High performance analytical tools for small molecules in biosamples

Dissertation for the degree Philosophiae Doctor

by

Elin Follaug Johnsen

Department of Chemistry

Faculty of Mathematics and Natural Sciences

University of Oslo

2016

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 1803

ISSN 1501-7710

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

Contents

Preface

The work presented in this thesis was carried out at the Department of Chemistry, University of Oslo in the period 2010-2016 under the supervision of Prof. Elsa Lundanes, Prof. Tyge Greibrokk and Ass. Prof. Steven Ray Wilson. I am truly grateful for their guidance and support through all these years, including their support in my non-scientific project which has been to create a family. These years have been challenging and sometimes frustrating, but most of all exciting, interesting and extremely educational.

A warm thanks to all my colleagues and students in the bioanalytical group, for good cooperation and interesting discussions, but also for all the fun and laughter and for creating an excellent working environment. I will not miss the taste of the terrible coffee we made, but I will miss drinking it with you! A special thanks to Marita Clausen and Inge Mikalsen for valuable support and help with technical questions. I am also very thankful to my co-authors for their contributions to this work and especially Siri Leknes who has introduced me to the world of neuroscience.

Thanks to all my friends for always being there. Many of you still don't have a clue about what I do and I really appreciate that, since it creates a perfect balance in my life. Although everything is chemistry, chemistry is not everything (!)

Thanks to my family for always supporting me and believing in me. Especially mum and dad who have taught me so much and who made it possible for me to study for such a long time. Thanks for being so patient with me while I was trying to figure out what I wanted to be when I grew up.

To my boys Jens and Ola, who were both born during this period; you have made my life complete, and I love you.

Finally, Magne, this thesis is dedicated to you. Thanks for all the support and for asking all the right (but sometimes tricky) questions. *All I know is I'm loving you for all the right reasons. In my sky you will always be my morning star.*

Elin Follaug Johnsen

Oslo, August, 2016

List of papers

I **Hydrophilic interaction chromatography of nucleoside triphosphates with temperature as a separation parameter.**

Elin Johnsen, Steven Ray Wilson, Ingvild Odsbu, Andreas Krapp, Helle Malerod, Kirsten Skarstad and Elsa Lundanes.

Journal of Chromatography A 1218 (2011) 5981-5986

II **A critical evaluation of Amicon Ultra centrifugal filters for separating proteins, drugs and nanoparticles in biosamples.**

Elin Johnsen, Ole Kristian Brandtzaeg, Tore Vehus, Hanne Roberg-Larsen, Vanya Bogoeva, Ornela Ademi, Jon Hildahl, Elsa Lundanes and Steven Ray Wilson. Journal of Pharmaceutical and Biomedical Analysis 120 (2016) 106-111

III **Liquid chromatography-mass spectrometry platform for both small neurotransmitters and neuropeptides in blood, with automatic and robust solid phase extraction.**

Elin Johnsen, Siri Leknes, Steven Ray Wilson and Elsa Lundanes.

Scientific Reports 5, 9308 (2015) DOI:10.1038/srep09308

IV **Proteomic tools reveal startlingly high amounts of oxytocin in plasma and serum.**

Ole Kristian Brandtzaeg, Elin Johnsen, Hanne Roberg-Larsen, Knut Fredrik Seip, Evan L. MacLean, Laurance R. Gesquiere, Siri Leknes, Elsa Lundanes and Steven Ray Wilson.

Scientific Reports 6, 31693 (2016) DOI:10.1038/srep31693

Additional paper related to this thesis

A1 Determination of 3'-Phosphoadenosine-5'-Phosphosulfate (PAPS) in cells and Golgi fractions using Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry (HILIC-MS).

Rua Kareem Dowood, Ravi Adusumalli, Emil Tykesson, Elin Johnsen, Elsa Lundanes, Kristian Prydz and Steven Ray Wilson.

Journal of Chromatography A 1470 (2016) 70-75

Abstract

This thesis describes the development of bioanalytical methods for determination of metabolites and other small molecules in biosamples. High performance analytical tools like hydrophilic interaction liquid chromatography (HILIC), various off-line and on-line sample preparation techniques and miniaturized LC columns were investigated with the aim of addressing important issues related to bioanalysis of small molecules.

P**aper I** describes the development of an LC-UV method for the determination of highly polar triphosphate nucleotides. When two 15 cm HILIC columns were coupled in series, all 8 nucleotides in a cell extract were successfully separated using a mobile phase consisting of 70 % acetonitrile (ACN) and 30 % 100 mmol/L (mM) ammonium carbonate. While changing the solvent composition did not lead to an improvement of the separation, temperature could be used to fine-tune the separation of the nucleotides. A **comprehensive 2-step off-line solid phase extraction (SPE) procedure** was employed after an initial protein precipitation (PPT) step. The sample was first loaded onto an RP SPE, before a strong anion exchange SPE was used. The eluate was evaporated to dryness and reconstituted twice; between the SPEs and before injection into the LC system. The advantage of this procedure was that all 8 nucleotides could be selectively determined in cell samples, using a (non-specific) UV detector. However, the procedure was time consuming and laborious, and all the manual steps increased the risk of errors, and make the method less reproducible.

Paper II describes the development of a simple RPLC-mass spectrometry (MS) method for determination of the non-polar drugs rifampicin and thioridazine, in limited amounts of cell lysate. **Off-line** centrifugal filters were used as a stand-alone sample preparation procedure, and no additional PPT was necessary. With only one centrifugation step, the recovery of the drugs was high when aqueous standards were filtrated, but decreased significantly for spiked cell samples. An additional washing step with the mobile phase was sufficient to disrupt the drug-protein bindings, which were assumed to be the reason for the low recovery in cell samples, and the filtrate could be directly injected into the LC-MS system. The procedure cannot be subjected to **on-line** coupling, but it is simple and robust, and at least some of the steps are possible to automate. The centrifugation filters are well suited for separating drugs and nanoparticles in simple aqueous solutions.

Attempts were hence made to modify the method for measuring drug release from nanoparticles in biological samples, but this was not successful since common drug-protein binding disruptors either dissolved the nanoparticles or were incompatible with the LC-MS instrumentation.

In **Paper III**, the knowledge of- and experience with HILIC, gained in **Paper I**, was used to develop a more advanced analytical platform for determination of neurotransmitters in biofluids, using **on-line** SPE. A HILIC stationary phase was used for both **on-line** SPE and capillary LC, and this approach enabled compounds from all categories of neurotransmitters to elute as narrow peaks, allowing precise quantification of analytes in small sample volumes of whole blood. The robust automatic filtration/filter back-flush (AFFL) feature was used to automate the platform, which, in combination with the use of **on-line** SPE, kept the manual sample preparation to a minimum. The methods' ability to identify and quantify a wide range of neurotransmitters simultaneously was demonstrated by analysing whole blood samples.

The neuropeptides oxytocin and vasopressin could also be successfully identified in whole blood using the validated neurotransmitter platform (**Paper III**), and hence, the possibility of incorporating neuropeptides into the method was investigated. The chromatography was satisfying, but a severe carryover was found for all the peptides, probably due to the peptides' low solubility in high amounts of ACN. The platform was hence found unsuited for determination of neuropeptides, and the goal in **Paper IV** was to develop an alternative LC-MS based method focusing only on oxytocin. Preliminary experiments had shown sufficient retention of neuropeptides on an RP-column, and hence RP was chosen for the method. A nano LC system was assessed to obtain sufficient sensitivity, and a silica monolithic RP column gave satisfactory retention and efficiency with a 20 min gradient. **On-line** SPE was utilized and the AFFL system was incorporated to avoid clogging of the columns. A PPT step with 80 % ACN was used to remove the plasma proteins, but a very strong protein binding made the determination of endogenous oxytocin difficult due to co-precipitation during PPT. A reduction/alkylation (R/A) step, prior to the PPT, resulted in a complete and stable derivatization of oxytocin, and when analysing unspiked R/A treated plasma samples, endogenous oxytocin was found to be present at high levels. A R/A step was hence included in the sample preparation procedure, prior to the PPT. The nano LC-MS methods ability to quantify endogenous oxytocin was shown by analysing human plasma and human cord serum.

Abbreviations

ACN	Acetonitrile
AFFL	Automatic filtration/filter back-flush
Cap	Capillary
cLOD	Concentration limit of detection
CNS	Central nervous system
CV	Coefficient of variation
CSF	Cerebrospinal fluid
dNTP	deoxynucleotide triphosphate
DSB	Disulfide[1] bridge
E.coli	*Escherichia coli*
ESI	Electrospray ionization
ELISA	Enzyme-linked immunosorbent assay
FA	Formic acid
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
ID	Inner diameter
IEX	Ion exchange
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase micro-extraction
MeOH	Methanol
MEPS	Micro-extraction by packed sorbent

[1] Sulfide/sulfate is the spelling recommended by IUPAC, but sulphide/sulphate is used in British English. Sulfide/sulfate is used in this thesis even though British English is used elsewhere.

MIP	Molecular imprinted polymer
MS	Mass spectrometry
MS/MS	Tandem MS
M.tb	*Mycobacterium tuberculosis*
NMR	Nuclear magnetic resonance
NP	Normal phase
NT	Neurotransmitter
NTP	Nucleotide triphosphate
PEA	Phenyl ethyl amine
PGC	Porous graphitic carbon
PLGA	poly(lactic-co-glycolic acid)
PPT	Protein precipitation
R/A	Reduction/alkylation
RAM	Restricted access medium
RIA	Radioimmunoassay
RIF	Rifampicin
RP	Reversed phase
RSD	Relative standard deviation
SALLE	Salting-out assisted LLE
SAX	Strong anion exchange
SBSE	Stir bar sorptive extraction
SCX	Strong cation exchange
SIL	Stable isotope labelled
S/N	Signal to noise ratio
SPE	Solid phase extraction
SPME	Solid phase micro-extraction
TCA	Trichloroacetic acid
TZ	Thioridazine
WAX	Weak anion exchange
WCX	Weak cation exchange

1. INTRODUCTION

Whereas measuring proteins/DNA/RNA give information of what *might* happen in the human body, measurements of metabolites (*metabolomics*) can reveal processes that have already occurred [1-3]. In contrast to proteins/DNA/RNA, which are macromolecules, metabolites are fairly small. Hence, different approaches have to be used for measuring small molecules in biosamples. This thesis is dedicated to the development of bioanalytical methods for determination of metabolites, but also other small molecules (e.g. drugs) in various biosamples, which can share analytical approaches with metabolites.

