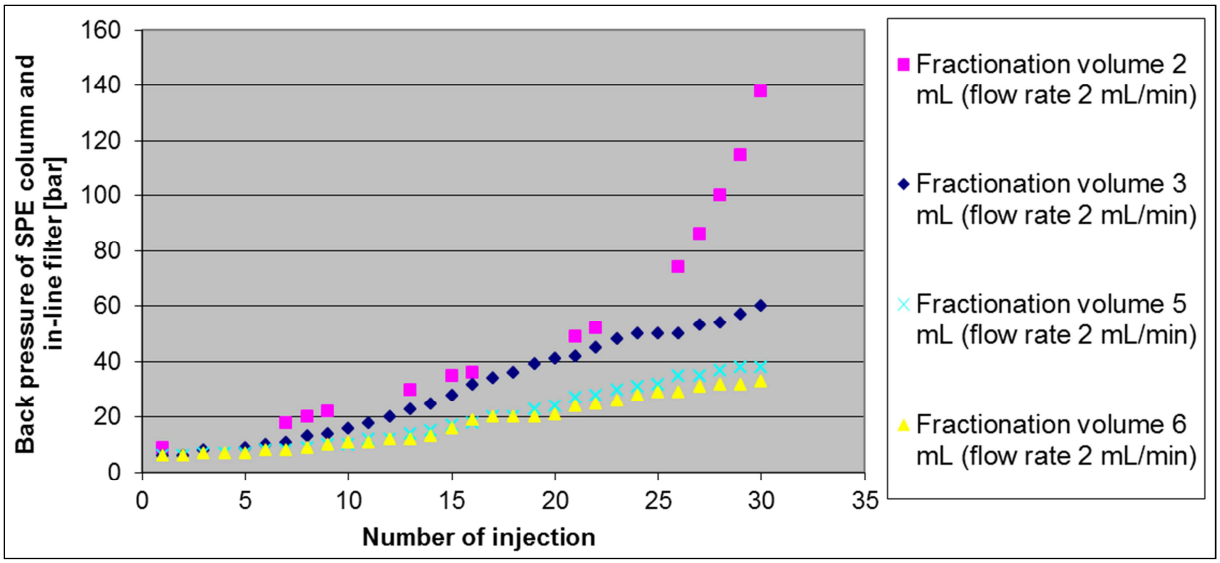


Figure 43: Back pressure of Oasis HLB SPE column (40 x 1mm ID) and in-line single sieve filter after repeated injection of heat-shock treated whole blood (20 µL) and fractionation with varying volumes of mobile phase (H_2O/ACN, 95/5, v/v).

Step 2: Varying the amount of organic modifier in mobile phases for fractionation- and transfer

The rise in back pressure also could be caused by partial denaturation of the blood protein matrix during the heat-shock treatment and during the transfer step due to the presence of an organic modifier. In this context, it should be noted that 2 to 5 vol% of ACN are required to displace target analyte(s) from their protein binding sites prior to Solid Phase Extraction (SPE).

As a reference sample, Cryo CDB prepared from the same whole blood sample by off-line cryogenic treatment was used.

Figure 44 depicts the resulting profiles of the back pressure. It is obvious that enhancing the amount of organic modifier (in this case acetonitrile) does not have a significant effect. This also holds when raising the amount of methanol from 50% to 100% in the transfer step.

The repeated injection of the reference sample (off-line snap-frozen whole blood, cryo CDB) did not cause any significant rise in back pressure, even after 50 injections.

From these findings it is obvious that the mobile phases used for the fractionation and transfer step have no significant influence in the built-up of back pressure. On

the other hand, the heat-shock treatment seems to generate larger particles/agglutinates compared to the cryogenic treatment of whole blood. These larger particles/agglutinates finally clog the sieve of the in-line filter and/or the SPE column.

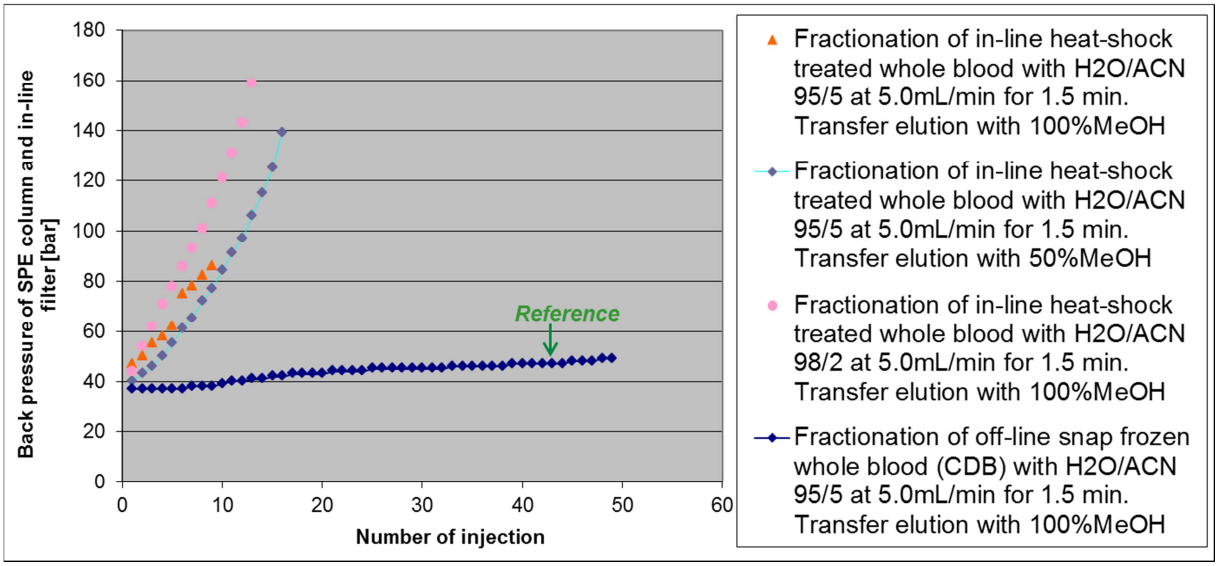


Figure 44: Back pressure of Oasis HLB SPE column (40 x 1mm ID) and in-line single sieve filter after repeated fractionation and transfer elution of heat-shock treated and snap-frozen whole blood samples (20 µL) using varying amounts of organic modifier.

Step 3: Varying the conditions for heat-shock treatment

As mentioned in 3.2.2, the in-line processing of anticoagulated whole blood by heat-shock treatment and the corresponding basic processing parameters (t_{min}, t_{max}, t_{heat}) have been described by Milojković [16]. For a mixture of whole blood and IS in 2.5 vol% DMSO, the optimal heating time is 13 seconds at 75 ℃. The minimum heating time at 75 ℃ is 9 seconds, and 30 seconds at 70 ℃ .

In order to see if the processing parameters influence the generation of larger particles/agglutinates, the absolute heating time at 75 ℃ and 70 ℃ was varied.

Figure 45 shows the resulting profiles of the rise in back pressure of the SPE column and the in-line single sieve filter during repetitive fractionation steps.

It is obvious that the reduction of the processing time from 13 seconds to 9 seconds at 75 ℃ does not have any significant effect on th e rise of the back pressure. The same holds true for reducing the processing temperature from 75 ℃ to 70 ℃.

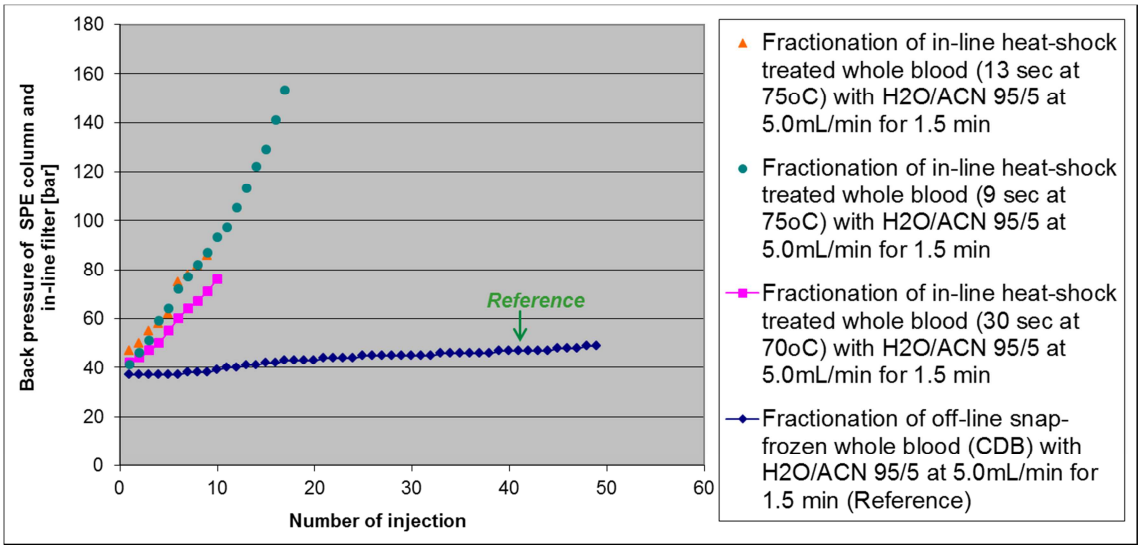


Figure 45: Back pressure of Oasis HLB SPE column (40 x 1mm ID) and in-line single sieve filter after repeated fractionation of snap-frozen and heat-shock treated whole blood samples (20 µL) applying varying conditions.

Step 4: Finding optimal hardware for in-line filter

In order to optimize the in-line filtration step two devices were compared.

The first one was a filter housing containing a woven three-layered metal sieve with smallest mesh size of 1 µm. This dead-volume free device is called in-line single sieve filter (see also 3.2.3).

The second one was a cartridge with varying length and inner diameter (in-line packed depth filter). The filter cartridge was dry packed with different porous materials, which possess electro-neutral and hydrophilic properties. The packing materials also varied in particle size 5-60 µm and pore size 60-120 nm. The purpose of the second filter device was to act as a depth filter for retaining cell nuclei and “cell debris” generated during the heat-shock treatment.

The profiles of the back pressure of the SPE column and the two different filters are shown in Figure 46.

Back pressure builds up much slower when using the depth filter instead of the single sieve filter.

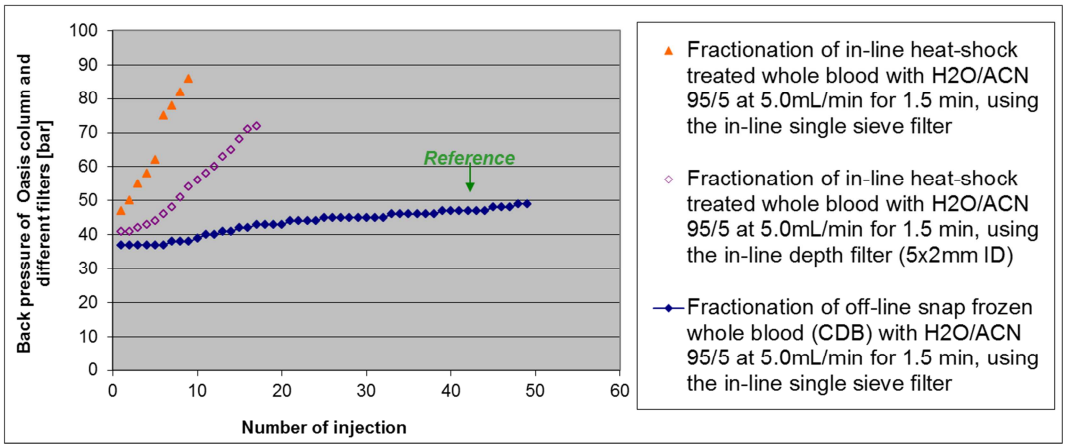


Figure 46: Back pressure of Oasis HLB SPE column (40 x 1mm ID) and filter (in-line single sieve filter or in-line depth filter) after repeated fractionation of snap-frozen and heat-shock treated whole blood (20 µL).

In a next step the inner diameter of the depth filter was extended from 2 to 4 mm. During the repeated injections of processed whole blood samples, i.e. CDB, the back pressure of the system was monitored at two different time points in the overall analysis cycle.

One was the back pressure generated by both, the SPE column and the in-line depth filter after the fractionation step. The other one was the back pressure of the depth filter during back flushing. From the profiles shown in Figure 47 it follows that the back pressure built up much slower in both cases when using a depth filter with a larger inner diameter.

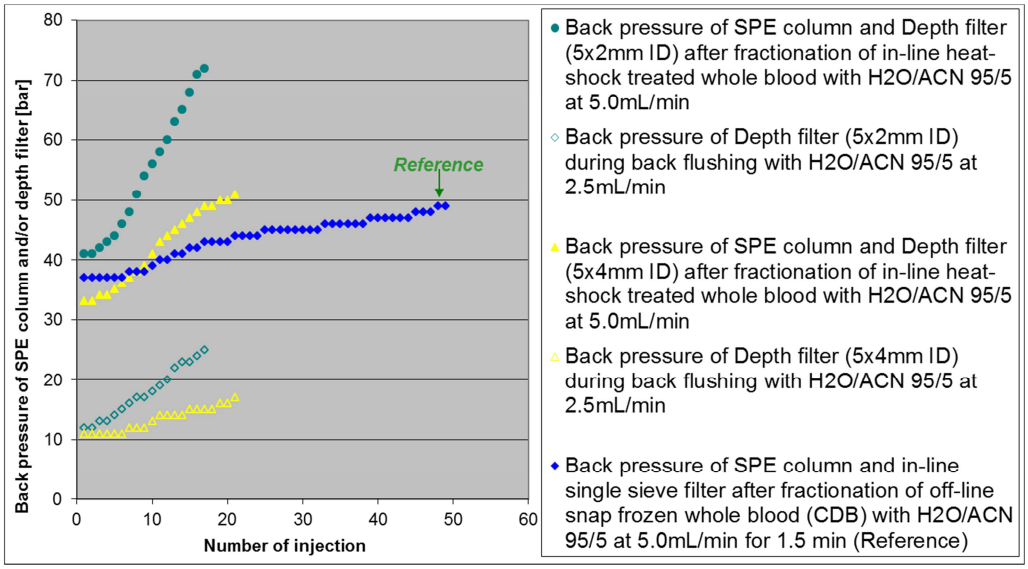


Figure 47: Back pressure of Oasis HLB SPE column (40 x 1mm ID) and/or depth filter (with different ID) after fractionation of in-line heat-shock treated whole blood samples (20 µL) and/or during back-flushing.

Finally, depth filter (5 x 4mm ID) packed with different modified silica materials (spherical silica, broken silica and large pore silica) were compared. The back pressure of the SPE column and depth filter was monitored during repeated injections (Figure 48). The depth filter packed with spherical silica particles provided the best results and was used for further studies.

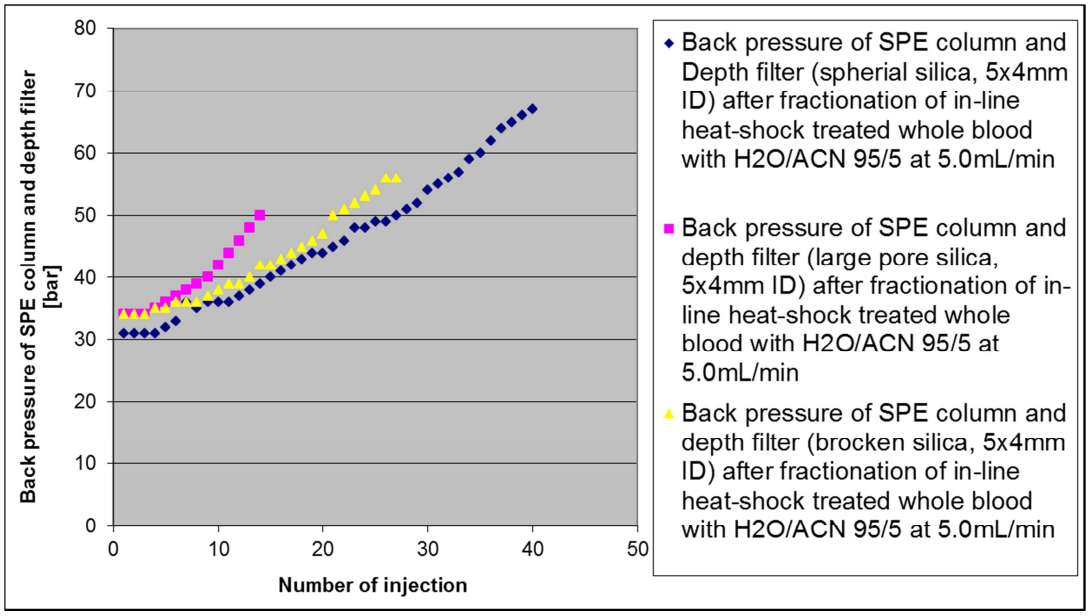


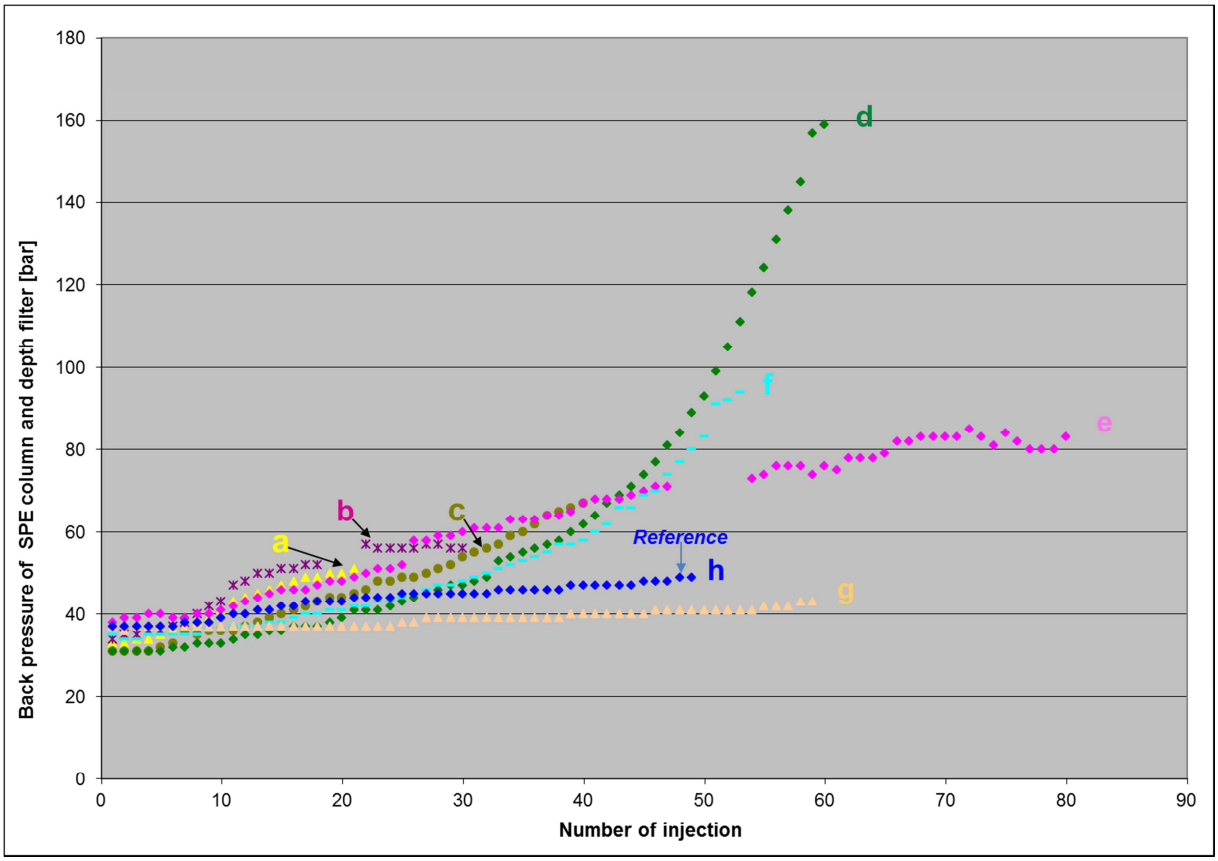
Figure 48: Back pressure of Oasis HLB SPE column (40 x 1mm ID) and depth filter packed with different modified silica materials (5 x 4mm ID) after fractionation of in-line heat-shock treated whole blood (20 µL).

Step 5: Finding optimal cleaning conditions for in-line depth-filter and SPE column

Another option to reduce the back pressure during repeated operation is to wash the depth filter and the SPE column properly in order to remove any residual matrix components. For this purpose, the system configuration was modified accordingly in order to wash the depth filter and the SPE column separately (see Figure 53).

15 % Acetic acid was used to remove acid-soluble proteins. A mixture of organic solvents was used to remove hydrophobic matrix components, such as lipids.

Figure 49 depicts the resulting profiles of the back pressure of the depth filter and the SPE column when using different wash programs. It is obvious that cleaning the depth filter with 15% acetic acid and the SPE column with 15% acetic acid as well as with a mixture of organic solvents gives the best result. The rise in back pressure is even less compared with the reference sample being repeatedly injected onto an in-line single sieve filter.



a) Back pressure of SPE column and depth filter (5x4mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 5.0mL/min. Filter and column washed with mixture of organic solvents

b) Back pressure of SPE column and depth filter (5x4mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 5.0mL/min. Filter and column washed with ACN/H2O 95/5

c) Back pressure of SPE column and depth filter (5x4mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 5.0mL/min. Filter washed with acetic acid, and column with ACN/H2O 95/5

d) Back pressure of SPE column and 2x depth filter (5x4mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 5.0mL/min. Filter washed with acetic acid, and column with H2O/ACN 95/5

e) Back pressure of SPE column and depth filter (10x4mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 5.0mL/min. Filter washed with acetic acid, plus filter and column with mixture of organic solvents

f) Back pressure of SPE column and depth filter (10x4mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 5.0mL/min. Filter washed with acetic acid, and column with mixture of organic solvents

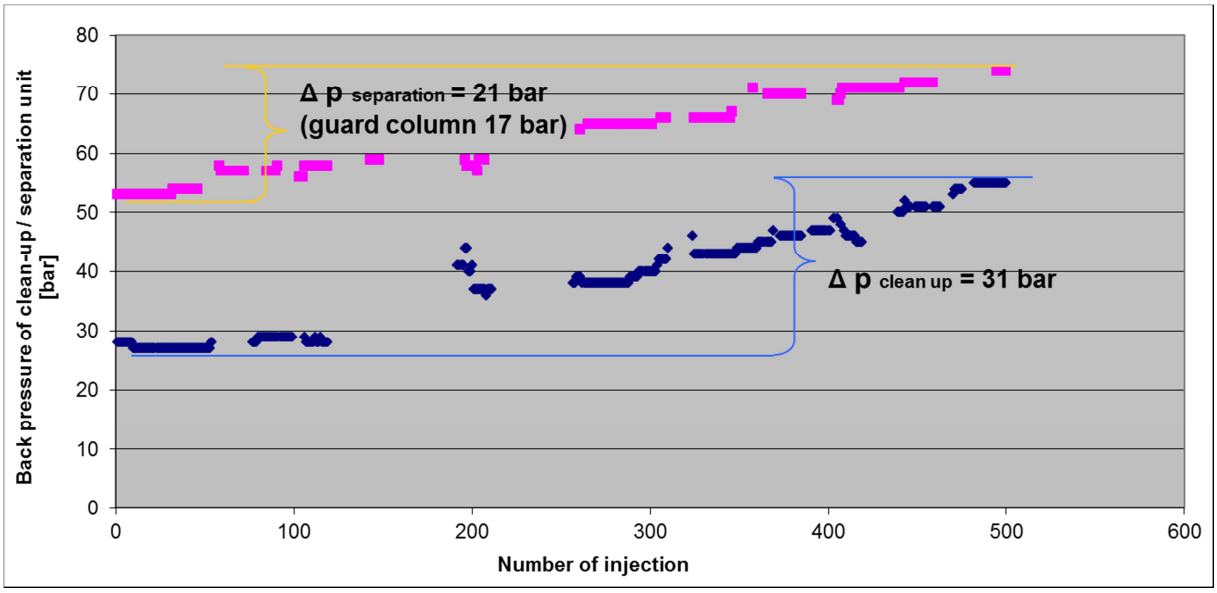
g) Back pressure of SPE column and depth filter (10x4mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 5.0mL/min. Filter washed with acetic acid, plus column with acetic acid and mixture of organic solvents

h) Back pressure of SPE column and in-line filter after fractionation of off-line snap frozen whole blood (CDB) with H2O/ACN 95/5 at 5.0mL/min. Filter and column washed with mixture of organic solvents

Figure 49: Effect of different wash programs on back pressure of Oasis HLB SPE column (40 x 1mm ID) and depth filter (5x4 and 10x4mm ID, packed with spherical, modified silica) after fractionation step of in-line heat-shock treated whole blood (20 µL).

Step 6: Determination of the robustness of the total analysis platform using the optimized in-line depth filter hard ware and wash program

The robustness of the total analysis platform was evaluated using the optimized hard ware (in-line depth filter), a larger fractionation volume (4.2 mL, corresponding to 430 times of the dead volume of the TFC SPE-column) and the optimal wash program (wash in-line depth filter with 15% acetic acid, wash TFC SPE-column with 15% acetic acid and a mixture of organic solvents).



- Back pressure of in-line depth filter (10x4mm ID) and TFC SPE-column (Cyclone-P 50x0.5mm ID) after fractionation of in-line heat-shock treated whole blood with H2O/ACN 95/5 at 2.5 mL/min. In-line depth filter was exchanged after every 200 analysis cylces
- Back pressure of guard column and analytical column (Hypersil Gold PFP, 10x2.1 and 50x2.1mm) during separation step with ACN/H2O 90/10 at 0.9mL/min and 70oC

Figure 50: Monitoring of the back pressure of clean-up and separation unit during repeated analysis of in-line processed whole blood sample (CDB, 10 µL) under optimized conditions.

The robustness of the total analysis platform finally was significant improved as can be seen from the pressure profiles shown in Figure 50:

The on-line clean-up system composed of the in-line depth filter (10 x 4 mm ID) packed with spherical, modified silica and the TurboFlow SPE column Cyclone-P (50 x 0.5 mm ID) built up a back pressure of less than 31 bar after 500 injections each of

10 µL of whole blood. The in-line depth filter contributed to a rise in back pressure of only 3-4 bar during 200 consecutive injections.

The separation system (guard column and analytical column, Hypersil Gold PFP 10 x 2.1 mm and 50 x 2.1 mm ID) built up a back pressure of 21 bar after 500 injections each of 10 µL of whole blood. The guard column contributed to back pressure of about 17 bar.

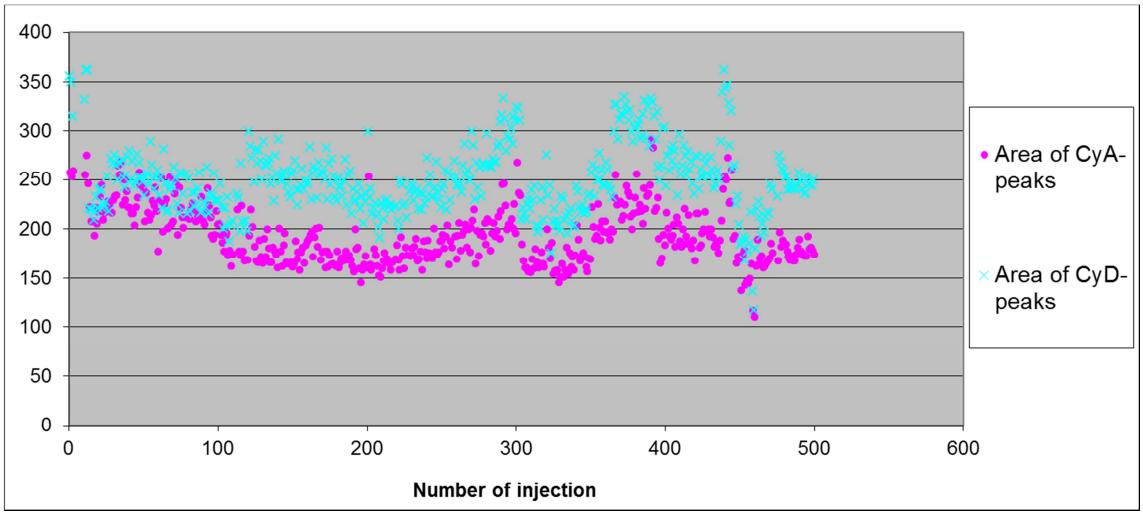


Figure 51: Monitoring of MS/MS response of direct analysis of in-line heat-shock treated whole blood sample (10 µL) using TFC-LC system.

In addition, the MS/MS responses of the analyte (Cyclosporine A) and the IS (Cyclosporine D) were monitored during the repeated analysis cycles, as shown in Figure 51. The RSD value for 500 injections counts to 15.6% for Cyclosporine A, and 15.2% for Cyclosporine D.

3.4 Description and evaluation of the improved, final total analysis platform for quantitation of Cyclosporine A after heat-shock treatment of whole blood

After the optimization of the hard ware and packing material as well as wash program for the in-line depth filter, a final overall analysis cycle has been composed (see Figure 52). This final total analysis platform for the detection and quantitation of Cyclosporine A after heat-shock treatment of whole blood has been evaluated with respect to the following:

- Validation parameters
- Robustness
- Comparison with an established, LC-MS/MS method for routine analysis of patient samples
- Applicability of commercial calibrators and quality control samples (ClinCal and ClinCheck from Recipe)

3.4.1 Final overall analysis cycle

The final overall analysis cycle – which relies on an in-line depth filter packed with spherical, modified silica and an optimal wash program– for the direct determination of Cyclosporine A in an anticoagulated whole blood sample (10 µL) lasts 14.4 min. This is documented in the representative chromatogram (Figure 52), where individual steps of the overall analysis cycle are color coded.

The detailed instrumental set-up for the analysis steps 4 – 9 is displayed in Figure 53. The mobile phases used in each step of the overall analysis cycle are listed in Table 6.

The software Chronos allows a time optimization of sequential analysis. The second analysis starts during the separation step of the analyte(s). Thus the duration for the

second analysis cycle as well as for the following cycles amounts to 10.9 minutes each (Step 4-9).