1.1 Bioanalysis and metabolomics

While bioanalysis is the common term for qualitative and quantitative determination of drug/metabolites/biomarkers in biological matrices [4], metabolomics can be defined as the comprehensive analytical approach for the study of all low-molar-mass species (the metabolome) present in a given biological system of interest, e.g. the human body [1, 5, 6]. The terms "metabolomics" and "metabonomics" are often used interchangeably [7], and in this thesis only the former will be used. Metabolites include peptides, lipids, amino acids, vitamins, minerals, food additivities, drugs, toxins and almost any other chemical (< 2000 Da) that humans ingest, metabolize, catabolize or come in contact with [8]. The human metabolome is not easily defined, in contrast to e.g. the proteome, since the metabolome consists of a mixture of endogenous and exogenous compounds [1, 8]. Endogenous metabolites are small molecules, e.g. amino acids and carbohydrates, synthesized by the enzymes encoded by our genome, while exogenous metabolites are "foreign" chemicals consumed as foods, drinks, drugs etc. [8] According to Pearson et al. the aim of metabolomics studies is to be able to take a sample of body fluid (e.g. urine or blood), scan it and find a profile of tens or hundreds of chemicals [5]. This approach is referred to as *untargeted metabolomics,* also called holistic, or global metabolomics. It is desirable to detect

as many metabolites as possible, known and unknown, in a single experiment, and then relate their concentrations to features or properties of the sample [6, 9], and untargeted metabolomics is a promising tool for biomarker discovery [2, 7]. In *targeted metabolomics*, a number of metabolites are recognised as analytical targets and a method is developed in order to quantify them in the samples of interest [7, 9, 10]. In this thesis the focus will be on targeted metabolomics.

As before mentioned; exogenous metabolites can originate from drugs, but drugs are not metabolites them self. However, small drugs and metabolites have a lot in common. Drugs are often based on endogenous compounds, and one example is nucleoside analogue compounds, which are used in anti-cancer, anti-viral and immunosuppressive therapy. The analytical techniques used in metabolomics studies are hence often well suited for determination of small drugs.

1.2 Analytical techniques in metabolomics studies

The number of metabolomics studies is increasing, and today applications can be found in the medical field, as well as in plant/food- and environmental sciences [6, 7].

Metabolites constitute a diverse set of atomic arrangements when compared to the proteome [11], with large variations in chemical (molar mass, polarity, solubility), and physical (volatility) properties [11]. The metabolome also extends over a wide range of possible concentrations (pM-mM), and no analytical technique can currently quantify all metabolites [1, 6, 7, 11]. Nuclear magnetic resonance (NMR) has been widely used, but due to low sensitivity and the spectral overlap that often occur, the number and variety of metabolites that can be simultaneously observed are limited [9, 10]. MS based methods most often provide greater sensitivity than NMR, and utilisation of MS in direct infusion mode has been proposed for screening purposes [7]. But without an efficient separation prior to the MS, extensive ion suppression can be a problem, and separation of isobaric and isomeric substances is impossible [7]. Gas chromatography (GC) is well suited for

measuring volatile and thermally stable metabolites, but a wider range of chemical species can be analysed with LC. LC offer the most versatile tools for the determination of a multitude of molecules which belong to different groups, have different molecular properties and coexist in the same sample in varying concentrations. Hence, LC has become an indispensable tool for the determination of metabolites in a very wide range of applications, and when combined with MS, this is currently the platform most frequently used in metabolomics [6, 7, 9].

1.2.1 RPLC vs HILIC

RP chromatography is the most reliable and robust LC separation principle [7], and is most often used for metabolite determination as it is directly compatible with the analysis of aqueous samples and can be utilized for a plethora of metabolites. However, RPLC has limitations regarding determination of polar molecules, due to their poor retention, and alternative separation principles are often needed for these compounds. Normal phase (NP)LC can be used, but is generally not desirable for routine applications due to poor reproducibility and compatibility issues with electrospray ionization (ESI)-MS [12].

HILIC is a good alternative for compounds with little or no retention on RP columns, as the elution order is more or less the opposite of what is seen in RP separations [7, 13]. The term “HILIC” was first suggested by Alpert in 1990 [14], and refers to the combination of a polar stationary phase and a mobile phase containing at least 60 % of water-miscible organic solvent [15]. The number of HILIC publications has increased substantially in the last few years [13, 15, 16], as illustrated in **Figure 1**.

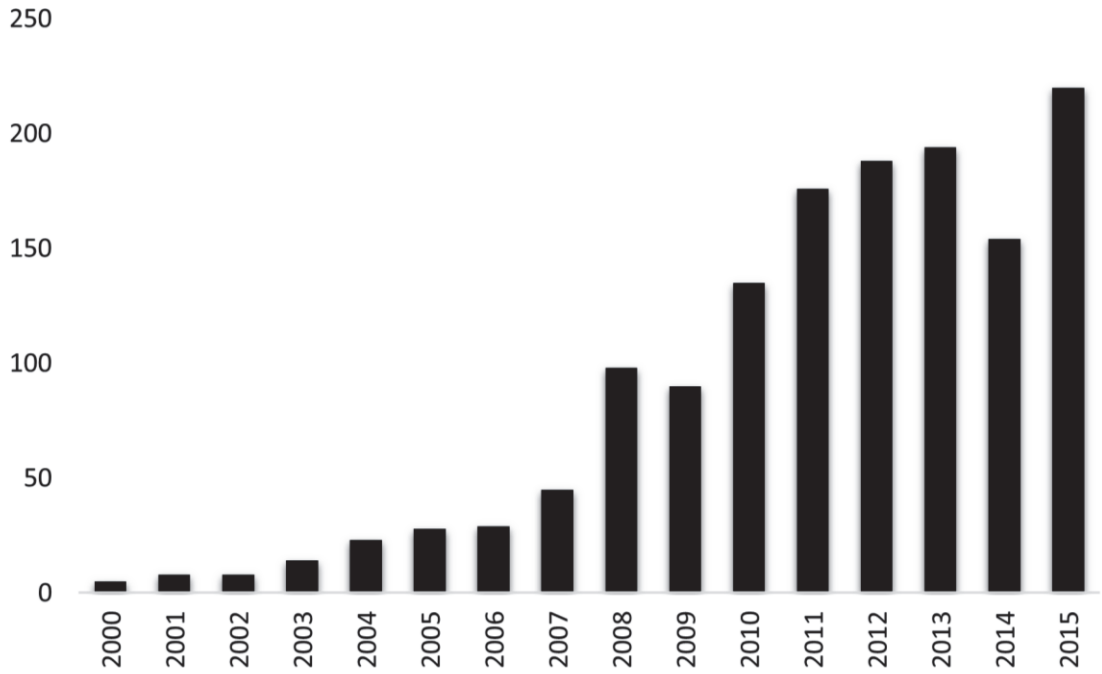


Figure 1. The number of publications published in 2000-2015 containing "hydrophilic interaction chromatography/hydrophilic interaction liquid chromatography in the title (Google Scholar, June 2016).

Alpert et al investigated the use of HILIC for separation of peptides, nucleic acids and other polar compounds, and Alpert proposed that the main mechanism in HILIC involves partitioning. Polar groups on the stationary phase attracts water molecules and an aqueous layer is formed over the surface. When a hydrophobic mobile phase with a high content (60–97 %) of organic solvent is used, the analytes will partition between the semi-immobilized aqueous layer and the mobile phase. Unlike RPLC, water is the strongest eluent in HILIC [15], and retention is proportional to the polarity of the analyte, and inversely proportional to the polarity of the mobile phase. Hence, more hydrophilic compounds will be more retained by the stationary phase than less hydrophilic compounds. Various types of buffers, such as ammonium acetate or ammonium formate, can be used to efficiently control the mobile phase pH, ionic strength, and ionization of the analytes [15].

In several more recent studies of HILIC separations [16-21], hydrogen-donor interactions and weak-electrostatic mechanisms have been observed as well, indicating that both adsorption and partition play important roles for retention in HILIC. This is due to intermolecular forces (e.g. electrostatic interactions, hydrogen bonding, dipole-dipole interactions and weak hydrophobic interactions).

Any polar chromatographic surface can be used for HILIC separations. Typical HILIC stationary phases consists of classical bare silica or silica gels modified with many polar functional groups (e.g. amino, amide, diol and cyanopropyl) [16]. Polymer-based stationary phases can also be used [22]. Zwitterionic stationary phases are available under the tradenames ZIC-HILIC [13, 23], and ZIC-cHILIC [23, 24] which are silica-based, or ZIC-*p*HILIC which is the polymer-based version of the former. ZIC-HILIC/ZIC-*p*HILIC has a sulfobetaine type functionality (quaternary ammonium part = positive charge, sulfonic acid part = negative charge), where the negative charge is the most accessible to interacting solutes, while ZIC-cHILIC has a phosphorylcholine type functionality (phosphate part = negative charge, quaternary ammonium part = positive charge), where the positive charge is the most accessible. The structures of both stationary phases are illustrated in **Figure 2**.

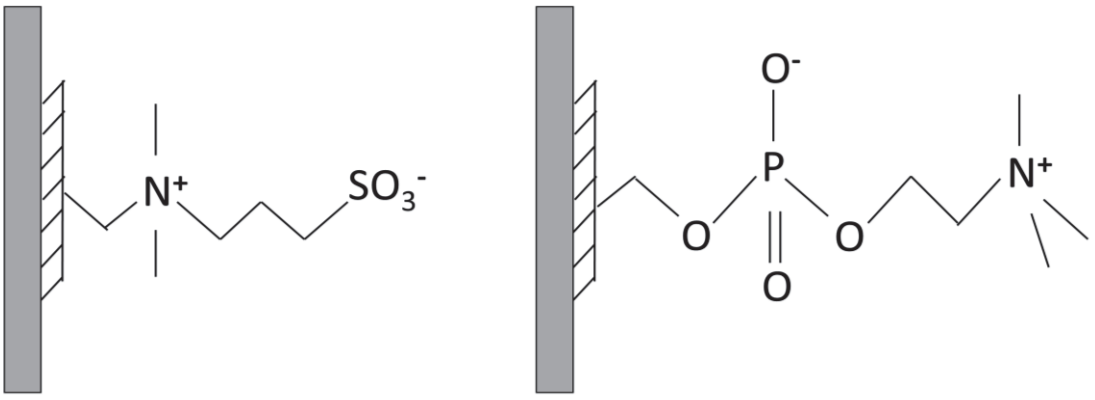


Figure 2. The structure of ZIC-HILIC/ZIC-pHILIC (left) and ZIC-cHILIC (right).

In addition to the possible retention of hydrophilic compounds, HILIC has several other benefits compared to RPLC [12, 15]. The low viscosity of the mobile phase, due to the high concentration of organic solvent, induces low column back pressure and hence longer columns can be used to increase the efficiency, or higher flow rates can be used to decrease the analysis time. The mobile phase is also highly volatile and provides good electrospray and desolvation efficiency, leading to improved sensitivity with e.g. ESI-MS. Finally, samples containing high organic solvent concentrations, obtained after SPE, liquid-liquid extraction (LLE) and PPT, can be directly injected without evaporation and reconstitution steps [15].

1.2.2 HILIC in metabolomics studies

Strong retention capability for highly polar compounds make HILIC an important alternative to RPLC in metabolomics studies [9], and is increasingly being used in metabolite profiling in either targeted or untargeted approaches. HILIC has been utilized for e.g. the simultaneous determination of highly polar sulfur-containing cellular metabolites [25, 26], amino acids and amine-containing metabolites [15, 27-30], lipids [31], nucleosides and nucleobases [32] and organic acids [33]. Since the retention mechanism is very different in HILIC compared to RPLC, these two chromatographic principles can be considered as complementary and orthogonal [15]. Several studies have proved that HILIC can provide complementary information to RP in a metabolomics study [9, 34, 35], and to screen as many metabolites as possible, a combination of the two principles will improve the metabolite coverage of the method [9]. Metabolites are well suited for multidimensional chromatography since the range of components varies widely with respect to polarity [9]. This can be done with off-line two-dimensional coupling of HILIC and RP, where fractions eluting from the first dimension is manually collected and injected onto the second dimension, or with column-switching systems where the analytes are automatically transferred from the first dimension to the second [36].

1.3 Miniaturization in LC

Miniaturization is essentially the reduction of the inner diameter (ID) of the LC column [37], and the typical column characterization, based on ID, can be seen in **Table 2**.

Table 1. IDs of columns in LC. Adapted from [38].

Column type	**ID (mm)**
Conventional	3-5
Narrow-bore	2
Micro	0.5-1
Capillary (cap)	0.1-0.5
Nano	0.01-0.10
Open tubular	0.005-0.05

If there is no limitation on the sample volume, conventional columns have a higher loading capacity and may therefore be preferable due to their better robustness, but for biological samples, often a limited amount is available (e.g. blood from infants or cerebrospinal fluid (CSF)). Hence, bioanalysis is continuously moving towards miniaturization (cap LC and nano LC) [39]. When the analytes of interest are low abundant, the method of choice has to be sensitive enough to detect the analytes against a complex matrix background. When using concentration-sensitive detectors, a high concentration in the eluting band gives high signal intensity. Due to less dilution of the analyte band, the signal-to-noise ratio (*S/N*), and hence the sensitivity, will improve substantially if the ID of the analytical column is reduced [37, 40-42].

Chromatographic dilution D is given by:

$$D = \frac{C_0}{C_{max}} = \frac{\varepsilon\pi r^2(1+k)\sqrt{2\pi LH}}{V_{inj}}$$

Here C_o is the initial compound concentration in a sample, C_{max} is the final compound concentration at the peak maximum, r is the column radius, k is the retention factor, L is the column length, H is the column plate height, ε is the column porosity and V_{inj} is the injected sample volume [37, 40]. D increases proportionally with the square of the column radius, so, if conditions are otherwise equal, by injecting the same amount of compound on a 2.1 mm ID column and a 0.1 mm ID nano column, the sensitivity can, in theory, be improved by a factor of approximately 400, using the nano column [43].

Decreasing the column ID increases the sensitivity of ESI-MS, which behaves as a concentration sensitive detector [38]. Nano columns are mostly used at flow rates of 1 µL/min or less, allowing a nano LC system to be coupled with a nano-spray interface. Nano-spray interfaces often provide higher sensitivity than regular electrospray interfaces because the initial droplets emitted from the narrow bore fused silica nano-spray emitter are significantly smaller in size compared to those emitted from normal stainless steel capillaries, providing a more efficient ionization [43].

One drawback of miniaturized systems (cap LC and nano LC) is the loss of detection sensitivity due to the small injection volumes these columns can handle [37, 40]. This problem can be solved by applying on-column focusing techniques, also called large volume injections [37, 44]. Large volume injections significantly increases detectability in miniaturized systems, but due to the low flow rates used, the loading time becomes an issue, and this technique is hence impractical for cap LC and nano LC [37]. A better solution is to load the samples onto a pre-column,

also called a trap column or an SPE column, since higher flow rates can be used during the sample-focusing step. Large volume injections were earlier done primarily for increasing the loading volume and for sample clean-up, but by selecting a specific stationary phase for the pre-column, the selectivity and isolation of the analytes of interest can be controlled as well [37]. The pre-column will then act as an **on-line** SPE, and this will be described in more detail later.

1.4 Sample preparation in LC-based targeted metabolomics

Despite significant advances in the development of efficient analytical instruments, providing high resolution, sensitivity, precision and practicability, LC analyses of biological samples are restricted by the sample preparation of the highly complex matrices [4, 42, 45, 46]. Biological matrices consist of numerous components including salts, acids, bases, proteins, lipids and cells, and the complexity differ from one matrix to another [4]. While urine often contains sufficient concentrations of metabolites to allow just a dilution before injection into the LC [1, 10], most biological samples only contain trace concentrations of the compounds of interest against a complex matrix background. Hence, direct injection onto an analytical column is often not possible, and a suitable sample preparation is needed prior to the chromatographic analysis.

Sample preparation, also known as sample treatment/sample clean-up/sample extraction, is an integral part of a bioanalytical method, and the aim is to make the sample suitable for the separation and detection technique of choice. In conventional targeted determinations, this is achieved by (i) a selective isolation of the analyte of interest from the matrix and/or matrix simplification, (ii) removal of interfering endogenous components, and (iii) pre-concentration of the analytes to a level above the concentration limit of detection (cLOD) of the analytical instrument [4, 47, 48]. An ideal sample preparation methodology should be fast, accurate and precise, and consume little organic solvent. A more recent demand is the possibility of automation, ideally of the whole procedure [49].

Sample preparation is probably the step most prone to error in most bioanalyses. Even though the trend is towards automatization and robotic systems, a large part of the sample preparation relies on humans, which increases the potential for the introduction of errors [7]. Sample preparation is also the most time consuming and labour-intensive step [4, 7], and in addition to more automation, and improvement of sensitivity and accuracy, efforts have been conducted towards increased speed [45].

In metabolomics studies, the choice of sample preparation method is extremely important because it affects both the observed metabolite content and biological interpretation of the data [6, 50]. Hence, different methods can lead to differences in the measured levels of the metabolites, which again can lead to erroneous conclusions if this is not accounted for. The sample preparation step should ideally retain metabolites of interest, despite differences in chemical and physical properties. It should also be fast, convenient and avoid conditions likely to lead to metabolite degradation or interconversion [10].

1.4.1 Off-line/on-line methodology in sample preparation

Sample preparation can be performed either **off-line** or **on-line**. In this thesis, **off-line** means that at least some steps in the sample preparation have to be done manually. This can be exemplified with SPE: if the sample preparation with SPE is done **off-line**, it means that there is no physical connection between the SPE and the LC. Even though the adding of solvents etc. can be done automatically, the operator has to collect the final eluent, and place it in the injector/auto-sampler by hand. When the sample preparation with SPE is done **on-line**, the SPE is connected to the LC system, and the eluent is transported directly from the SPE to the analytical column. There are several advantages of doing the sample preparation **on-line,** but also some additional challenges, and this will be discussed in more detail after the more conventional off-line sample preparation methods have been introduced.

1.4.2 Off-line sample preparation approaches

PPT, LLE and SPE have been widely used for bioanalytical sample preparation [47], and are still popular methods, though the methodologies are evolving in response to the need for simpler processes and more automation/higher throughput [51]. Ultrafiltration, which has been used extensively for the study of macromolecules, has recent years gained popularity in the study of small molecules as well, and is an alternative to the more traditional approaches [6].

Protein precipitation (PPT)

The matrix of most bioanalytical samples contain a large amount of proteins, which have to be removed or at least reduced before injection into the LC system [47]. For this purpose, PPT is an effective sample preparation method, and even though it is an old technique, PPT is still one of the most used methods in bioanalysis [1, 4, 6, 10, 11, 47, 52]. PPT disrupts any binding between metabolites and proteins (protein binding), so the total metabolite concentration can be measured, which is equal to the sum of bound and free metabolite concentration [6]. PPT involves denaturation of proteins in the matrix by external stress from adding strong acid/base/heat or, most commonly, adding an organic solvent like ACN [4]. PPT can also be performed with salts and metal ions such as zinc sulfate and ammonium chloride [50]. The sample is mixed with the precipitant and centrifuged, and this leads to formation of a protein pellet, and the supernatant is collected for analysis. Depending on the type of precipitant used for extraction, supernatants can either be injected directly, or evaporated to dryness and re-suspended in a solvent compatible with the LC system [1]. PPT is applicable to both hydrophilic and hydrophobic compounds [52], and it can be performed in a 96-wells format which makes it possible to automate the procedure for higher through-put and less manual handling [4]. The drawback of PPT is that the extracted samples are still relatively complex [52], and often an additional sample clean-up step is necessary, e.g. employing LLE or SPE.