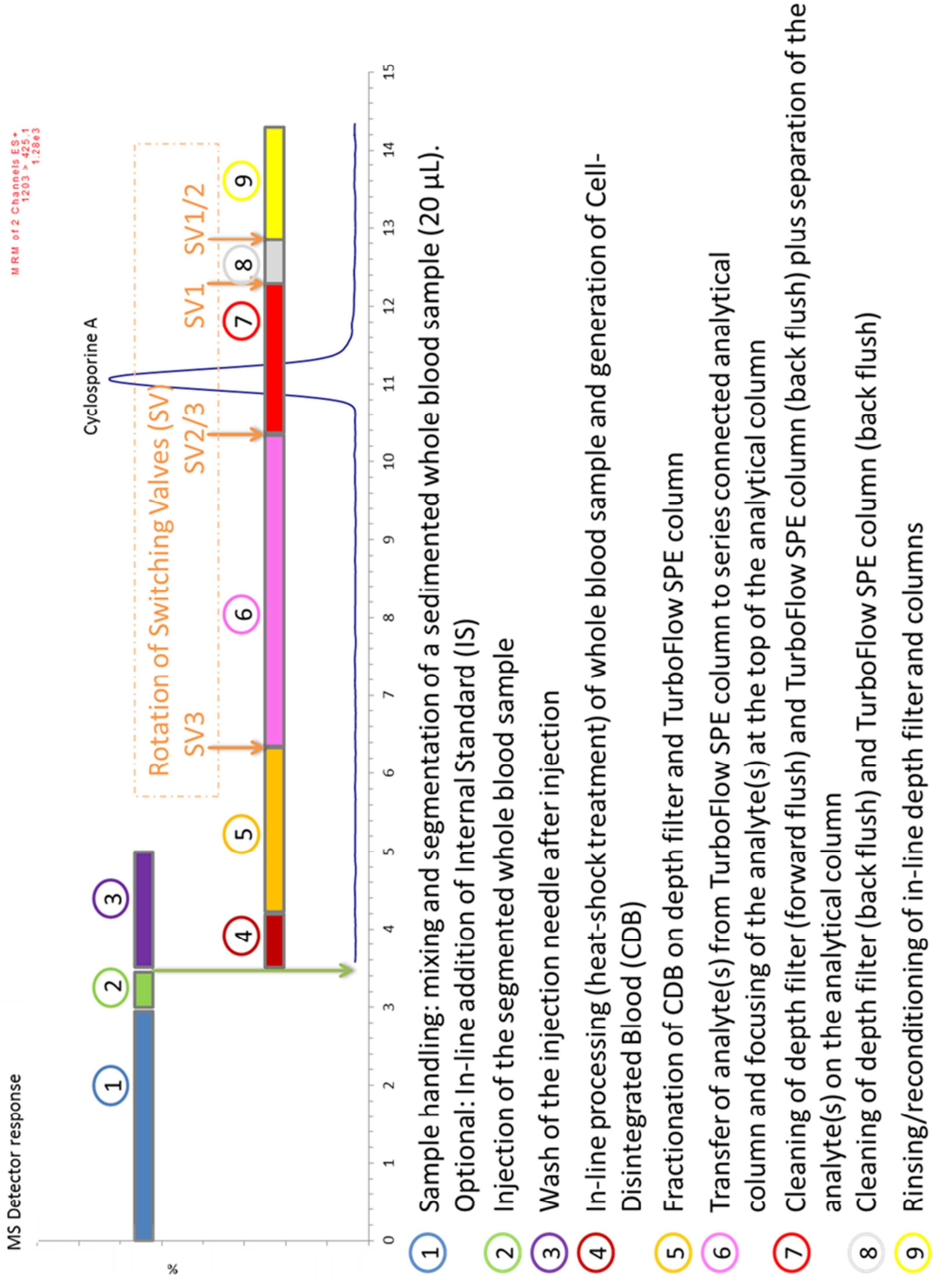
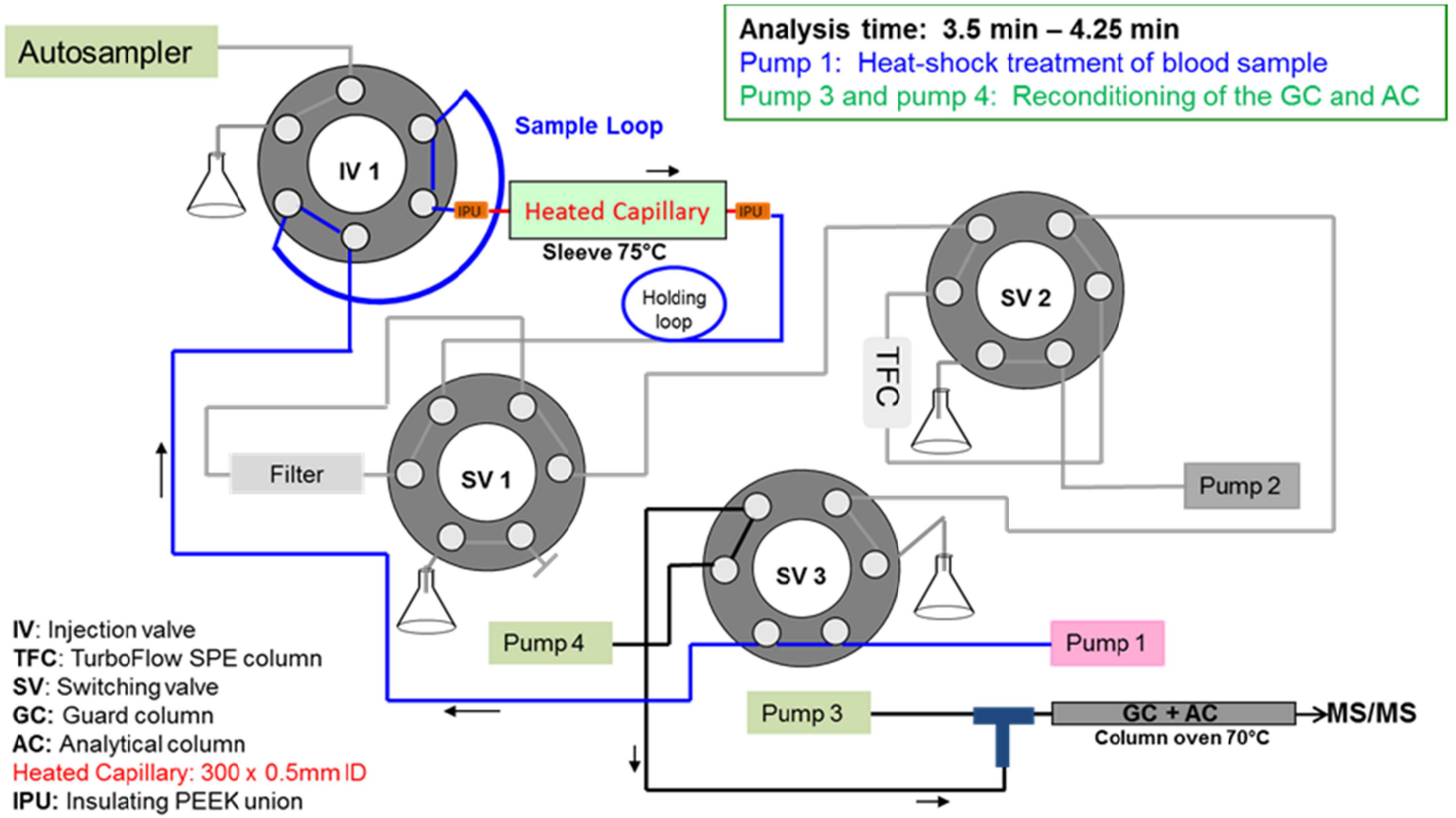
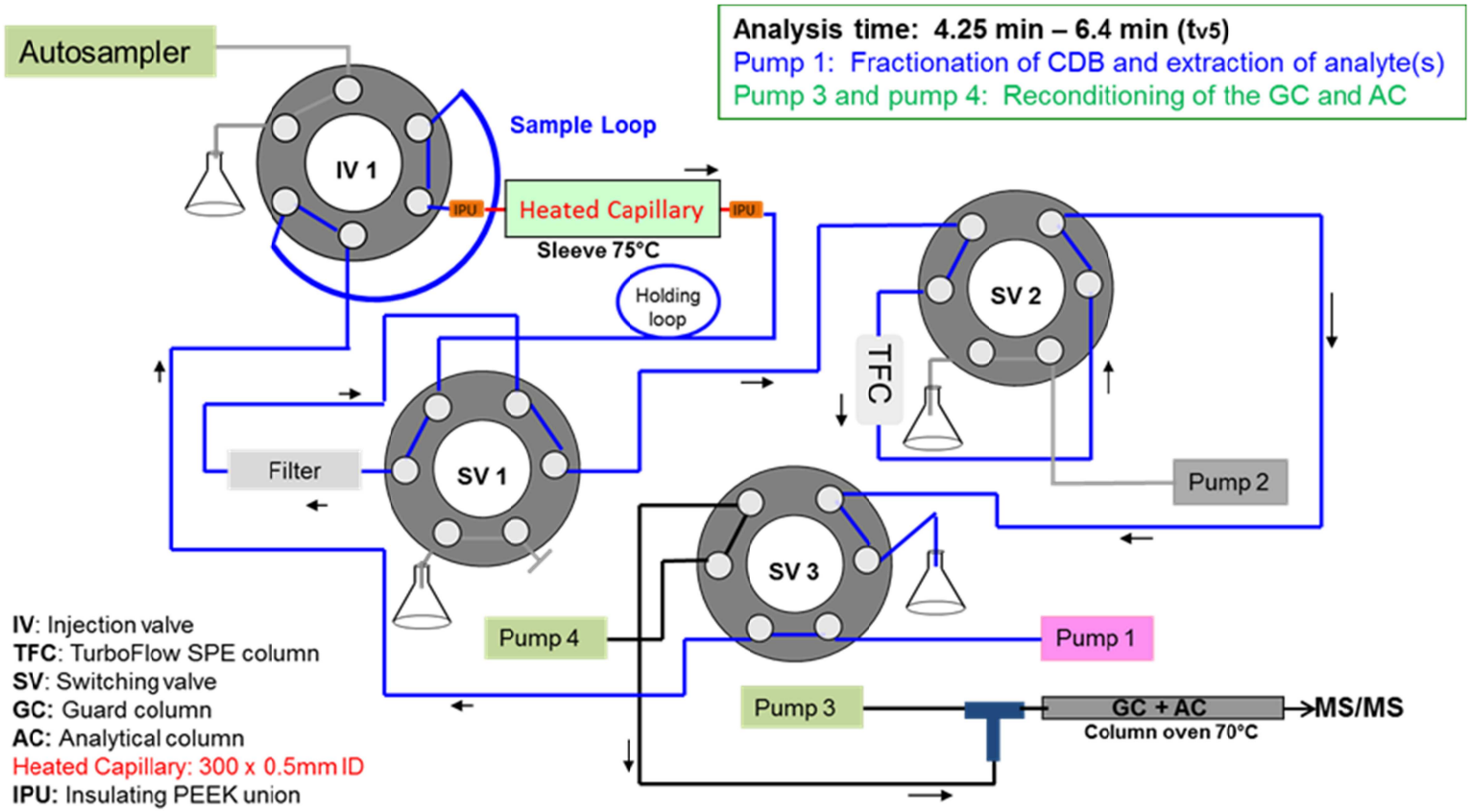


Figure 52: Final overall analysis cycle for fully automated quantitation of Cyclosporine A in whole blood.

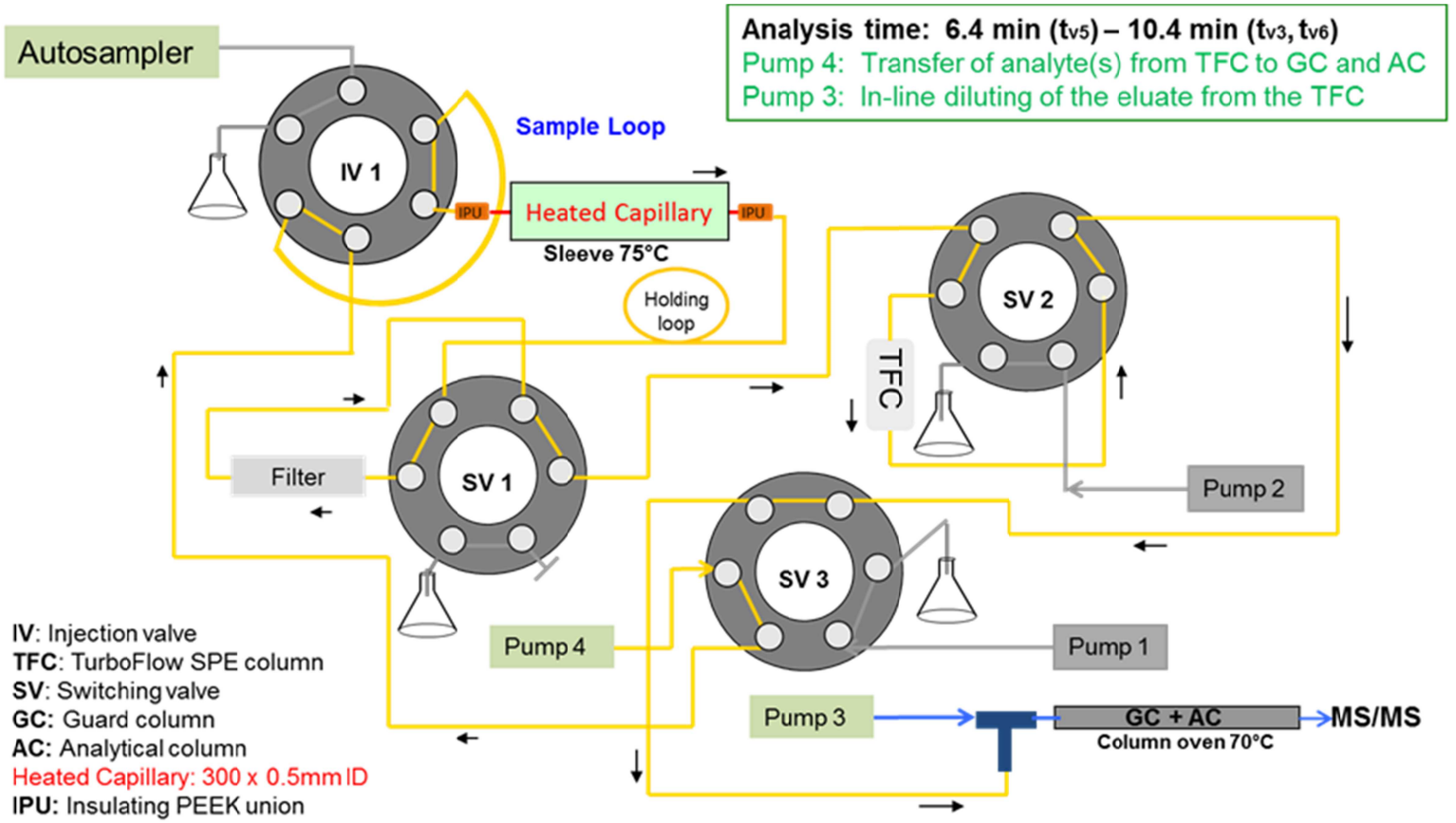
Step 4


Analysis time: 3.5 min – 4.25 min
Pump 1: Heat-shock treatment of blood sample
Pump 3 and pump 4: Reconditioning of the GC and AC
Autosampler
IV 1
Sample Loop
IPU
Heated Capillary
IPU
Sleeve 75°C
Holding loop
SV 2
TFC
Filter
SV 1
Pump 2
SV 3
Pump 4
Pump 1
Pump 3
GC + AC
Column oven 70°C
MS/MS
IV: Injection valve
TFC: TurboFlow SPE column
SV: Switching valve
GC: Guard column
AC: Analytical column
Heated Capillary: 300 x 0.5mm ID
IPU: Insulating PEEK union