Liquid-liquid extraction (LLE)

LLE is the oldest sample preparation technology used in metabolomics [47] and is still used frequently today. LLE is a simple technique which uses the differential distribution of an analyte between the aqueous matrix and an immiscible organic solvent [47], which are mixed together in a container. Some compounds diffuse readily between the aqueous phase and the organic phase, but some need vigorous mixing before gaining an acceptable recovery. The phases may be separated by gravity, centrifugation, a semi-permeable membrane, or by adsorbing one of the phases to a support. Physical separation such as pipetting at a height above the meniscus may also be required [47]. The extracts are generally very clean, but LLE suffers from several limitations such as low/variable recovery, the need for a large sample volume, poor selectivity and matrix effects when using LC-MS [4]. LLE is also almost inapplicable for hydrophilic compounds [52], and might be labour intensive and difficult to automate [47, 51]. An improved version of LLE is "salting-out assisted LLE" (SALLE), where an inorganic salt is added to a mixture of water and a water-miscible organic solvent [4]. This creates a phase separation between the water-miscible organic solvent and water, and can be used to improve the recoveries of hydrophilic analytes in LLE, by enhancing the extraction of the analytes into the organic phase [4, 53]. The method is applicable to a broad range of compounds ranging from low to high hydrophilicity, and the effectiveness depends on the physiochemical properties of the analyte and the type of salt used. SALLE methods are simple and cost-effective [4, 52], and since the first publication in 1973 [53], the interest of the method has increased significantly. SALLE has been used in bioanalysis of drugs and metabolites [52, 54-56], but has also been applied to plant, environmental samples and food products [57-59].

Solid phase extraction (SPE)

SPE is an effective sample preparation method for removal of interfering compound, but also for enrichment/up-concentration of the analytes, which will

result in a more sensitive method [49]. SPE can be used with all chromatographic principles, and in RP systems the analyte is distributed between the aqueous phase moving though the extraction cartridge and the organic phase bound to the solid support [47]. The goal is to fully retain the analytes of interest on the SPE cartridge, while interferences/contaminants, which are less retained by the SPE material, can be washed off. The analytes are then eluted off the SPE column with an appropriate solvent, and by using a smaller eluting volume than the original sample volume, the analytes will be enriched as well [6]. In addition to conventional SPE cartridges it is also possible to use SPE disks, which are made up of a monolithic based stationary phase, available in 96-well format, and is easy to automate [4, 48].

SPE was traditionally available in NP, RP and ion exchange (IEX) mode. The RP mode is the most popular, but these traditional modes are not always suitable, and significant efforts have been made for development and characterization of both new formats and new sorbent materials. The aims have been to improve selectivity towards target analytes, and the sorptive capacity and to enhance the physicochemical or mechanical stability of sorbents, to achieve sorbents that can be applied to a wide range of matrices and analytes [48]. Research has mainly focused on materials with improved stability and/or high affinity for polar analytes. The isolation of highly polar species from aqueous samples is a general problem in bioanalysis, since most traditional non-selective sorbents either lack the ability to retain polar compounds or retain them too strong (irreversible). Generally, sorbents for SPE can be divided into three categories: inorganic oxides (e.g. silica, alumina, magnesium silicate), low-specific sorbents (surface-modified silica, poly(styrene-co-divinylbenzene) resins and carbon-based materials), and compound-specific and class-specific sorbents [48, 51]. An example of the last category is «mixed mode SPE», which refers to a material where two separation principles are combined. The combination of IEX and RP are often used [60-62], and mixed-mode SPEs have the possibility to reduce multiple step purification processes to one step, and to increase retention and recoveries of a wide range of compounds [61]. Other approaches towards developing more specific and selective stationary phases for SPE are molecularly imprinted polymer (MIP) SPEs,

immunosorbents, magnetic nanoparticles, ion imprinting polymers, restricted access medium (RAM) [48], dispersive SPE (also known as QuEChERS, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe), disposable pipette extraction, micro-extraction by packed sorbent (MEPS), solid-phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE) [4].

Due to the wide range of available SPE materials, SPE can be used for most compounds, and it is possible to automate the whole procedure. However, even though SPE offers several advantages over LLE and PPT, the off-line SPE steps can be complicated and laborious, prone to contamination, and it can be difficult to use this approach for small, limited amount samples. The reproducibility can be poor, and the one-time-use SPE columns are expensive [52]. Another limitation is that many matrix constituents can be adsorbed as well, resulting in matrix effects when LC-MS is utilized. This is especially a problem for polar analytes, since they often co-elute with matrix compounds [63, 64]. To summarize; since the introduction of the SPE technique, a significant progress has been observed, including simplification, automation and miniaturization of the original concept [48], and many new approaches have been designed, developed and validated to overcome various limitations or to improve the performance of the SPE technique [4]. The main advantage of new sorbents is their high selectivity and enrichment capability, and the possibility of reducing the number of steps in the extraction procedure.

Ultrafiltration

Ultrafiltration is a simple sample preparation procedure where a sample is filtered through a special filter which only allows passage of molecules of specific mass [6]. Common cut-offs are 3000, 10 000 and 30 000 Da, and filtration is achieved by applying pressure or centrifugation [6]. The latter is most common, and the technique is then called centrifugal ultrafiltration, with the use of centrifugal filters. Since the method relies on separation by the size of the molecule, rather

that the solubility, a major advantage is the removal of lipoproteins, which other methods are not able to remove [65]. Another advantage is the possibility to study macromolecules, which is not the case for precipitation methods where they become denatured [65]. Centrifugal filters are mostly used for desalting, protein enrichment and deproteinization, and since the ultrafiltration results in separate fractions of small molecules and macromolecules, both fractions can be studied and analysed separately. Centrifugal filters have been extensively used for the isolation and enrichment of proteins [66, 67], but is becoming popular for small molecule determination as well, since centrifugal filters can be used as a clean-up step following LLE [68] or PPT [69], and also as a stand-alone sample preparation procedure [66, 70-72]. When centrifugal filters are used as the only sample preparation it is a simple method, with a minimal consumption of sample (usually 100 µL), and easily applicable in a routine analysis laboratory [71]. Centrifugal filters have also been shown to be useful in drug release studies, by being able to separate poly(lactic-co-glycolic acid) (PLGA) nanoparticles and drug, in simple aqueous solutions [73-75]. However, centrifugal filters can have recovery/selectivity issues [65, 69, 76, 77] and they can differ in performance after product re-design. Performance can also vary between brands [77].

1.4.3 On-line sample preparation

One way to improve the efficiency and accuracy of the analytical procedure is on-line coupling of the sample preparation techniques with the LC system. Advantages of this can be improved sensitivity due to reduction of analyte loss, reduction in time- and labour used, reduction of the sample contamination and possible degradation of analytes (due to a closed system), improved operation repeatability and further enhancing the analytical precision and accuracy owing to the automated on-line process with less human error, potentially reducing the consumption of organic solvents and samples [45]. Additionally, on-line systems are often easily automated.

The on-line coupling of sample preparation techniques with LC can be traced from the hyphenation of SPE and LC in the early 1980s [78], and since then, on-line coupling techniques have been widely applied [45]. SPE or SPME have been mostly used, but also MEPS, SBSE, liquid phase microextraction (LPME), microdialysis and dialysis have been reported in on-line systems for liquid samples [45].

On-line solid phase extraction (SPE)

Although on-line coupling of micro-extraction techniques such as SPME, SBSE and LPME are increasing in popularity, SPE is by far the most commonly used on-line sample preparation technique and will be the main focus in this introduction. When searching in the literature there has been an increasing trend of using on-line SPE the last ten years, and this is illustrated in **Figure 3**.

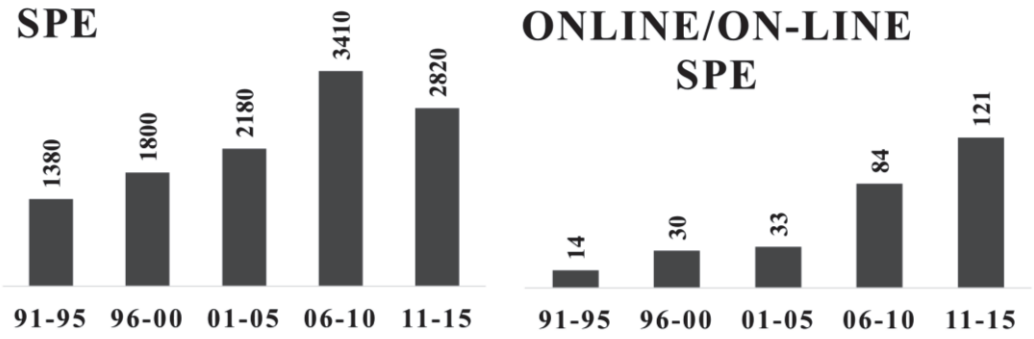


Figure 3. The number of publications published in 1991-2015 containing "online/on-line solid phase extraction/SPE" in the title (left), and those only containing "solid phase extraction/SPE" (right) (Google Scholar, June 2016). Although more papers are published on the use of off-line SPE, the relative increase in the last years is significantly larger for on-line SPE.

SPE is quite easily coupled on-line with LC systems, and this is often referred to as *column switching* [45, 64, 79-83]. On-line SPE-LC has been used for decades [79-81], and has primarily been used for drug and pollutant determination with non-miniaturized systems [49, 81-83]. Today on-line SPE is becoming a common feature in cap LC and nano LC systems as well [45]. On-line SPE-LC is easily automated, and hence offer the potential for automation of the total analytical procedure [43]. This can allow preparation of less amount of sample or improve the total method's limit of detection (LOD), in addition to saving time and labour [43]. By reducing the manual preparation steps, the risk of human errors is also reduced. An additional benefit is that larger volumes can be quickly injected into an SPE-LC switching system, substantially improving the sensitivity in cap LC and nano LC, without compromising the chromatographic performance [39, 43]. A schematic view of a traditional column switching system using a two-position six-port valve system is shown in **Figure 4**.

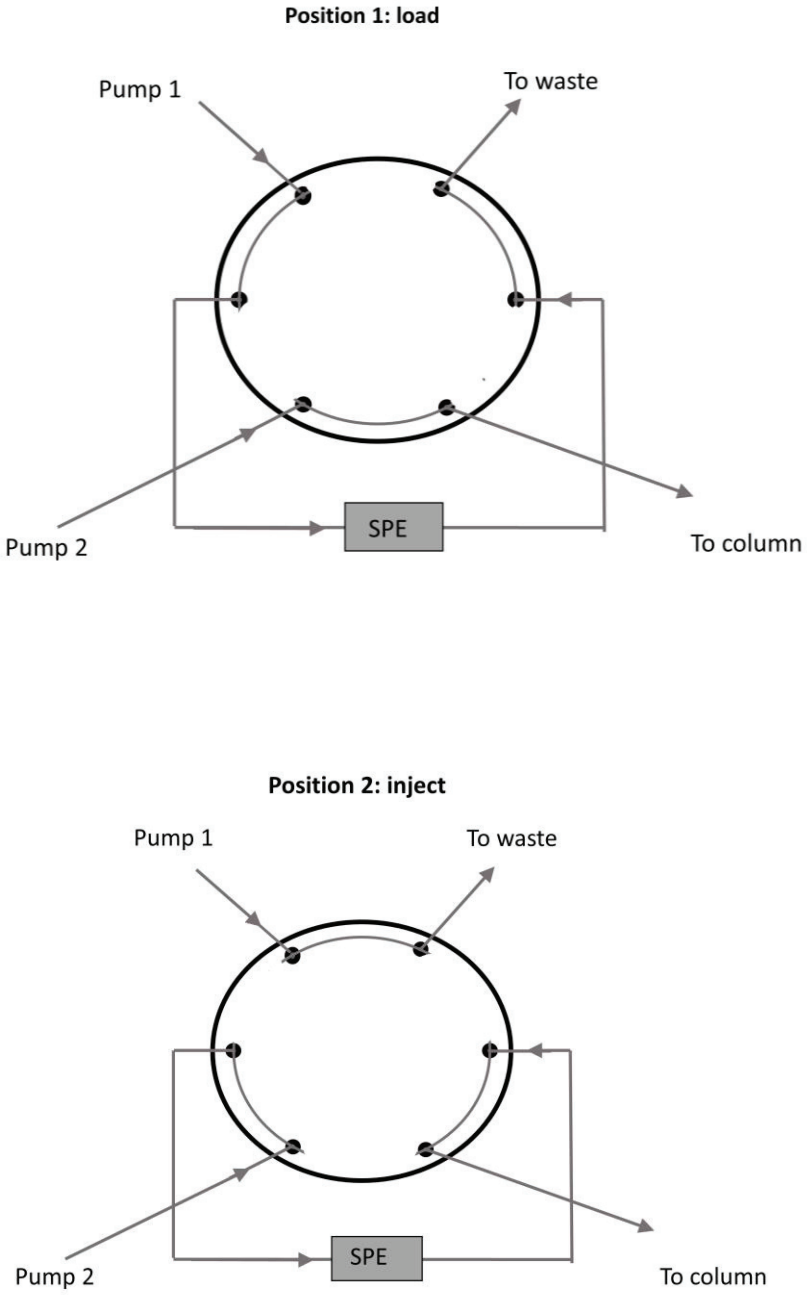


Figure 4. A traditional column switching set-up. The two switching positions (i) "load" and (ii) "inject" are illustrated. The valve set-up is shown in front-flush mode.

The system is based on a valve-switching approach (hence, the name *column switching*) and consists of a loading devise (injector or auto-sampler), typically a six-port valve and two pumps. When the valve is in "load" position, a loading pump transfers the sample from the injector onto the SPE column, where the analytes are trapped, while non-retained compounds and solvent are washed out to waste. When the valve is switched to "inject" position another pump elutes the analytes from the SPE and onto the analytical column for separation. This can be done in either front-flush, or back-flush mode. The latter is normally used since it allows the analytes retained at the front of the SPE to be easily transferred onto the LC column, minimizing peak broadening [45]. When using an auto-sampler, the sample loading, trapping on SPE, washing and transfer to the analytical column can be controlled by the LC software, and the analysis can be completely automated [45].

The most common switching system contains an RP SPE column and an RP LC column, and this allows for trapping and separation of relatively hydrophobic analytes [39]. However, this combination is not very selective, and it is not applicable for all compounds, e.g. highly polar metabolites. To obtain the desired selectivity, alternatives can be e.g. HILIC, IEX, adsorption on porous graphitic carbon (PGC), MIPs, RAM and even mixed-mode SPE [39]. When combining two different phases, e.g. HILIC and RP, issues concerning solvent incompatibilities between enrichment/separation principles and sample solvent requirement can arise. This can lead to low recovery and poor resolution [39].

On-line SPE-LC can be a very effective tool, but unfortunately pressure build-up and column clogging are well-known issues [39], especially for miniaturized systems. The reason is often accumulation of particles on the SPE column [84], and in addition to pressure build up, these particles can also be back-flushed onto the LC column. Accumulation of particles severely reduces the lifetime of the columns, and sometimes the SPE column has to be changed after only 10-30 injections [39]. Changing the SPE is often acceptable for limited studies, but the

lack of robustness limits the use of on-line systems in routine laboratories. To protect the on-line SPE-LC system, it is possible to perform an off-line filtration or off-line SPE prior to injection. This is however time consuming and makes the procedure more expensive, and can in worst cases increase the possibility of sample loss, contamination or reduced precision [84].

Automatic filtration/filter back-flush (AFFL)

Another solution to the issue of clogging is to use AFFL-SPE-LC [84]. This set-up allows for injection of fairly dirty samples using regular SPE-LC hardware and an example of the set-up is shown in **Figure 5**.

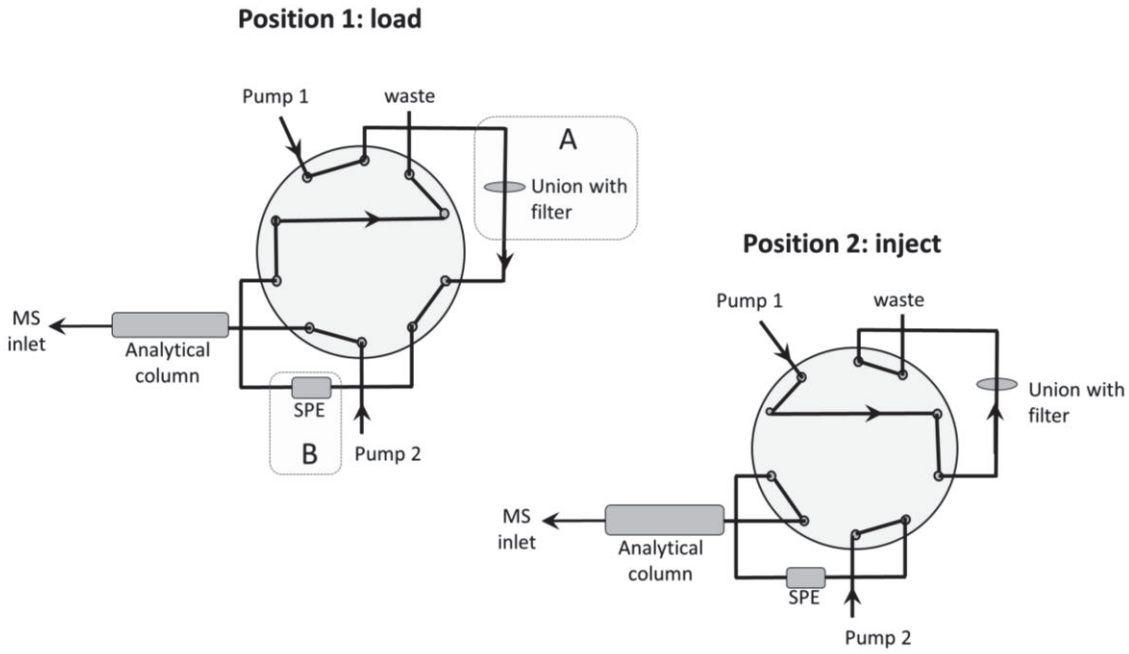


*Figure 5. Illustration of an AFFL-SPE-cap LC-MS system, consisting of a 10 port valve and two pumps. The two switching positions of the system (i) "load" and (ii) "inject" are illustrated. The dotted areas A and B highlight the AFFL filter and the SPE, respectively. The set-up is shown in front-flush mode (**Paper III**).*

The injected sample passes through a union containing a stainless steel filter, prior to the SPE trapping, and the filter stops any particulate matter from reaching the SPE. When the valve is switched, and the SPE is connected to the LC column, the filter is back-flushed, removing all particles before next injection. The AFFL-SPE set-up allows for injections of e.g. precipitated plasma and cell lysates [84, 85], and is compatible with miniaturized systems [86].

1.5 Validation of bioanalytical methods

Mistakes in sample processing or minor instrumental variations can have large impacts on the measurements by a bioanalytical method, and should thus be eliminated as far as possible. By validating the whole method, mistakes can be revealed and measures taken to avoid them. Method validation is a necessary process to demonstrate that an analytical method is suitable for its intended use [87], and according to the FDA guidance for industry [88], bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, is reliable and reproducible for the intended analytical applications. The fundamental parameters for a bioanalytical method validation are defined in **Textbox 1**.

Textbox 1. Definitions of the fundamental bioanalytical method validation parameters according to the FDA guidance for industry [88].

Selectivity: Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Confirmed by analysing a blank sample of appropriate biological matrix (n = 6). Ensure selectivity at lower limit of quantification (LLOQ) (defined under sensitivity).