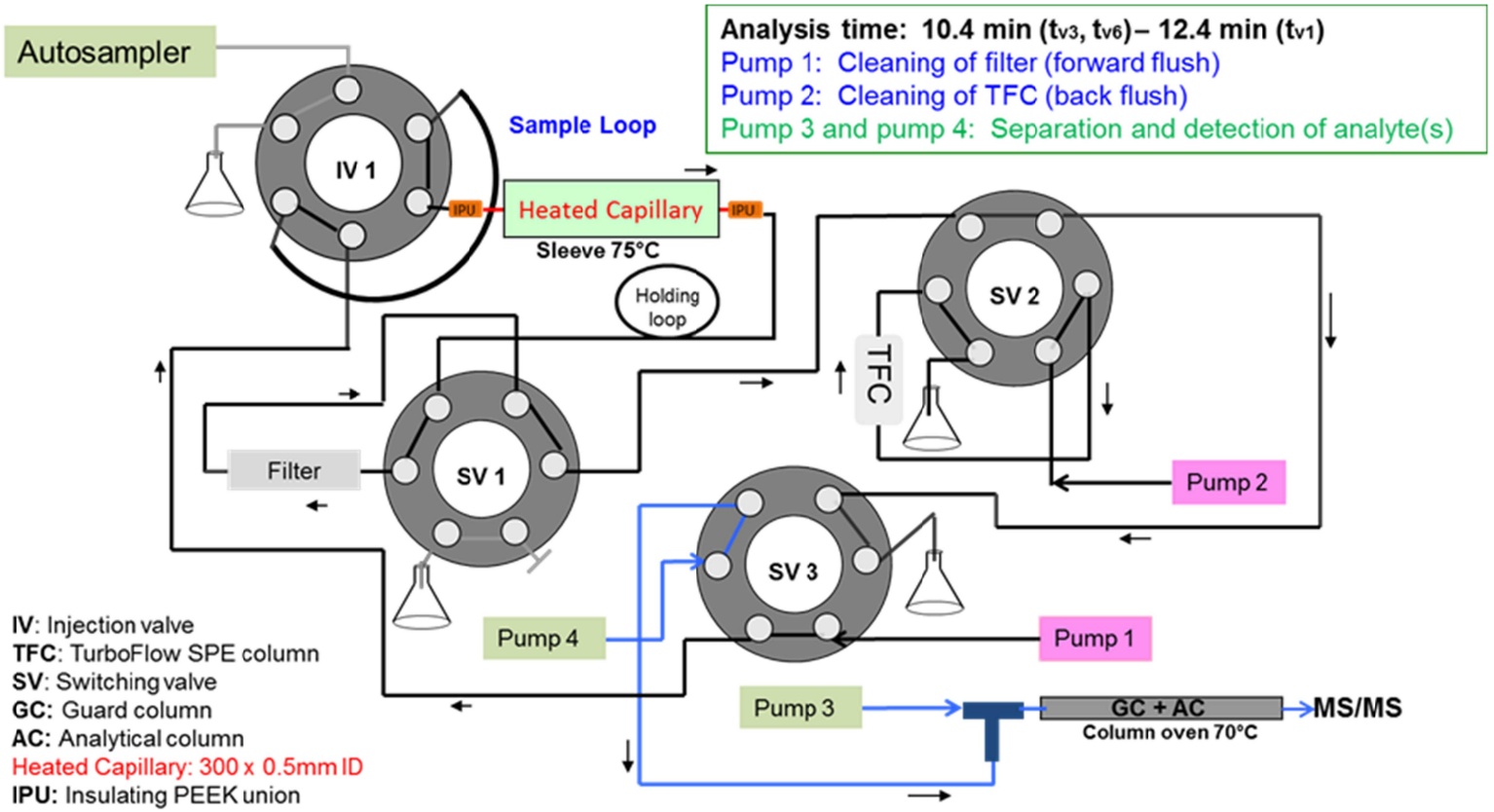
Step 5


Analysis time: 4.25 min – 6.4 min (tv5)
Pump 1: Fractionation of CDB and extraction of analyte(s)
Pump 3 and pump 4: Reconditioning of the GC and AC
Autosampler
IV 1
Sample Loop
IPU
Heated Capillary
IPU
Sleeve 75°C
Holding loop
SV 2
TFC
Filter
SV 1
Pump 2
SV 3
Pump 4
Pump 1
Pump 3
GC + AC
Column oven 70°C
MS/MS
IV: Injection valve
TFC: TurboFlow SPE column
SV: Switching valve
GC: Guard column
AC: Analytical column
Heated Capillary: 300 x 0.5mm ID
IPU: Insulating PEEK union

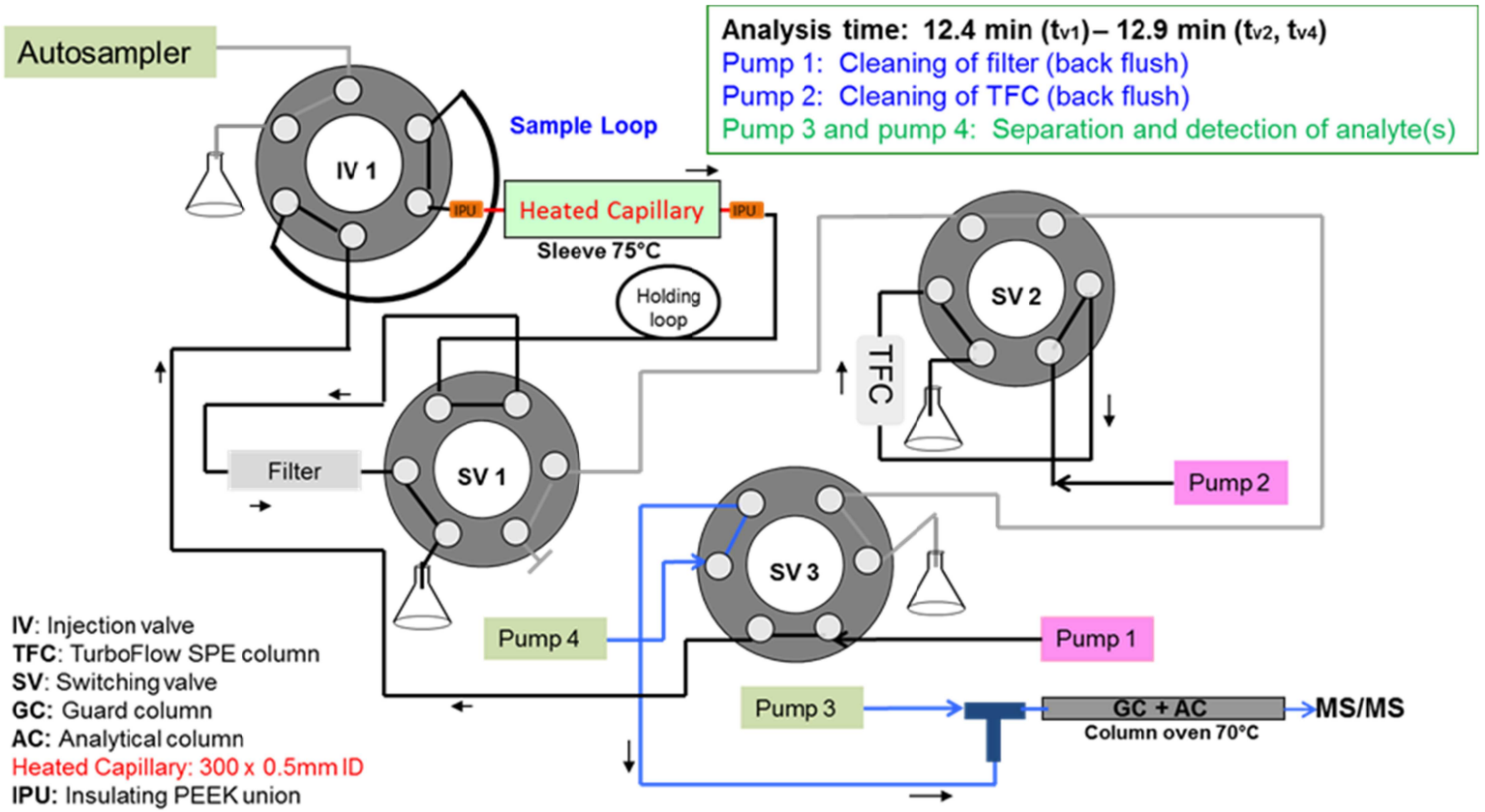
Step 6


Analysis time: 6.4 min (tv5) – 10.4 min (tv3, tv6)
Pump 4: Transfer of analyte(s) from TFC to GC and AC
Pump 3: In-line diluting of the eluate from the TFC
Autosampler
IV 1
Sample Loop
IPU
Heated Capillary
IPU
Sleeve 75°C
Holding loop
SV 2
TFC
Filter
SV 1
Pump 2
SV 3
Pump 4
Pump 1
Pump 3
GC + AC
Column oven 70°C
MS/MS
IV: Injection valve
TFC: TurboFlow SPE column
SV: Switching valve
GC: Guard column
AC: Analytical column
Heated Capillary: 300 x 0.5mm ID
IPU: Insulating PEEK union

Step 7


Analysis time: 10.4 min (tv3, tv6)– 12.4 min (tv1)
Pump 1: Cleaning of filter (forward flush)
Pump 2: Cleaning of TFC (back flush)
Pump 3 and pump 4: Separation and detection of analyte(s)
Autosampler
IV 1
Sample Loop
IPU
Heated Capillary
IPU
Sleeve 75°C
Holding loop
SV 2
TFC
Filter
SV 1
Pump 2
SV 3
Pump 4
Pump 1
Pump 3
GC + AC
Column oven 70°C
MS/MS
IV: Injection valve
TFC: TurboFlow SPE column
SV: Switching valve
GC: Guard column
AC: Analytical column
Heated Capillary: 300 x 0.5mm ID
IPU: Insulating PEEK union

Step 8


Analysis time: 12.4 min (t_{v1}) – 12.9 min (t_{v2}, t_{v4})
Pump 1: Cleaning of filter (back flush)
Pump 2: Cleaning of TFC (back flush)
Pump 3 and pump 4: Separation and detection of analyte(s)
Autosampler
IV 1
Sample Loop
IPU
Heated Capillary
IPU
Sleeve 75°C
Holding loop
SV 2
TFC
Filter
SV 1
Pump 2
SV 3
Pump 4
Pump 1
Pump 3
GC + AC
Column oven 70°C
MS/MS
IV: Injection valve
TFC: TurboFlow SPE column
SV: Switching valve
GC: Guard column
AC: Analytical column
Heated Capillary: 300 x 0.5mm ID
IPU: Insulating PEEK union

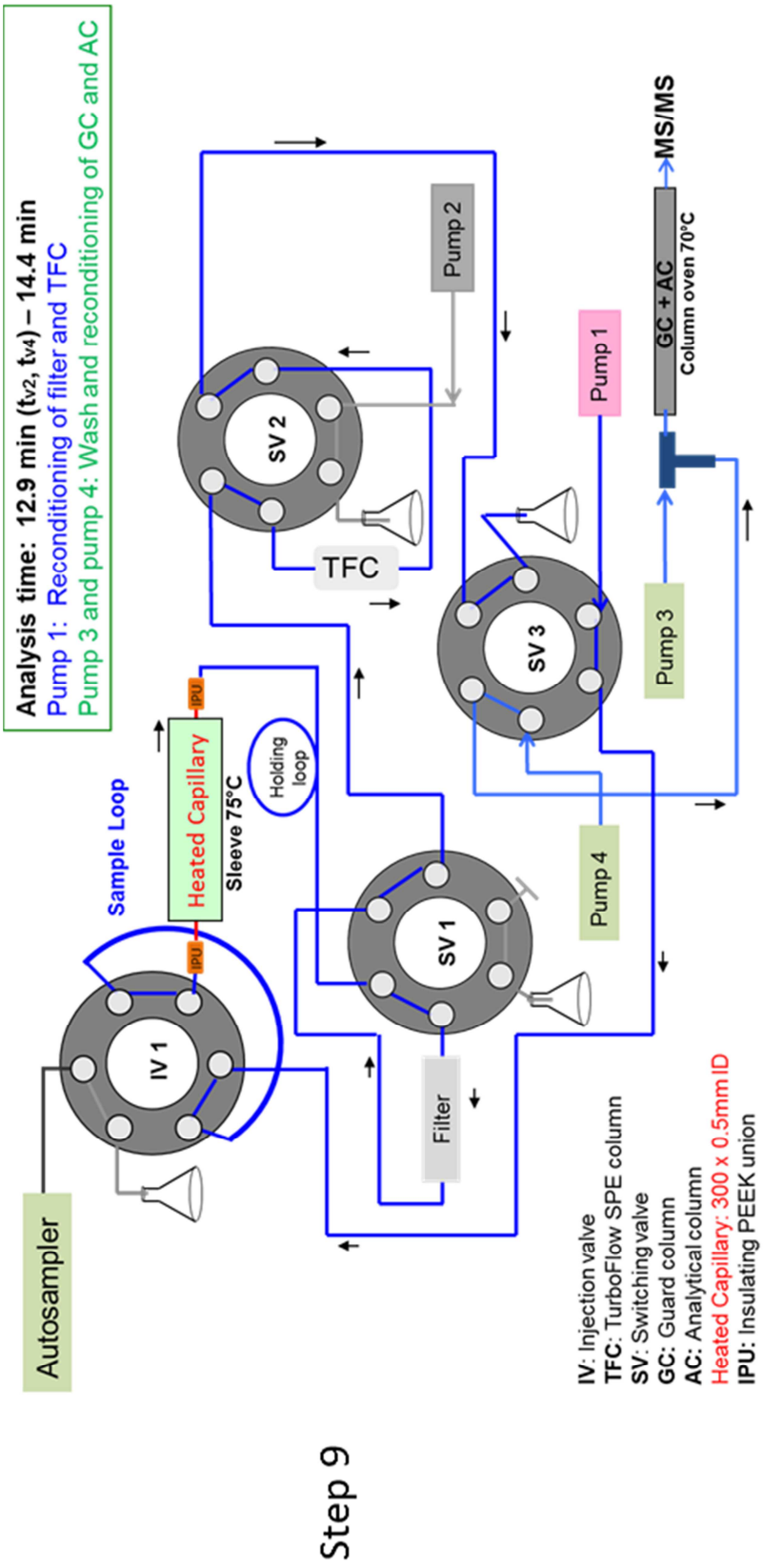


Figure 53: Final system configuration for in-line processing of a whole blood sample by heat-shock treatment and on-line SPE-LC-MS/MS analysis of the processed sample (CDB): Analysis cycle steps 4-9.

A	Analysis Time [min]	Pump 1		
		Mobile Phase	Flow rate [mL/min]	Function
Step 1	0 - 3	H_2O/ACN (95/5, v/v)	1.0	Reconditioning of in-line depth filter and SPE column
Step 2	3 – 3.5			
Step 4	3.5 – 4.25	H_2O/ACN (95/5, v/v)	0.27	In-line processing (heat-shock teratment) of whole blood
Step 5	4.25 – 6.4	H_2O/ACN (95/5, v/v)	2.0	Fractionation of the processed whole blood (CDB) and SPE extraction of analyte(s)
Step 6	6.4 – 10.4	H_2O/ACN (95/5, v/v)	0	------
Step 7	10.4 – 11.4	15 % acetic acid	5.0	Forward flushing of in-line depth filter with acetic acid
	11.4 – 12.4	H_2O/ACN (95/5, v/v)	3.0	Forward flushing of in-line depth filter with aqueous mobile phase
Step 8	12.4 – 12.9	H_2O/ACN (95/5, v/v)	5	Back flushing of in-line depth filter with aqueous mobile phase
Step 9	12.9 – 14.4	H_2O/ACN (95/5, v/v)	2.0	Reconditioning of in-line depth filter and SPE column

B	Analysis Time [min]	Pump 2		
		Mobile Phase	Flow rate [mL/min]	Function
Step 1	0 - 3	15 % acetic acid	0	------
Step 2	3 – 3.5			
Step 4	3.5 – 4.25	15 % acetic acid	0	------
Step 5	4.25 – 6.4	15 % acetic acid	0	------
Step 6	6.4 – 10.4	15 % acetic acid	0	------
Step 7	10.4 – 11.3	15 % acetic acid	2.5	Back flushing of SPE column with acetic acid
	11.3 – 12.4	Mixture of organic solvents	3.0	Back flushing of SPE column with organic mobile phase
Step 8	12.4 – 12.9	Mixture of organic solvents	3.0	Back flushing of SPE column with organic mobile phase
Step 9	12.9 – 14.4	15 % acetic acid	0	------

C	Analysis Time [min]	Pump 3		
		Mobile Phase	Flow rate [mL/min]	Function
Step 1	0 - 3	H_2O/ACN (90/10, v/v)	0.7	Reconditioning of analytical column
Step 2	3 – 3.5			
Step 4	3.5 – 4.25	H_2O/ACN (90/10, v/v)	0.7	Reconditioning of analytical column
Step 5	4.25 – 6.4	H_2O/ACN (90/10, v/v)	0.7	Reconditioning of analytical column
Step 6	6.4 – 10.4	H_2O	0.5	In-line dilution of the eluate from SPE column
Step 7	10.4 – 10.8	H_2O/ACN (10/90, v/v)	0.4	Focusing of the analyte(s) on the analytical column
	10.8 – 12.4	H_2O/ACN (10/90, v/v)	0.9	Separation of analyte(s)
Step 8	12.4 – 12.9	H_2O/ACN (10/90, v/v)	0.9	Separation of analyte(s)
Step 9	12.9 – 14.4	H_2O/ACN (10/90, v/v)	2.0	Wash analytical column with organic mobile phase

D	Analysis Time [min]	Pump 4		
		Mobile Phase	Flow rate [mL/min]	Function
Step 1	0 - 3	H_2O/ACN (10/90, v/v)	0	------
Step 2	3 – 3.5			
Step 4	3.5 – 4.25	H_2O/ACN (10/90, v/v)	0	------
Step 5	4.25 – 6.4	H_2O/ACN (10/90, v/v)	0	------
Step 6	6.4 – 10.4	H_2O/ACN (10/90, v/v)	0.4	Transfer of analyte(s) from TurboFlow SPE column to analytical column
Step 7	10.4 – 10.8	H_2O	0.5	Focusing of the analyte(s) on the analytical column
	10.8 – 12.4	H_2O/ACN (10/90, v/v)	0	------
Step 8	12.4 – 12.9	H_2O/ACN (10/90, v/v)	0	------
Step 9	12.9 – 14.4	H_2O/ACN (10/90, v/v)	0	------

Note: Mobile phases of H_2O and ACN contain 10 mM ammonium formate and 0.05 vol% formic acid
Blue shaded area = start of next analysis cycle
SPE = Solid Phase Extraction

Table 6: Time table of final overall analysis cycle: Flow rates, compositions of mobile phases and functions of pump settings.