Accuracy: The closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Determined by analysis of replicate concentrations (n = 5) at min three concentration levels covering the dynamic range of the method. Mean value should be ≤ 15 % of true value except LLOQ which should be ≤ 20 %.

Precision*: The closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Determined by analysis of replicate concentrations (n =5) at min three concentration levels covering the dynamic range of the method. Should be determined within-day and between-day. Generally expressed as % coefficient of variation (% CV) or relative standard deviation (RSD). At LLOQ, CV ≤ 20%, and at other points ≤ 15%.

Sensitivity: Expressed at the LLOQ**, which is the lowest concentration of the analyte, which can be determined with acceptable accuracy (≤ 20) and precision (80-120%) – and response at least 5 times as compared with the blank signal. It is not required to determine the LOD, but it is often useful to ensure significant difference between signal and noise. A S/N = 3 is acceptable for demonstrating LOD, while a S/N = 10 is acceptable for the limit of quantification (LOQ). LOD and LOQ can be determined visually.

Recovery: (or extraction efficiency) Ability of a method to extract the analyte from the biological matrix; % of the known amount of analyte carried thought the sample extraction and processing steps of the method. Established by comparing results from extracted samples with un-extracted samples (analyte added AFTER extraction), which represent 100 % recovery. Do not need to be 100%, but should be consistent, precise and reproducible over the dynamic range of the method.

Stability: Should reflect the situations during sample handling and analysis. Stability of stock and working solutions should be investigated with freeze/thaw cycles (min 3), short term/bench top stability (4-24 h) and long term/freezer stability. Post preparative stability is the stability of analyte and internal standard after completion of sample preparation and should be investigated in the auto-sampler for longer time than expected between first sample collection and last sample analysis.

*Precision is called repeatability in validation data from **Paper II** and **Paper III**.

The LLOQ definition is not used in the method validation of the papers related to this thesis. In **Paper II, the term LOQ is used in the validation, while in **Paper III** the terms cLOD/LOQ are used.

1.5.1 Calibration curve

The general approach in quantitative bioanalysis utilize a calibration curve (sometimes called a standard curve), which is the mathematical equation that relates the instrument response to the analyte concentration and refers to the ability of the method to produce signal proportional to the concentration of the analyte present in the sample [87]. The calibration curve is generally assessed by analysing series of samples known as calibration standards, and the regression equation obtained can be used to calculate the concentration of analyte(s) in unknown samples based on the instrument response [89]. The calibration curve should cover the entire range of expected concentrations, starting with the LLOQ. A coefficient of regression (R^2) of 0.99 is often sufficient for accepting a calibration curve [89], and the calibration standards should be prepared by spiking known concentrations of the analyte in the same biological matrix as the samples [89]. This method is known as the addition calibration method (standard is added to the matrix), and is the most commonly used method for quantification of endogenous compounds [90]. Since the endogenous compound (analyte) is already present in the matrix of interest, this has to be accounted for when the calibration curve is used to calculate the analyte concentration in unknown samples [91]. The addition calibration method can be challenging if the matrix has a large variability in analyte concentration [90]. Alternatively, a surrogate matrix approach [92] or a surrogate analyte approach can be used [93]. It is also possible to use matrix stripping to prepare analyte-free matrix [94].

1.5.2 Internal standard

Internal standards are compounds added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s) [89]. Internal standards are used (i) to compensate for sample to sample recovery differences during preparation and sample extraction, (ii) discrepancies in actual injection volume between injections and (iii) compensation for variability in instrument performance or response due to matrix effects [95, 96].

Internal standards can be structurally similar analogues or stable isotope labelled (SIL) compounds. When using LC-MS, the latter is preferred since they will have the same retention time, and behave in the same way as the target analytes. SIL internal standards are however expensive and not available for all analytes [95]. Chemical analogues are cheaper, more flexible (can often be used with other detectors than MS), but since they are not identical to the target analyte(s), the physicochemical properties can be different, and this can affect e.g. the extraction efficiency and the matrix effects [96].

In LC-based methods, when an internal standard is used the calibration curve is constructed by using the equation:

$$\frac{A}{A_{is}} = \frac{C}{C_{is}}$$

where A is the peak area of the analyte of interest, A_{is} is the peak area of the internal standard, C is the concentration of the analyte of interest and C_{is} is the concentration of the internal standard.

The recovery of the internal standard should be determined and compared to the recovery of the analyte(s), since good agreement ensures the suitability of the internal standard for quantification of the analyte(s) [89]. It is also advised to monitor the internal standard response across an analytical run to identify variability in the internal standard response [95].

1.5.3 Matrix effects

The FDA guideline states that when LC-based procedures using ESI-tandem MS (MS/MS) are validated, matrix effects should be investigated to ensure that precision, selectivity and sensitivity will not be compromised [89]. Matrix effects occur when molecules co-eluting with the analytes of interest alter the ionization efficiency of the electrospray interface [96-98], and can result in either a decrease (ion suppression) or an increase (ion enhancement) of the efficiency of formation of the analyte ions. Matrix effects are compound dependent, and polar compounds

are known to be subjected to the largest ion suppression [97]. The two main techniques for assessing matrix effects are *post-extraction addition* and *post-column infusion* [96-98]. In *post-extraction addition* sample extracts are spiked with the analyte of interest post-extraction and the signals compared with those of aqueous standards containing equal amounts of the analyte of interest. The degree of matrix effects is determined by the difference in response between the two divided by the aqueous standard response. In *post-column infusion* a constant flow of analyte is delivered into the mobile phase at a point after the column, but before the ESI inlet, leading to a constant baseline. A blank sample extract (matrix without added analyte) is injected into the system, and any endogenous compound that elutes from the column and causes a variation in ESI response of the infused analyte is seen as a suppression/enhancement in the response of the infused analyte. Hence, chromatographic regions most likely to experience matrix effects are identified [96]. The drawbacks of the post-infusion technique is that analytes are infused at concentrations higher than LLOQ, and matrix effects are therefore not investigated for low concentration samples, and if several analytes are determined in one method, all analytes should be infused separately [96]. An alternative approach is multicomponent post-column infusion of several internal standards [99].

To overcome matrix effects, appropriate design of bioanalytical methods is suggested, such as (i) ensuring analyte peaks elute in a region where ion suppression is not observed, (ii) improving sample extraction to eliminate interferences and (iii) the use of pure SIL internal standards since the matrix effect will be identical for the analyte and internal standard [89, 96, 100]. However, it has been demonstrated that the SIL internal standards may not always account for matrix effect, due to slight differences in molar masses and retention times [89]. Even if the matrix effects are corrected for by the use of SIL internal standards, problems with accuracy and precision are not necessarily solved if the matrix effects significantly reduces the signal of the analyte or the internal standard, since the S/N ratio may be compromised [100]. If more than one compound is

determined in the method, a SIL internal standard for each analyte would be required [96]. However, this is not always practically feasible.

1.5.4 Carryover

Even though not mentioned in the FDA guidance from 2001 [88], carryover effects can affect the precision and accuracy of a bioanalytical method [89]. Carryover is generally caused by residual analyte from the previously analysed sample, and can affect the next sample or several subsequent samples [87, 89]. Problems with carryover is of great importance in LC-MS/MS based bioanalytical methods, due to their high sensitivity and broad calibration ranges. The carryover effect influences to a greater extent the precision and accuracy of low concentration samples, and is of special concern when the study includes samples with large differences in analyte concentration [87, 89]. The carryover can also be random, e.g. if late eluting compounds are accumulated and eluted several injections later [87, 89]. According to other validation guidelines [87], the carryover cannot be higher than 20 % of LLOQ or 5% of the internal standard response. The extent of carryover should be investigated during method development and eliminated or minimized by optimizing the composition of washing solvents. If significant carryover is observed, blank samples after expected high concentrations should be injected [87, 89].

2. AIM OF STUDY

There is a consistent need for high performance analytical methods for metabolite/small molecule measurements in biosamples. In this thesis, an analytical toolkit featuring RPLC and HILIC as separation principles, and various off-line and on-line sample preparation approaches are to be developed and assessed. The methods are to be used for determination of various metabolites/small molecules in cells, whole blood, plasma and serum.

3. RESULTS AND DISCUSSION

By using different high performance analytical tools four methods for determination of various small molecules have been developed, and included in this thesis. Each method will be presented and discussed separately. The two methods based on conventional sized LC columns and off-line sample preparation techniques (**Paper I** and **Paper II**) will be presented first and then the two more advanced methods, using miniaturized LC columns and on-line sample preparation techniques (**Paper III** and **Paper IV**) will follow.

3.1 Off-line SPE and HILIC – determination of nucleotides

Endogenous nucleotides constitute an important group of metabolites. In addition to being the precursors of DNA and RNA, they also fulfil central roles in metabolism, such as storage and transport of metabolic energy and cellular signalling [94, 101, 102]. Determination of nucleotides is therefore of fundamental interest in genetic and molecular biology research, but also in the understanding of the mechanisms of nucleoside analogue compounds, which are used in anti-cancer, anti-viral and immunosuppressive therapy [103]. The determination of the corresponding generated nucleotides in cells and tissues may provide valuable information on understanding the mechanism of action of these analogues [103, 104].

Nucleotides are composed of a phosphate moiety containing one to three phosphate groups bound to a pentose sugar that is, in turn, linked to a purine or a pyrimidine base [102]. The overall charge of nucleotides depends on the number of phosphate groups, but above pH 2, all nucleotides are negatively charged [102]. When having three phosphate groups the compounds are called nucleoside triphosphates (NTPs) or deoxynucleoside triphosphates (dNTPs). The structures are shown in **Figure 6**.

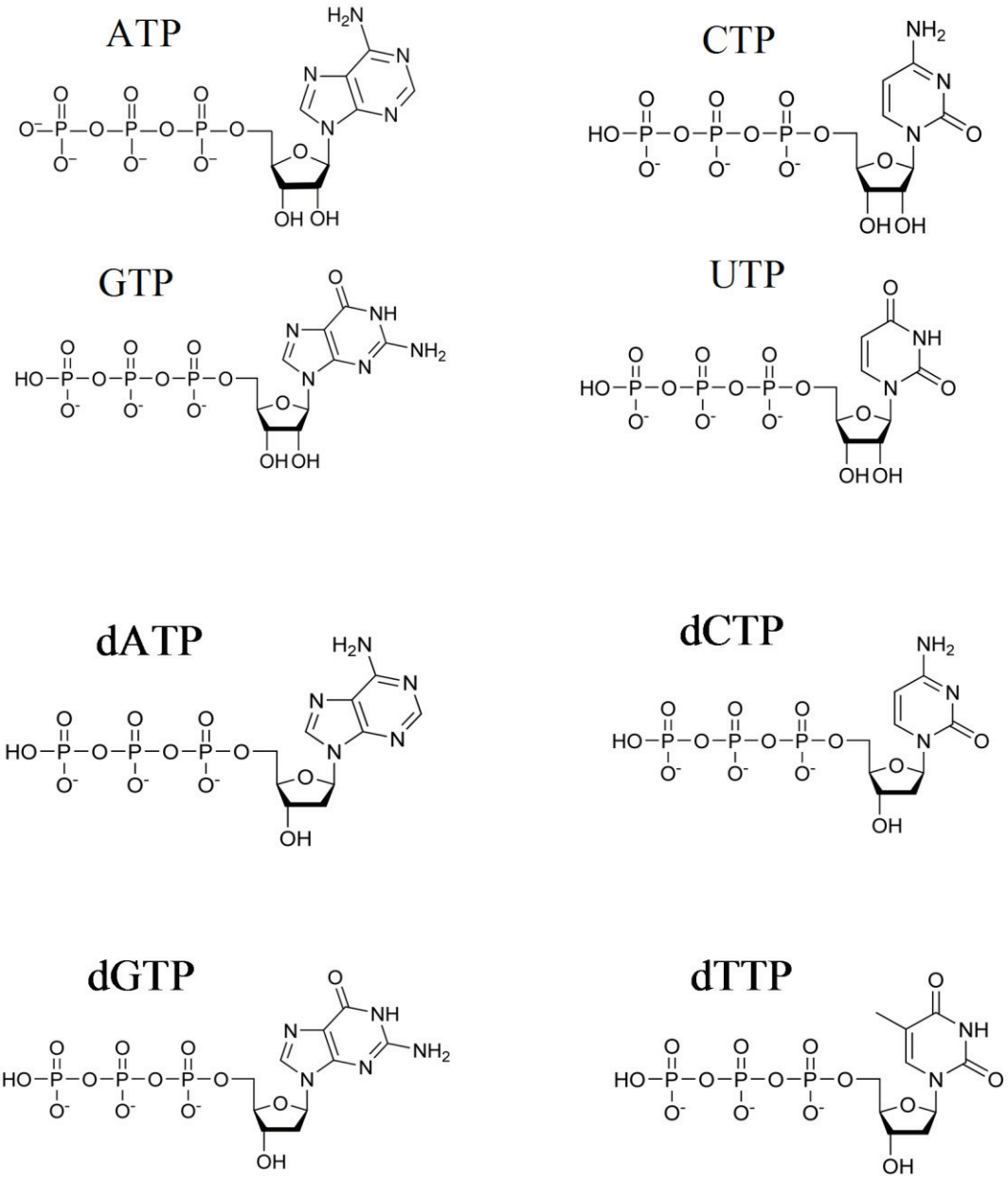


*Figure 6. Molecular structures of the four NTPs; ATP, CTP, GTP and UTP, and the four dNTPs; dATP, dCTP, dGTP and dTTP (Adapted from **Paper I**).*

Determination of intracellular dNTPs/NTPs is challenging due to their low levels, the difference in concentrations between dNTPs and NTPs, their ionic nature and their chemical similarities [101, 103]. dNTPs/NTPs are highly hydrophilic compounds, with little or no retention on RP columns using conventional mobile phases [103]. Several methods assessing strong anion exchange (SAX) columns have been developed [105-109], but these methods can not satisfactory separate all 8 dNTPs/NTPs, or have very long analysis times. SAX methods also suffer from poor compatibility with MS [90, 102] due to the high concentration of competing ions in the mobile phase. Validated LC-MS methods have been presented, using a volatile ion pair agent, and either a silica based RP column [91, 102] or a PGC column [90, 103]. However, these methods cannot separate all dNTPs/NTPs with chromatography alone.

3.1.1 HILIC for separation of dNTPs/NPTs

HILIC has in recent years been extensively used for separating hydrophilic compounds [13] and is a logical alternative for compounds with poor or no retention on RP columns, like the dNTPs/NTPs. In **Paper I**, a silica based ZIC-HILIC column was initially examined for separating the dNTPs/NTPs, but gave extremely broad and asymmetrical peaks. The column had stainless-steel frits, and problems with phosphorylated compounds and this type of frits were already reported by Alpert in 1990 [14] and more recently by Sakamaki et al [110]. Other studies have demonstrated that stainless steel surfaces in an LC-MS system can adsorb, and even trap, phosphorylated analytes under acidic conditions [111, 112]. This leads to loss of analyte or severe peak tailing unless the interactions are avoided by e.g. treating steel parts with phosphoric acid [113] or using an ion pair agent [103]. Adsorption of phosphorylated compounds to silica tubing used in the LC system has also been reported, though this can be avoided by using a higher pH [112]. In **Paper I**, an organic polymer ZIC-HILIC column (ZIC-pHILIC) gave markedly improved chromatographic performance for the dNTPs/NTPs, probably due to the lack of stainless steel frits, but also because the column could be used

with a higher pH. A mobile phase consisting of 70 % ACN and 30 % 100 mM ammonium carbonate ($(NH_4)_2CO_3$), with a pH of 8.9, gave symmetrical peaks and suitable retention. The addition of carbonate (CO_2^{3-}) to the mobile phase has also been reported as highly effective in suppressing the interaction between phosphate groups and stainless steel, thus minimizing peak tailing [114]. Despite the improvement, the dNTPs/NTPs were not satisfactory separated by this mobile phase composition (**Figure 7**, top chromatogram). Weaker mobile phases (higher % of ACN) did not solve this issue (**Figure 7**, middle chromatogram), neither did the use of solvent gradients (**Figure 7**, bottom chromatogram).

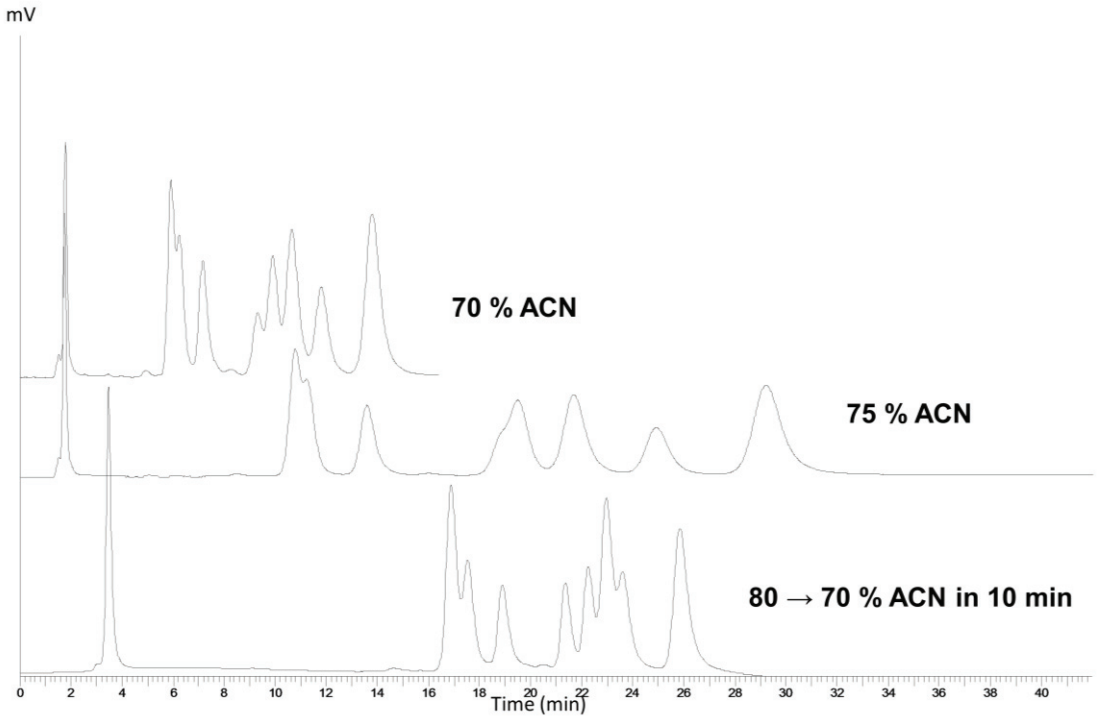


*Figure 7. Separation of 8 dNTP/NTP standards on a ZIC-pHILIC column (2.1 x 150 mm, 5 µm) at 25 ° C. The mobile phase consisted of 100 mM $(NH_4)_2CO_3$ (aq), pH 8.9 (A) and ACN (B). The two upper chromatograms show isocratic elution with 70 % B and 75 % B at 200 µL/min. The lower chromatogram shows a gradient from 80 to 70 % B in 10 min at 100 µL/min. The injection volume was 5 µL and the UV detection was performed at 254 nm (**Paper I**).*

It was assumed that the selectivity of the HILIC columns changed during the gradient, since the various interactions involved in HILIC may vary based on the solvent composition. These interactions can therefore counteract each other during a gradient, resulting in poorer separation. Effects on HILIC selectivity as a function of organic solvent content have also been reported by others [115, 116]. In **Paper I**, the effect of ionic strength was investigated, as a parameter to optimize the separation [12, 22], and a higher concentration of ammonium carbonate gave longer retention times, as expected due to shielding of negatively charged sites on the stationary phase, decreasing electrostatic repulsion [117]. However, the separation did not improve. Since HILIC conditions normally results in lower back pressure than RP conditions, this allows the use of longer columns to increase separation efficiency [15, 35]. Thus, in **Paper I**, two identical ZIC-*p*HILIC columns were coupled in series to increase the plate number, and this resulted in satisfactory separation of 6 of the 8 dNTPs/NTPs, but dATP and dTTP were still not baseline separated. The column temperature was initially 25° C, but was increased to 40° C in an attempt to further improve the separation of dATP and dTTP. The efficiency increased as expected, but the separation actually worsened, and the retention times increased (**Figure 8**). This revealed an inverse relationship between temperature and retention, which has also been reported by others [35, 116, 118]. Temperature clearly affected the HILIC selectivity for dNTPs/NTPs (**Figure 8**), and could be used for fine-tuning the separation. By decreasing the temperature to 15° C, all 8 dNTPs/NTPs were satisfactory separated isocratically in 35 minutes with a flow rate of 200 µL/min.