3.4.2 Validation of the improved, final method

As the experimental conditions, i.e. hard ware, packing material and wash program of in-line depth filter for the improved method (see also 3.3.2.2) have been changed, a new validation was required. The validation parameters are described in 3.3.1, and the results are presented in the following.

3.4.2.1 Linearity and Range

A new calibration curve was constructed with seven different analyte concentrations in the rage of 10 to 1000 ng/mL.

The calibration curve shown in Figure 54 proved to be linear over the tested concentration range. The regression coefficient for the calibration curve was 0.9977.

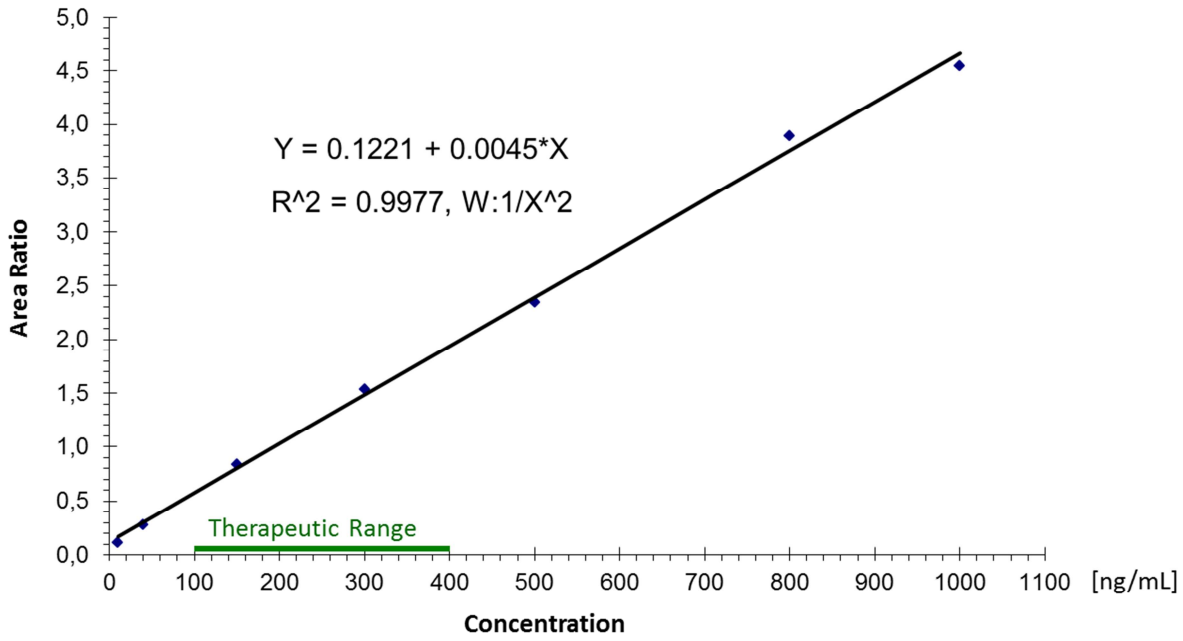


Figure 54: Calibration curve for Cyclosporine A in 10 µL whole blood using the final analysis protocol.

3.4.2.2 Sensitivity

The LOD value was found to be 5.4 ng/mL for CyA in 10 µL of anticoagulated whole blood sample. The LLOQ turned out to be 10 ng/mL.

3.4.2.3 Accuracy and Precision

Table 7 and Table 8 document the data obtained for intra- and inter-day accuracy and precision respectively. The intra-day accuracy ranges from 0.06 to 7.93 %, and the intra-day precision ranges from 2.68 to 4.53 %RSD. The average values of all three concentration levels from three consecutive runs on three separate days are within 15 % of the expected concentration. The inter-day precision ranges from 3.46 to 4.96 %RSD.

CyA Level in blood	Spiked Concentration [ng/mL]	Calculated Concentration [ng/mL]	Accuracy [%Diff]	Precision %RSD (n=5)
Low 1	100	102.07	2.07	4.53
Low 2	100	92.07	-7.93	
Low 3	100	100.36	0.36	
Low 4	100	98.45	-1.55	
Low 5	100	103.68	3.68	
Middle 1	200	192.49	-3.75	2.83
Middle 2	200	196.62	-1.69	
Middle 3	200	185.44	-7.28	
Middle 4	200	191.53	-4.23	
Middle 5	200	199.89	-0.06	
High 1	400	383.96	-4.0	2.68
High 2	400	400.78	0.19	
High 3	400	410.25	2.56	
High 4	400	395.15	-1.21	
High 5	400	408.46	2.11	

Table 7: Determination of intra-day accuracy and precision at 3 different concentration levels of Cyclosporine A in 10 µL of whole blood using the final analysis protocol.

CyA Level in blood	Spiked Concentration [ng/mL]	Accuracy [%Diff]			Precision %RSD (n=15)
		Day 1	Day 2	Day 3	
Low 1	100	2.07	-2.79	7.67	4.96
Low 2	100	-7.93	2.42	-2.17	
Low 3	100	0.36	-1.62	4.22	
Low 4	100	-1.55	-0.58	-1.36	
Low 5	100	3.68	-3.42	12.85	
Middle 1	200	-3.75	-0.16	12.58	4.82
Middle 2	200	-1.69	3.12	-3.05	
Middle 3	200	-7.28	1.74	2.73	
Middle 4	200	-4.23	-0.74	-1.02	
Middle 5	200	-0.06	6.69	-2.13	
High 1	400	-4.0	0.91	10.38	3.46
High 2	400	0.19	0.65	1.15	
High 3	400	2.56	3.10	-0.80	
High 4	400	-1.21	6.03	2.42	
High 5	400	2.11	4.32	-2.52	

Table 8: Determination of inter-day accuracy and precision at 3 different concentration levels of Cyclosporine A in 10 µL of whole blood using the final analysis protocol.

3.4.2.4 Recovery

The matrix-independent recovery was only measured for the middle level (200 ng/mL) of Cyclosporine A solved in EtOH. The EtOH standard solution was analyzed in triplicate. The matrix-independent recovery was 98.2 % with standard deviation of 4.1 %. The matrix-dependent recovery value ranges from 100.5 % to 106.1 % with standard deviation from 1.8% to 4.3%, as shown in Table 9. It is obviously that the matrix-dependent recovery was significantly improved by optimization of the in-line filter hard ware, packing material and wash program.

CyA Level in blood	QC EtOH: measured concentration of on-line analysis [ng/mL]	QC Whole Blood: measured concentration of on-line ananlyis [ng/mL]	Matrix-dependent recovery
Low 1	92,82	102.07	106.1 ± 3.5 %
Low 2	91,49	92.07	
Low 3	95,59	100.36	
Low 4	93.31	98.45	
Low 5	94.78	103.68	
Middle 1	193.03	192.49	100.5 ± 1.8 %
Middle 2	190.94	196.62	
Middle 3	188.76	185.44	
Middle 4	191.91	191.53	
Middle 5	196.79	199.89	
High 1	401.71	383.96	101.4 ± 4.3 %
High 2	381.53	400.78	
High 3	391.62	410.25	
High 4	403.59	395.15	
High 5	394.18	408.46	

QC = Quality Control

Table 9: Mean values and standard deviations for matrix-dependent recovery of Cyclosporine A at 3 different concentrations in 10 µL of whole blood using the final analysis protocol.

3.4.3 Robustness of the improved, final total analysis platform

As documented in chapter 3.3.2.2 (step 6), the robustness of the improved total analysis platform was significantly improved by optimization of the hard ware, packing material and wash program for the in-line depth filter:

The on-line clean-up system composed of the in-line depth filter (10 x 4 mm ID) packed with spherical, modified silica and the TurboFlow SPE column Cyclone-P (50 x 0.5 mm ID) built up a back pressure of 31 bar after 500 injections each of 10 µL of whole blood. The in-line depth filter contributed to a rise in back pressure of only 3-4 bar during 200 consecutive injections.

The separation system (guard column and analytical column, Hypersil Gold PFP 10 x 2.1 mm and 50 x 2.1 mm ID) built up a back pressure of 21 bar after 500 injections

each of 10 µL of whole blood. The guard column contributed to a back pressure of 17 bar.

In addition, the MS/MS responses for the analyte (Cyclosporine A) and the IS (Cyclosporine D) were monitored during the 500 analysis cycles. The relative standard deviation (RSD) of 500 injections of each of 10 µL of whole blood amounts to 15.6 % for Cyclosporine A, and 15.2 % for Cyclosporine D.

3.4.4 Applicability of commercial calibrators and quality control samples

The optimized total analysis platform was further evaluated with regard to the use of commercial available calibrators and quality control samples. The lyophilized calibrator ClinCal from RECIPE Chemicals + Instruments GmbH was used to construct the calibration curve. The lyophilized quality control samples ClinCheck (low, middle and high level) were used for internal quality assurance. In this context, it was found that the protein matrix of the lyophilized samples precipitates upon heating. Therefore, these samples were treated at room temperature.

The calibration curve was constructed from double runs with six different analyte concentrations and the blank sample of the ClinCal kit. The calibration curve shown in Figure 55 proved to be linear over the range of 0 ng/mL to 1264 ng/mL. The regression coefficient for the calibration curve is 0.9996.

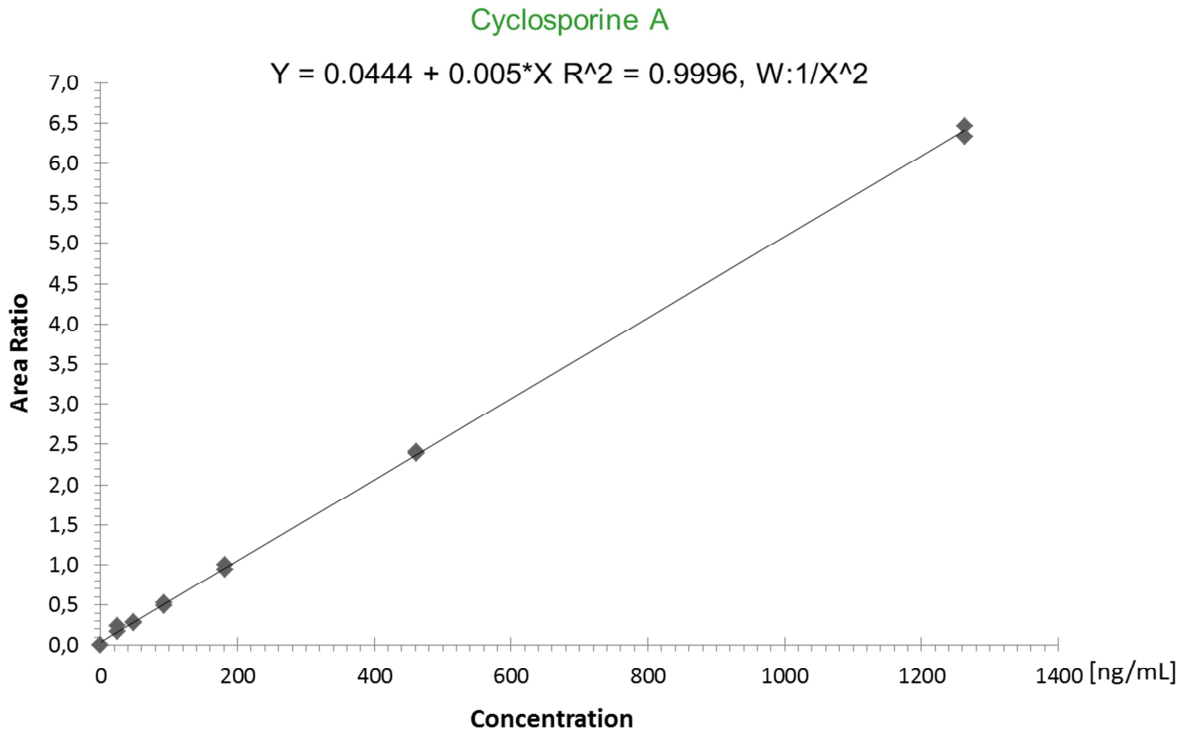


Figure 55: Calibration curve for Cyclosporine A in 10 µL ClinCal samples using the final analysis protocol.

The intra-day accuracy and precision were determined from triple analysis of ClinCheck samples, which are available in three different levels of concentration of Cyclosporine A.

As shown in Table 10 the intra-day accuracy ranges from 1.11 to 14.53 %, and the intra-day precision ranges from 3.57 to 4.64 % RSD.

CyA in ClinCheck	Mean Value [ng/mL]	Measured Concentration [ng/mL]	Accuracy [%Diff]	Precision %RSD (n=3)
Level 1	55.9	52.79	-5.56	4.04
Level 1	55.9	56.52	1.11	
Level 1	55.9	56.79	1.59	
Level 2	113	96.58	-14.53	3.57
Level 2	113	103.24	-8.64	
Level 2	113	97.84	-13.42	
Level 3	223	198.07	-11.18	4.64
Level 3	223	216.02	-3.13	
Level 3	223	213.42	-4.30	

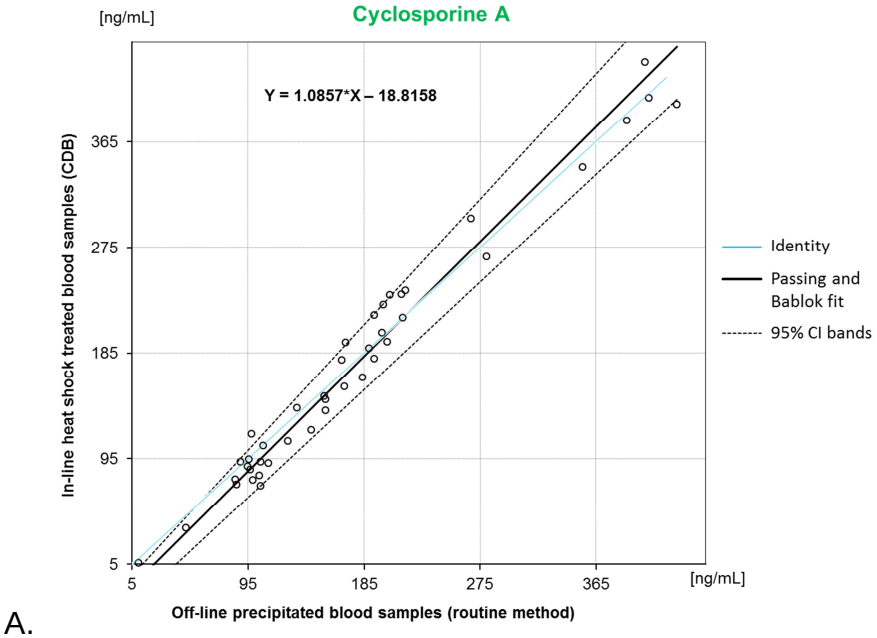
Table 10: Determination of intra-day accuracy and precision at 3 different concentration levels of Cyclosporine A in 10 µL of ClinCheck sample applying the final analysis protocol

3.4.5 Comparison with an established method for routine analysis of patient samples

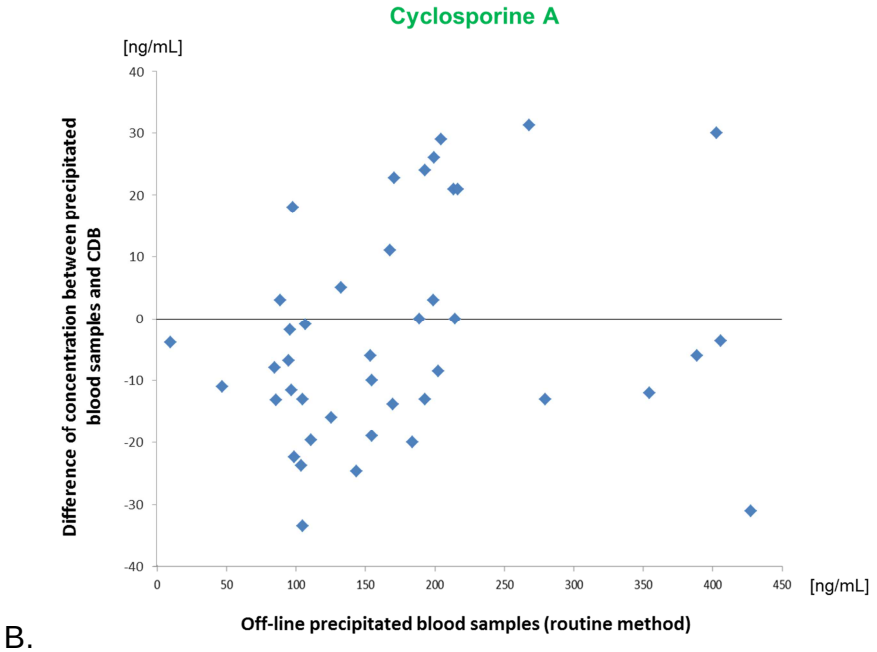
The final total analysis platform was compared with the established method of the Institute of Laboratory Medicine, Medical Center of the University of Munich, for routine LC-MS/MS analysis of Cyclosporine A in patient samples. As a matter of routine, the whole blood samples of patients treated with Cyclosporine A were off-line (i.e. manually) precipitated prior to on-line SPE-LC-MS/MS analysis. The results of the routine method were used as reference values. After having been stored at 4 ℃ for at least 3 days, the same patient samples were in-line heat-shock and/or off-line cryogenic treated prior to on-line SPE-LC-MS/MS analysis as described in the final protocol (see 3.4.1).

The inter-laboratory method comparison is based on Passing and Bablok plots.

Figure 56 shows the comparison of SPE-LC-MS/MS determination of Cyclosporine A in 42 patient blood samples, after off-line protein precipitation and in-line heat-shock treatment, respectively. The equation for the Passing and Bablok regression line is Y = 1.0857 X – 18.8158. The 95 % confidence interval (CI) for the slope is from 1.0188 to 1.1885. This small proportional difference indicates a good agreement between both methods. The 95 % confidence interval (CI) for the intercept is from -34.7705 to -10.4387. This constant difference indicates an average abatement of the measured concentration of in-line heat shock treated blood samples of patients. In general, the Passing and Bablok regression analysis shows a very good agreement of the two methods compared.



A.



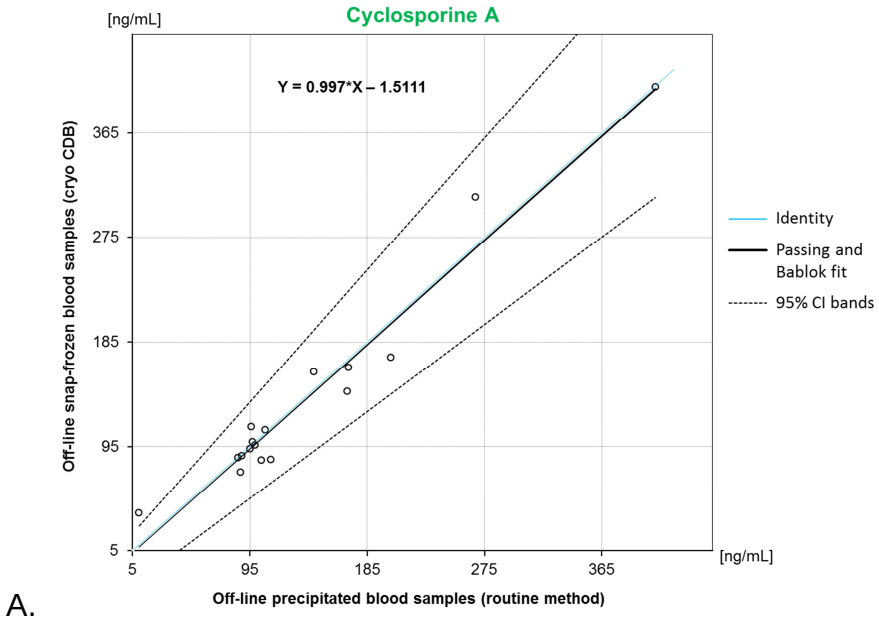
B.

Figure 56: Inter-laboratory method comparison (Passing and Bablok regression analysis), n=42 SPE-LC-MS/MS-determination of Cyclosporine A in off-line protein precipitated and in-line heat shock treated blood samples of patients.

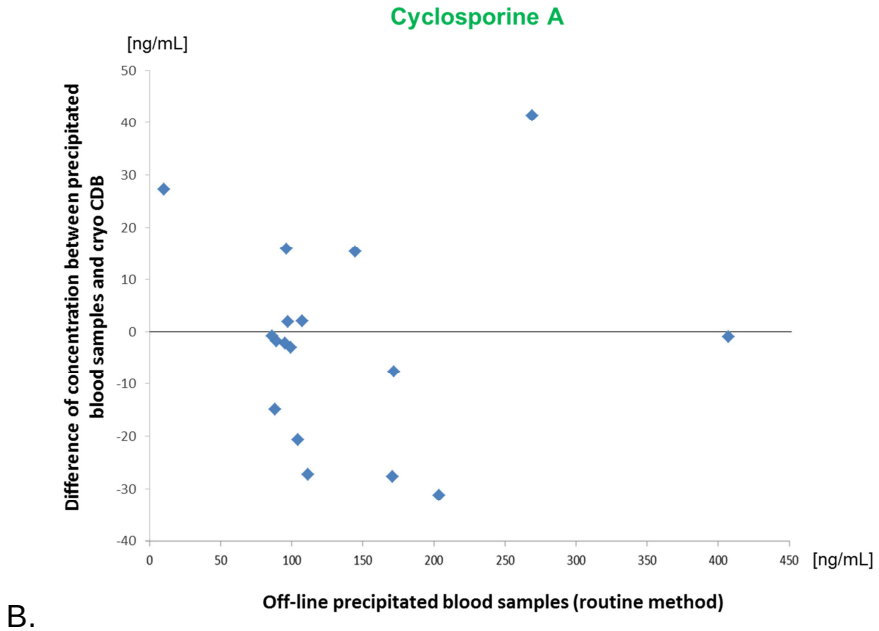
A. Scatter diagram with regression line and confidence bands for regression line
B. Residual plot of distribution of difference around fitted regression line

Figure 57 shows the comparison of SPE-LC-MS/MS determination of Cyclosporine A in 17 patient blood samples, after off-line protein precipitation and off-line snap-freezing, respectively. The equation for the Passing and Bablok regression line is Y = 0.997 X – 1.5111. The 95 % confidence interval (CI) for the slope is from 0.8330 to -

1.2631. The 95 % confidence interval (CI) for the intercept is from -29.0592 to 13.7629. In general, the Passing and Bablok regression analysis shows again a very good agreement of the two different methods compared.



A.

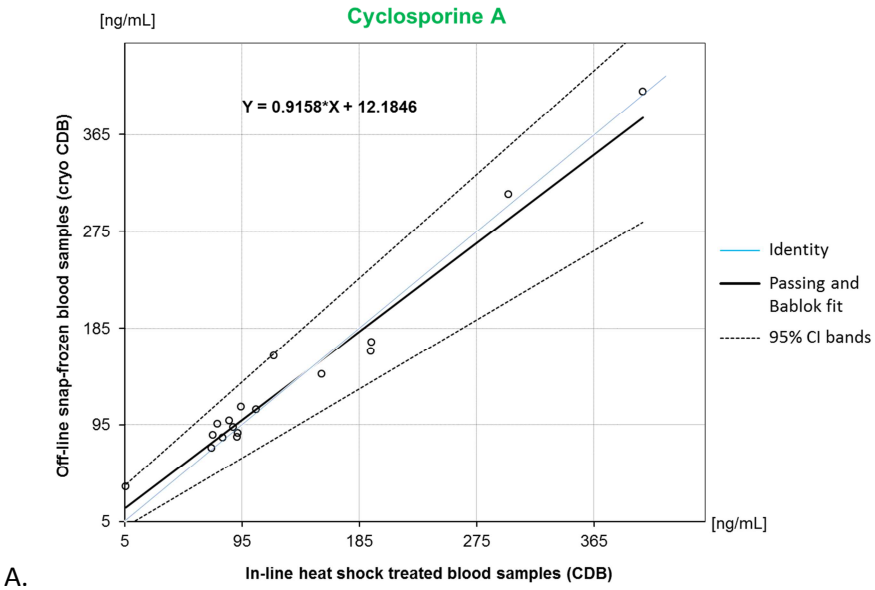


B.

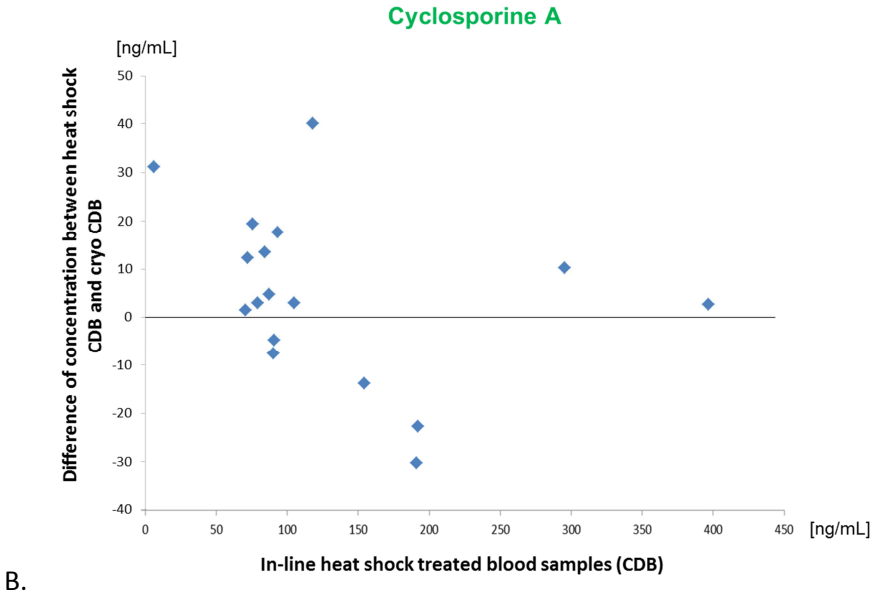
Figure 57: Inter-laboratory method comparison (Passing and Bablok regression analysis),n=17. SPE-LC-MS/MS-determination of Cyclosporine A in off-line protein precipitated and off-line snap-frozen (cryo CDB) blood samples of patients.

A. Scatter diagram with regression line and confidence bands for regression line
B. Residual plot of distribution of difference around fitted regression line

Figure 58 shows the comparison of SPE-LC-MS/MS determination of Cyclosporine A in the 17 patient blood samples, after in-line heat shock treatment and off-line snap freezing, respectively. The equation for the Passing and Bablok regression line is Y = 0.9158 X + 12.1846. The 95 % confidence interval (CI) for the slope is from 0.7137 to 1.0722. The 95 % confidence interval (CI) for the intercept is from -3.6691 to 32.9425. In general, the Passing and Bablok regression analysis shows a very good agreement of the two methods compared.



A.



B.

Figure 58: Method comparison (Passing and Bablok regression analysis), n=17. SPE-LC-MS/MS-determination of Cyclosporine A in in-line heat shock treated (in-line CDB) and off-line snap-frozen (cryo CDB) blood samples of patients.

A) Scatter diagram with regression line and confidence bands for regression line
B) Residual plot of distribution of difference around fitted regression line

In addition, 25 patient samples have been in-line heat-shock treated and on-line analyzed using SPE-LS-MS/MS on three consecutive days. The inter-day accuracy and precision of the measured patient samples are shown in Table 11.

The inter-day accuracy ranges from 0.22 to 28.51 %, and the inter-day precision ranges from 2.45 to 15.55 % RSD. The average values of all the patient samples from three consecutive runs on three separate days are within 15 % of the expected concentration, except one patient sample 31-2717.

Patient sample	Reference Concentration [ng/mL]	Accuracy [%Diff]			Precision %RSD (n=3)
		Day 1	Day 2	Day 3	
31-2704	403	4.81	5.34	12.80	4.13
31-2708	200	-0.22	22.06	12.04	8.19
31-2709	98	13.31	15.01	28.21	11.73
31-2713	205	27.75	20.99	-5.90	15.55
31-2716	155	-0.28	-5.09	-14.12	7.51
31-2717	47	-28.51	-18.46	-23.40	6.53
31-2720	280	-10.31	-3.90	-0.36	5.32
31-2721	193	-8.73	-13.07	1.42	8.01
31-2722	215	0.23	-14.74	14.35	14.67
31-2737	154	-1.93	9.18	-19.43	15.02
31-2751	126	-19.37	-8.87	-11.91	6.09
31-2755	355	-1.3	-7.55	-2.01	3.56
31-4006	85	-12.79	-2.51	-12.60	6.75
01-2706	105	-8.32	-15.85	-13.56	4.41
01-2710	133	-10.09	11.58	8.89	11.61
01-2717	389	-1.35	2.00	-5.30	3.79
01-2722	199	0.26	4.21	-0.56	2.45
01-2724	189	7.89	-1.78	-5.65	7.03
01-2725	184	-5.01	-12.18	-14.84	5.32
01-2726	193	19.28	11.73	6.39	5.77
01-2727	155	-9.85	-14.31	-13.52	2.79
02-2713	428	-6.66	-4.28	-11.23	3.83
02-2716	214	3.42	16.46	10.07	5.95
02-2717	217	16.03	9.50	3.73	5.67
02-2719	168	3.87	18.70	-2.31	9.98

Table 11: Determination of inter-day accuracy and precision of Cyclosporine A measurement in 10 µL of whole blood of patients using the final analysis protocol

3.4.6 Evaluation and elimination of matrix effects

The matrix effects on the ionization response of the analytes were tested by two different approaches:

1) Comparison of the absolute MS peak area(s) of the analyte(s) obtained from spiked matrix samples, i.e. whole blood and spiked neat samples, i.e. EtOH. The samples were spiked with the same concentration of analyte(s) and underwent the same processing and SPE-LC-MS/MS analysis procedures.

2) Post-column infusion of a solution of the analyte into the eluent of LC introduced to the ion source. After reaching a steady baseline, i.e. ionization, a blank matrix sample is injected, heat-shock treated, fractionated and separated. Any matrix components of the sample which affects the ionization process, causes either a decline of the baseline, i.e. ion suppression, or a raise of the baseline, i.e. ion enhancement.

The result of the first approach is documented in a plot of the peak area versus the concentration of the analyte for spiked matrix samples and spiked neat samples (Figure 59).

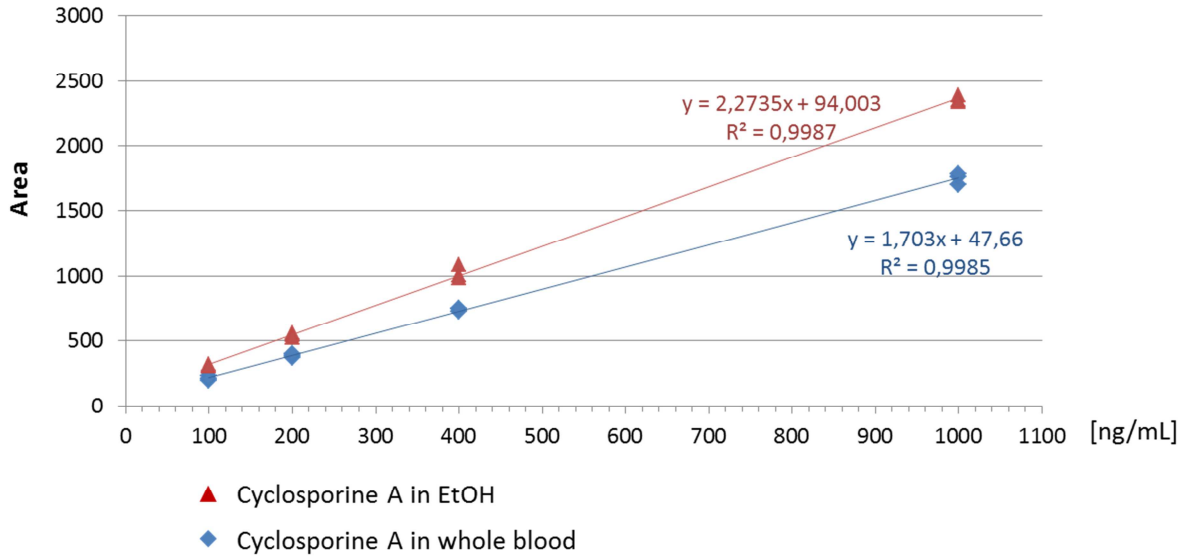


Figure 59: Evaluation of matrix effects: Comparison of the MS peak area of the analyte Cyclosporine A obtained from spiked matrix samples and spiked neat samples.

The difference in the peak areas at the same concentration is due to a matrix effect. In this case the ionization of Cyclosporine A is suppressed.

The extend of matrix effects can be given in percent and is calculated according to Equation 1. The calculation of the matrix effects at each of the different concentrations revealed a value of about minus 25%.

Inter-individual matrix effects have been monitored by measuring blood samples from seven healthy people. All these blood samples were spiked with Cyclosporine A to a concentration of 500 ng/mL. As reference, a spiked neat sample of EtOH with a concentration of 500 ng/mL was used. The results for inter-individual matrix effects are listed in Table 12. The magnitude of matrix effects in 7 different blood samples is around minus 22%. This means that the matrix effects are independent from the concentration of the analyte and are similar in individual samples.

Blood sample	Spiked Concentration [ng/mL]	Peak area of blood sample	Mean peak area of EtOH sample (n=7, reference)	Matrix Effect (%)
WB-1	500	972	1162	- 16.4 %
WB-2	500	863		- 25.7 %
WB-3	500	806		- 29.9 %
WB-4	500	998		- 14.1 %
WB-5	500	917		- 21.1 %
WB-6	500	860		- 25.9 %
WB-7	500	891		- 23.3 %

Table 12: Monitoring of inter-individual matrix effects by measuring 7 different blood samples spiked with 500 ng/mL Cyclosporine A

Figure 60 shows the post-column infusion LC-MS/MS chromatograms of both, blank matrix sample (processed whole blood, i.e. CDB) and blank neat sample (EtOH). Both chromatograms are overlayed with a chromatogram obtained after injection of a solution of Cyclosporine A in EtOH.

The peaks at 4.5 min in both infusion chromatograms are due to valve switching for transfer step. The peaks at 10 min are due to valve switching for separation step. The fluctuation of the baseline during the transfer step (4.5 - 10 min) is due to the varying composition of the mobile phase and/or coeluting matrix components. The varying

composition of the mobile phase also contributes to the rise of the baseline during the separation step in post-column infusion chromatogram of blank neat sample (EtOH). Compared to that, no rise of the baseline during the separation step in the post-column infusion chromatogram of the blank matrix sample (CDB) was observed. This indicates that ion suppression is caused by the sample matrix.

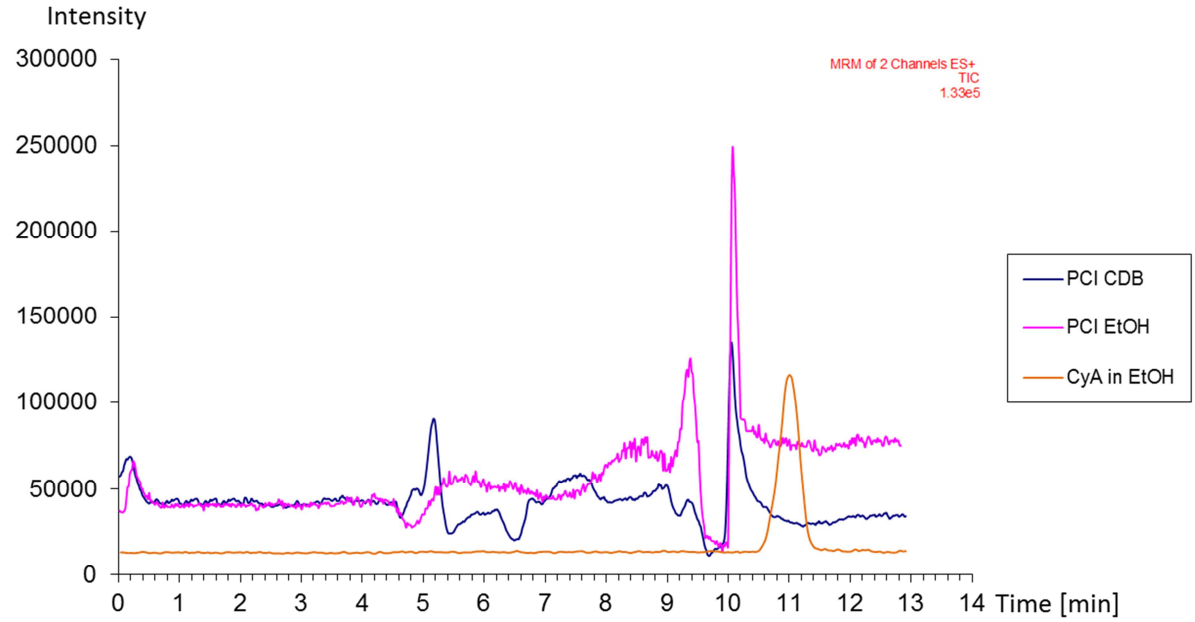


Figure 60: Evaluation of matrix effects: Post-column infusion (PCI) LC-MS/MS chromatograms of blank matrix sample (processed whole blood, i.e. CDB), blank neat sample (EtOH) and a regular chromatogram of Cyclosporine A in EtOH.

In order to eliminate the observed matrix effect, the wash program for on-line SPE-LC has been partially optimized, as described in 3.3.2.2. An optimal wash program could be achieved using an additional pump channel. A mixture of H_2O/ACN (75/25, v/v) could be used to flush the in-line depth filter and SPE column at the end of fractionation step. However, this could not be performed in our method because our HPLC pump does not have such an additional channel for the additional wash solvent.

3.5 Development of a SPE-LC-MS/MS method for analysis of Cyclosporine A using in-line cryogenic treatment of whole blood

The SPE-LC-MS/MS based analysis method for in-line snap-frozen whole blood (cryo CDB) was exactly the same as described in 3.4.1. Two additional steps, however, had to be optimized, namely the sampling of the blood sample by a special processing needle and the snap-freezing/thawing process.

3.5.1 Optimization and standardization of sampling of whole blood for cryogenic treatment

After mixing of a sedimented blood sample (cf. 3.2.1.1), an aliquot (up to 40 µL) of homogenized blood sample is aspirated into the processing needle. The processing needle has a relative large inner diameter and thus the syringe speed was set to 2 µL/s in order to aspirate reproducible blood volumes.

During the freezing process the sample volume expands. Therefore, the blood sample is air-segmented in such a way, that it is positioned in the center of the stainless steel processing needle. This ensures that no sample is lost during the freezing process. The optimal aspiration and segmentation sequence turned out to be:

20 µL air gap (pre sample) – 40 µL whole blood – 30 µL air gap (post sample).

It was found, that due to the large inner diameter, residual wash solution remained in the lumen of the processing needle. Therefore, the processing needle was dried with a nitrogen stream at the end of the wash step.

3.5.2 Optimization of in-line processing of whole blood by cryogenic treatment

A sample of 40 µL of anticoagulated whole blood was in-line processed under different conditions with respect to freezing rate, freezing time, warming rate and thawing time. The resulting cryo CDB samples were examined by light microscopy. All the produced cryo CDB samples were mixed with Trypan Blue (1:100), and inspected at a magnification of one thousand. Under optimal conditions for in-line cryogenic treatment, no intact erythrocytes could be detected.

It turned out that the optimal processing parameters for in-line cryogenic treatment of whole blood (40 µL) are as follows:

Freezing rate: 1296 °C/min

Freezing time: 10 s

Warming rate: 200 °C/min

Thawing time: 60 s

In addition, it could be shown that these process parameters are independent of both, the hematocrit and the blood species.

4. Discussion

Prerequisites for an optimal LC-MS/MS method for clinical-chemical / pharmacological quantitation of small molecules, e.g. drugs and endogenous compounds in body fluids are, that such a procedure

- should have a short analysis time
- should be selective and sensitive
- should be robust
- should not be disturbed by ion suppression when applying an ESI-mode in MS/MS detection
- should be fully automated and thus allow the direct injection and integrated processing of the body fluid investigated.

In this context, it has been shown recently that anticoagulated whole blood can be converted into so-called Cell-Disintegrated Blood (CDB). CDB represents a homogenous blood matrix which can be further pretreated and analyzed using any combination of high performance liquid fractionation, separation and detection systems [17].

One procedure for such a system-integrated, i.e. in-line conversion has been described, namely the heat-shock treatment [15,16]. With regard to a second procedure, i.e. the cryogenic treatment of whole blood, only preliminary results have been published [109].

A major aim of this thesis was to implement both procedures into an autosampler and hyphenate them with an on-line SPE-MS/MS system.

For this purpose, a dedicated XYZ– autosampler was configured in cooperation with LEAP Technologies (Carrboro, NC, USA) and Axel Semrau GmbH (Sprockhövel, Germany).

This special autosampler was programmed to mix a sedimented whole blood sample in a vortexer. The two liquid handling / injection units implemented in the platform were programmed to aspirate a blood sample and / or an Internal Standard, thereby

segmenting both by, e.g. an air gap. After sampling of whole blood, the injection needle can be washed from outside using a pump-driven wash module in order to remove any contamination. The newly developed DLW module of CTC Analytics AG (Zwingen, Schweiz) / LEAP Technologies (Carrboro, NC, USA) was found to be a unique liquid handling system, as it can aspirate and dispense highly viscous samples such as whole blood. As shown, this device guarantees a highly reproducible and contamination-free injection of whole blood. This is because that the fluid to be analyzed is aspirated not into the lumen of a conventional stainless-steel injection needle or syringe body, but into the lumen of a sample holding loop made of Tygon. In combination with the optimized air / liquid / sample segmentation, as well as wash programs, a near-zero carryover / memory effect could be achieved. After aliquoting, the blood sample is processed in-line in two ways and converted into a homogeneous matrix, i.e. Cell-Disintegrated Blood (CDB), by heat-shock treatment for 13 seconds at 75 °C or by snap-freezing with liquid nitrogen followed by slow thawing.

The heat-shock treatment takes place in a heated stainless-steel HPLC capillary (300 x 0.5 mm ID), which is directly connected to the injection valve. This is achieved by using a heated sleeve (250 x 1.587 mm ID) instead of a column oven. This configuration minimizes the dead volume between the injection port and the heated capillary. Combined with an optimal segmentation of the blood sample in the holding loop of the injection needle, a dilution of the blood sample and thus a lysis of blood cells by the aqueous mobile phase are also minimized.

The cryogenic treatment could be optimized with regard to sample volume, sample segmentation, freezing rate, freezing time, warming rate and warming time. The optimal processing parameters for in-line cryogenic treatment of a 40 µL air segmented whole blood sample turned out to be 10 seconds for freezing by contact with liquid nitrogen and 60 seconds for thawing at room temperature.

It was found that for on-line SPE-LC-MS/MS analysis of processed whole blood, i.e. CDB, a replaceable in-line filter has to be inserted between the sample inlet and SPE column in order to prevent clogging of capillaries, sieves and columns by cell nuclei and “cell debris”.

In this work, two different in-line filters were investigated. The single sieve filter consists of a special three-layered stainless-steel sieve in a dead volume free housing. The filter device is sealed by a Teflon ring and a conical pressure screw. Because of the small pore diameter of the sieve (approximately 1 µm), all the components and agglomerates larger than 1 µm formed during the in-line processing, especially during the heat-shock treatment of blood samples, could be withheld by this in-line filter. Sample components of smaller size, however, were retained by the serial connected SPE column and then transferred to the analytical column. This caused a rise in back pressure of both SPE and analytical column after 220 injections / processing cycles each of 20 µL whole blood.

Therefore, the filter device was optimized by converting it into a depth filter. Different porous packing materials, which possess electro-neutral and hydrophilic properties, were tested in depth filters of varying length and inner diameter. We discovered that spherical hydrophilic silica is the material of choice for retaining cell nuclei and “cell debris” generated during the heat-shock treatment. In addition, the conditions for forward and backward flush of the filter after fractionation of the blood sample and transfer of the analyte(s) could be optimized.

For a rapid and effective sample clean-up, i.e. depletion of the macromolecular sample matrix, a TurboFlow SPE column (Cyclone-P, 50 x 0.5 mm ID) was investigated. Due to the high linear flow velocity during operation and the resulting very short residence time of the sample in the column, macromolecular sample components have no time to diffuse into the pores and thus are eluted very effectively in the void volume to waste.

The installed configuration of the switching valves and tubings allows that the TurboFlow SPE column can be back flushed separately from the in-line depth filter after fractionation of the CDB sample and transfer of the analyte(s). By this measure, the life-time of both the depth filter and the SPE column could be extended significantly.

The described heat-shock treatment of a blood sample requires a relatively low flow rate of the sample and mobile phase, respectively. On the other hand, the SPE-based fractionation of the processed sample, i.e. CDB, requires a relatively high flow rate. To solve this problem, a holding loop with a volume of 200 µL was inserted between the outlet of the heated capillary and the TurboFlow SPE column. This

allowed the heat-shock treatment of the blood sample to proceed at a flow rate of 270 µL/min and the operation of the SPE column at a flow rate of 2000 µL/min.

The final total analysis platform for quantitation of the model analyte Cyclosporine A after heat-shock treatment of anticoagulated whole blood was evaluated with regards to different aspects.

First, the developed method was validated. It was shown that the method is linear in the range of 10 (LLOQ) to 1000 ng/mL. The intra-day and inter-day accuracy and precision are within the limits required by the FDA for a bioanalytical method. A matrix-independent and a matrix-dependent recovery of around 100% demonstrate that the on-line SPE-step is very efficient. By comparing the MS-response of the model analyte Cyclosporine A in a spiked sample of whole blood with a sample of Cyclosporine A dissolved in EtOH with the same concentration, it was shown that the ionization process is suppressed by approximately 25 %. However, the addition of an Internal Standard, i.e. Cyclosporine D, can totally compensate for these matrix effects.

The robustness of the total analysis platform could be optimized by improving the hardware for the in-line filter and the wash programs for the in-line filter and the SPE column. After 500 injections each of 10 µL of whole blood, the on-line clean-up system (composed of the in-line packed depth filter and the TurboFlow SPE column) built up a back pressure of 31 bar, and the separation system (guard column and analytical column) built up 21 bar. Furthermore, the MS/MS responses for the analyte (Cyclosporine A) and the Internal Standard (Cyclosporine D) were monitored during 500 analysis cycles. The relative standard deviation (RSD) of 500 injections amounts to 15.6 % for Cyclosporine A and 15.2 % for Cyclosporine D. These findings prove that this analysis platform is robust and thus can be used in routine.

The optimized analysis method was further evaluated by an inter-laboratory method comparison. The routine method established in the Institute of Laboratory Medicine, Medical Center of the University of Munich, was used as a reference. It relies on a manually performed precipitation of a whole blood sample prior to on-line SPE-LC-MS/MS analysis of the resulting supernatant obtained after centrifugation. A method comparison applying Passing and Bablok plots shows very good agreement between the described method and an established routine method controlled by proficiency test. The measured concentrations of Cyclosporine A in heat-shock treated patient

samples (n = 42) are slightly lower than those routinely determined. One possible reason for this might be that the same patient samples have been measured after at least three days storage at 4 °C, while in the routine laboratory the patient samples are analyzed on the first day. Compared to heat-shock treated samples, snap-frozen samples revealed a smaller difference. However, fewer samples have been analyzed after cryogenic treatment (n = 17). Therefore, it is difficult to estimate which processing method is better from a statistical point of view. In addition, the inter-day accuracy and precision of 25 heat-shock treated patient samples has been determined. The average values of all 25 patient samples (with only one exception) from three consecutive runs on three separate days are within 15 % of the reference concentration measured in routine.

Finally, the applicability of commercial calibrators (ClinCal, Recipe, Munich) and quality control samples (ClinCheck, Recipe, Munich) was tested. The protein matrix of these lyophilized samples turned out to precipitate upon heating. Processing these samples at room temperature, however, solved this problem. Also, a very good linearity ranging from 0 to 1264 ng/mL for the model analyte Cyclosporine A as well as very good accuracy and precision were found.

In conclusion, the described SPE-LC-MS/MS platform fulfills the criteria needed in clinical-chemical analysis as it

- has a short total analysis time (< 11minutes)
- is selective and sensitive with a LLOQ of 10 ng/mL for the model analyte Cyclosporine A
- is robust and tolerates at least 500 injections each of 10 µL of whole blood
- is slightly affected by ion suppression. These matrix effects, however, can be totally compensated for by addition of the Internal Standard Cyclosporine D
- is comparable with an established, semi-automated method in routine use
- is fully automated and allows for the direct injection and integrated processing of anticoagulated whole blood samples.

5. Summary

The thesis describes the configuration, optimization and evaluation of a novel instrumental platform for fully automated SPE-LC-MS/MS analysis of small molecules, such as drugs, in whole blood.

The immunosuppressant Cyclosporine A was chosen as a model analyte, as this drug is predominantly bound to erythrocytes.

First, anticoagulated blood is converted into so-called Cell-Disintegrated Blood (CDB) by heat-shock or cryogenic treatment. CDB represents a homogenous blood sample and consists of subcellular particles which do not sediment on standing and do not clog capillaries, sieves or HPLC column packings.

For in-line treatment of anticoagulated whole blood, i.e. generation of CDB, a sample mixing unit, two special liquid handling units and two home-made sample processing modules were embedded into a XYZ-autosampler.

The module for heat-shock treatment consists of a stainless-steel capillary jacketed with a heating sleeve. Under optimal conditions for sampling and in-line processing of 20 µL of whole blood, it takes 13 seconds at 75 ℃ to generate CDB. The latter is stored in a holding loop before further treatment.

For cryogenic treatment of a blood sample, a stainless-steel processing needle with a large inner diameter was installed in one of the liquid handling units. The autosampler was programmed to introduce the processing needle containing the blood sample (40 µL) into a stand-pipe, which is located in a thermo-flask filled with liquid nitrogen. The processing needle therefore contacts liquid nitrogen and the blood sample is snap-frozen. Optimal conditions were found to be 10 seconds for snap-freezing at -196 ℃ and 60 seconds for thawing at room temperature.

A CDB sample obtained either by heat-shock or cryogenic treatment is further processed by being pumped via a switching-valve through an in-line filter to retain cell nuclei and “cell debris”. It was found that a depth filter packed with spherical hydrophilic silica is optimal. This filter allows at least 200 analysis cycles before it has to be replaced.

Next, the CDB sample is pumped on-line via another switching-valve through a SPE

column (50 x 0.5 mm ID) at a high flow rate. Due to the special packing material and the very small inner diameter, a high linear flow velocity is achieved and turbulent flow is generated. By this, high-molecular matrix components such as proteins are eluted in the void volume to waste. The low-molecular weight target analyte Cyclosporine A and the Internal Standard Cyclosporine D are retained and extracted by reversed phase partitioning chromatography (RPC).

After fractionation of CDB on the SPE column, the analyte and the IS are transferred to a series-connected analytical column and separated from residual matrix components by RPC. Finally, the analyte is detected by a tandem mass spectrometer applying electrospray ionization (ESI) and multiple reaction monitoring (MRM).

The optimized method has a total analysis time of less than 11 minutes. The analytical procedure and the instrumental platform were validated for heat-shock treated blood samples with respect to linearity, range (10 - 1000 ng/mL), lower limit of quantitation (10 ng/mL), intra-day and inter-day accuracy and precision, as well as matrix-independent and matrix-dependent recovery (around 100 %). It was shown that the electrospray induced ionization is suppressed by approximately 25 %. These matrix effects, however, can be totally compensated for by the addition of an Internal Standard, i.e. Cyclosporine D.

A comparison with a semi-automated SPE-LC-MS/MS method, established in the Institute, revealed a very good agreement. This was shown by Passing and Bablok plots.

The robustness of the fully automated SPE-LC-MS/MS analysis platform was monitored during 500 consecutive analysis cycles with heat-shock treated blood samples. The relative standard deviation for the signal response was 15.6 % for Cyclosporine A and 15.2 % for Cyclosporine D. The back pressure of the total system rose only by 52 bar.

These findings show that, despite its instrumental and chromatographic complexity, the described analysis platform fulfills the prerequisites to be used in routine clinical-chemical analysis.

6. Zusammenfassung

Die Doktorarbeit beschreibt die Konfiguration, Optimierung und Evaluierung einer neuartigen instrumentellen Plattform für die vollständig automatisierte SPE-LC-MS/MS Analyse von kleinen Molekülen, wie beispielsweise Arzneistoffe, im Vollblut.

Das Immunsuppressivum Cyclosporin A wurde als Modellanalyt gewählt, da dieser Arzneistoff vorwiegend an Erythrozyten gebunden ist.

Zunächst wird antikoaguliertes Blut durch eine Hitze- oder Kälteschock Behandlung in sogenanntes Zell-desintegriertes Blut (Cell-Disintegrated Blood, CDB) überführt. CDB stellt eine homogene Blutprobe dar und besteht aus subzellulären Partikel, die beim Stehen nicht sedimentieren und keine Kapillaren, Siebe und HPLC-Packungsmaterialien verstopfen.

Für die in-line Behandlung von antikoagulierten Vollblut, d.h. für die Herstellung von CDB, wurde ein Gerät zum Mischen der Probe, zwei spezielle Bauteile für die Handhabung von Flüssigkeiten und zwei selbst-gebaute Module für die Probenprozessierung in einen XYZ-Probengeber eingebaut.

Das Modul für die Hitze-Schock Behandlung besteht aus einer Edelstahlkapillare, die mit einer Heizmanschette ummantelt ist. Unter optimalen Bedingungen für die Probenahme und in-line Prozessierung von 20 µL Vollblut werden 13 Sekunden und 75 ℃ benötigt um CDB herzustellen. Letzteres wird vor einer weiteren Behandlung in einer Rückhalteschleife gelagert.

Für die Tieftemperatur Behandlung einer Blutprobe wurde eine weitlumige Edelstahlnadel zur Prozessierung in eines der Bauteile für die Handhabung von Flüssigkeiten eingebaut. Der Probengeber wurde so programmiert, dass die Nadel, welche die Blutprobe (40 µL) enthält, in ein Steigrohr, welches sich in einem mit flüssigem Stickstoff gefüllten Isolierbehälter befindet, eingeführt wird.

Hierdurch wird die Nadel mit flüssigem Stickstoff kontaktiert und die Blutprobe schockgefroren. Als optimale Bedingungen wurden 10 Sekunden für das Schockgefrieren bei -196 ℃ und 60 Sekunden für das Auftauen bei Raumtemperatur gefunden.

Eine CDB Probe, die entweder durch Hitze- oder Kälteschock-Behandlung

gewonnen wurde, wird weiter prozessiert, indem sie über ein Schaltventil durch einen in-line Filter gepumpt wird, um Zellkerne und „Zellbruchstücke“ zurückzuhalten. Es stellte sich heraus, dass ein Tiefenfilter, der mit sphärischem hydrophilem Kieselgel gepackt ist, optimal ist. Dieser Filter ermöglicht mindestens 200 Analysen-Zyklen bevor er ausgetauscht werden muss.

In einem weiteren Schritt wird die CDB Probe on-line über ein weiteres Schaltventil mit einer hohen Flussrate durch eine SPE Säule (50 x 0.5 mm ID) gepumpt. Aufgrund des speziellen Packungsmaterials und dem sehr kleinen Innendurchmesser wird eine hohe lineare Flussgeschwindigkeit erreicht und eine turbulente Strömung erzeugt. Hierdurch werden hochmolekulare Matrixkomponenten wie beispielsweise Proteine im Totvolumen in den Abfall eluiert. Niedermolekulare Zielanalyte wie Cyclosporin A und der interne Standard Cyclosporin D werden über Umkehrphasen-Verteilungschromatographie (RPC) reteniert und extrahiert.

Nach der Fraktionierung von CDB auf der SPE Säule, wird der Analyt und der interne Standard auf eine in Serie geschaltete analytische Säule überführt und von restlichen Matrixbestandteilen über RPC abgetrennt. Zum Schluss wird der Analyt in einem Tandem-Massenspektrometer über eine Elektrospray Ionisation (ESI) und Multiple Reaction Monitoring (MRM) detektiert.

Die optimierte Methode weist eine Gesamtanalysezeit von weniger als 11 Minuten auf.

Das Analysenverfahren und die instrumentelle Plattform wurden für Hitzeschock behandelte Blutproben hinsichtlich Linearität, Messbereich (10 – 1000 ng/mL), unterer Bestimmungsgrenze (10 ng/mL), Richtigkeit und Präzision innerhalb eines Tages und von Tag zu Tag, sowie Matrix-unabhängiger und Matrix-abhängiger Wiederfindung (um 100 %) validiert. Es konnte gezeigt werden, dass die über Elektrospray induzierte Ionisation um ca. 25 % unterdrückt wird. Diese Matrixeffekte können jedoch durch Zugabe des internen Standards Cyclosporin D vollständig kompensiert werden.

Ein Vergleich mit einer teilautomatisierten SPE-LC-MS/MS Routinemethode, die im Institut etabliert ist, ergab eine sehr gute Übereinstimmung. Dies konnte anhand von Passing und Bablok Plots aufgezeigt werden.

Die Robustheit der vollständig automatisierten SPE-LC-MS/MS Analysenplattform

wurde während 500 aufeinander folgenden Analysezyklen mit Hitzeschock behandelten Blutproben überprüft. Die relative Standardabweichung für das MS-signal betrug 15.6 % für Cyclosporin A und 15.2 % für Cyclosporin D. Der Rückdruck des gesamten Systems stieg nur um 52 bar an.

Diese Ergebnisse zeigen, dass – trotz der instrumentellen und chromatographischen Komplexität – die beschriebene Analysenplattform die Anforderungen, die in der klinisch-chemischen Routineanalytik gestellt werden, erfüllt.

7. Appendix

7.1 Equipment and Materials

SPE-LC-MS/MS (-UV):

Autosampler	CTC Analytics (CTC Analytics, Zwingen, CH)
HPLC pumps	Agilent Technologies 1100 Series (Agilent, Santa Clara, CA, USA)
	Merk-Hitachi L 6200 intelligent pump (Merck KGaA, Darmstadt, Germany)
Degasser	Agilent Technologies 1100 Series (Agilent, Santa Clara, CA, USA)
Column oven	HotDog 5090 (Prolab GmbH, Reinach, CH)
Capillary heater	MultiSLEEVE and AgileSLEEVE Plus (Analytical Sales and Services, Inc., NJ, USA)
Mass Spectrometer	Quattro Micro TM (Waters, Manchester, UK)
	LTQ Velos (ThermoFisher Scientific, USA)
UV-Detector	Agilent Technologies 1100 Series (Agilent, Santa Clara, CA, USA)
Software	Chronos (Axel Semrau GmbH & Co. KG, Sprockhövel, Germany)
	ChemStation Rev.B.04.03 (Agilent, Santa Clara, CA, USA)
	MassLynx 4.1 (Waters Corporation, Milford, USA)
	Xcalibur (ThermoFisher Scientific, USA)
In-line Filter	(Recipe Chemicals + Instruments GmbH, Munich, Germany)

SPE columns

Cyclone-P	50 x 0.5 mm ID, ThermoFisher Scientific, USA
Home-made column	40 x 1 mm ID, packed with Oasis HLB material (dp 10 µm, Waters Corporation, Milford, USA)

Analytical columns

Accucore PFP	50 x 2.1mm ID, ThermoFisher Scientific, USA
Hypersil Gold PFP	10 x 2.1 mm and 50 x 2.1 mm ID, ThermoFisher Scientific, USA

Other Equipment

Centrifuge 5415 D	Eppendorff AG, Hamburg, Germany
Balance Sartorius CP 225 D-OCE	Sartorius AG, Göttingen, Germany
pH-meter Metrohm 691	Deutsche Metrohm GmbH & Co.KG, Filderstadt, Germany
Microscope	Olympus Deutschland GmbH, Hamburg, Germany
Ultrasonic Bath	Bandelin electronic GmbH Co. KG, Berlin, Germany
Sieves	Haver & Boecker OHG, Oelde, Germany

7.2 Chemicals and Reagents

Cyclosporine A	Novartis International, Basel, CH
Cyclosporine D	Novartis International, Basel, CH
Cyclosporine A – D12	Novartis International, Basel, CH
Clin Cal ®	RECIPE Chemicals + Instruments GmbH, Munich, Germany
Clin Check ®	RECIPE Chemicals + Instruments GmbH, Munich, Germany
Dimethyl Sulfoxide	Sigma-Aldrich, Steinheim, Germany
Acetonitrile	J. T. Baker, Deventer, Netherlands
Ethanol	J. T. Baker, Deventer, Netherlands
Methanol	J. T. Baker, Deventer, Netherlands
Water	J. T. Baker, Deventer, Netherlands
Formic Acid	Merck KGaA, Darmstadt, Germany
Ammonium Acetate	Sigma-Aldrich, Steinheim, Germany
Sodium Chloride	Merck KGaA, Darmstadt, Germany
Acetic Acid	Merck KGaA, Darmstadt, Germany
BCA™ Protein Assay	Thermo Scientific, Rockford, IL, USA

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