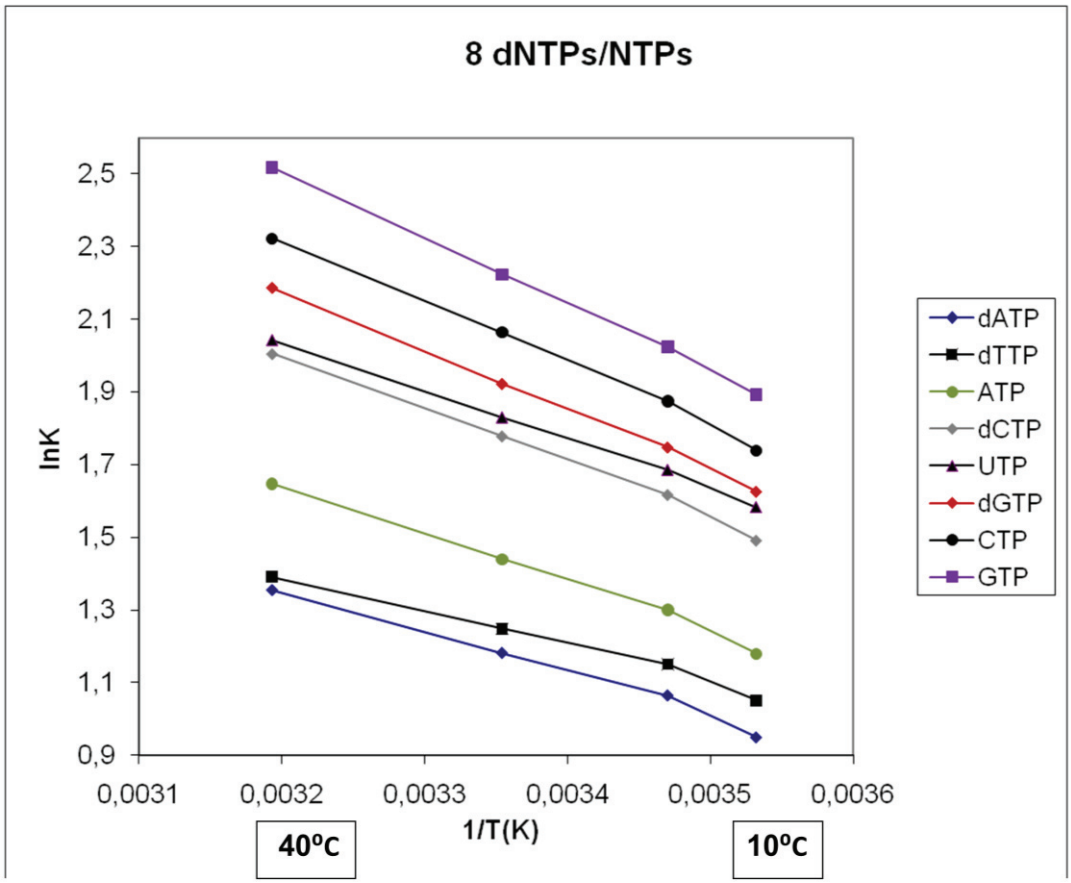


*Figure 8. A Van't Hoff plot of the 8 dNTPs/NTPs chromatographed on two ZIC-pHILIC columns at 40, 25, 15 and 10 ° C. The plot illustrates the inverse relationship between temperature and retention. The selectivity of the dNTPs/NTPs were affected by temperature and the plot shows that dATP and dTTP were better separated at low temperatures, while the separation of UTP and dGTP increased with higher temperature (Adapted from **Paper I**).*

3.1.2 Sample preparation for dNTPs/NTPs in cell samples

Cellular samples constitute a complex analytical matrix with a large number of endogenous compounds. The first step in the analytical workflow for the study of intracellular nucleotides is a PPT, which is often followed by a SAX or weak anion exchange (WAX) SPE procedure to selectively isolate the nucleotides [101, 103]. In **Paper I**, *e.coli* cell extracts were received from the Norwegian Radium Hospital, and pellets from 150 mL cell culture were dissolved in 200 µL of

methanol (MeOH)/H_2O (60/40, v/v) and vortexed for 2 min to precipitate the proteins, before a 2-step **off-line** SPE procedure was employed (for details, see **Textbox 2**).

*Textbox 2. The 2-step SPE procedure used for extraction of dNTPs/NTPs in **Paper I**.*

SPE step 1: C18-endcapped cartridge

1. Condition the SPE cartridge with
    a. 1 mL of ACN
    b. 1 mL of H_2O/ACN (99/1, *v/v*),
2. Load 200 µL of sample
3. Elute the dNTPs/NTPs with 1 mL of H_2O
4. Evaporate eluate to dryness with N_2
5. Reconstitute in 200 µL MeOH
6. Vortex for 2 min
7. Centrifuge for 10 min at 13k rpm

SPE step 2: WAX cartridge

- Condition the SPE cartridge with
    - 2 mL MeOH and
    - 2 mL of 50 mM ammonium acetate (NH_4OH, pH 4.5)
- Load 200 µL of sample solution (from SPE step 1)
- Wash SPE cartridge with 2 mL of 50 mM NH_4OH (pH 4.5),
- Elute the dNTPs/NTPs with 2 mL of MeOH/H_2O/50 mM NH_4OH (80/15/5, *v/v/v*).
- Evaporate eluate to dryness with N_2
- Reconstitute in 50 µL of mobile phase
- Vortex for 2 min
- Centrifuge for 10 min at 13k rpm

The comprehensive two-step SPE procedure used in **Paper I** was necessary due to the use of (a non-specific) UV detector. LC methods using MS detection for determination of nucleotides have been reported with a sample preparation consisting of a PPT step and a single SPE step (WAX) [90] and even with only PPT [94, 119, 120]. Kamceva et al. developed an LC-MS method for dNTPs/NPTs where they were able to avoid the SPE step completely [94]. Even though a lower

recovery was obtained due to a higher ion suppression from the complex matrix, the use of SIL internal standards corrected for the matrix effects and accurate quantification could still be performed. In **Paper I**, it was investigated if only PPT (and filtration), or PPT and a single SPE step (SAX) could be used instead of the multi-SPE procedure, but large amounts of interfering peaks were observed in the chromatograms and it was impossible to identify the dNTPs/NTPs. Hence, these approaches (only PPT and PPT + single SPE) most likely require the use of MS detection.

With the two-step SPE procedure used in **Paper I**, the samples had to be evaporated to dryness and reconstituted in an appropriate solvent twice. This is both time consuming and involves great sources of error, e.g. due to loss of or contamination of the samples. The two-step SPE procedure can also cause variations in repeatability and reproducibility [6, 103]. To decrease the manual labour and hence the time used and the errors possibly introduced, automation, e.g. with the use of SPE discs, is a possibility, but this is challenging with a multistep SPE procedure. An alternative approach can be the use of multi-mode SPE [61], where two separation principles, e.g. RP and SAX, are combined in the sorbent material. This would both simplify the SPE procedure dramatically and also make it more easy to automate.

An on-line SPE method would also reduce the manual preparation steps, increase the ease of automation, in addition to improve accuracy and precision [49]. Machon et al. describes the development of an on-lin**e** SPE LC-MS/MS method for the quantification of nucleoside mono- and triphosphates, using a WAX SPE and a PGC analytical column. To get an acceptable recovery on the SPE, the sample had to be loaded with an aqueous mobile phase. An aqueous loading phase is however not compatible with an analytical HILIC column since a water plug would elute from the SPE, most likely ruining the retention of the dNTPs/NTPs on the HILIC column. This was indeed experienced for hydrophilic neurotransmitters in **Paper III**, and has also been reported by others [121].

One of the main difficulties with on-line coupling of SPE and LC is the need for compatibility of solvents between the extraction step and the chromatographic separation [39, 103]. To avoid this issue, a HILIC SPE was used in **Paper III**, but the SPE cartridges are made with stainless steel frits, which are known to retain phosphorylated compounds, like the dNTPs/NTPs [14, 110-112, 114]. Another difficulty with on-line SPE is, as mentioned before, the pressure build-up and column clogging [39]. To avoid this, Machon et al washed the SPE for 6 hours after each series of analysis (daily procedure) [103]. An alternative approach could have been to incorporate the AFFL feature [84] used in **Paper III** and **Paper IV**, but this feature also contains stainless steel frits (and silica tubing[112]) so modification of the AFFL feature would have been necessary before it could be used for phosphorylated compounds like dNTPs/NTPs. A de-phosphorylation of the dNTPs/NTPs is possible [112], but then an additional sample preparation step has to be performed, and the method would probably be just as time consuming and laborious as the two-step off-line SPE procedure. The de-phosphorylation would also make it impossible to distinguish between nucleotides with the same base, but different number of phosphate groups.

3.1.3 Application of the method – dNTPs/NTPs in cell culture

To demonstrate the methods' suitability for measuring dNTPs/NTPs, *e.coli* cell culture samples were analysed in **Paper I**. The column temperature was set to 15° C, which gave the overall best separation and the flow rate was decreased from 200 µL/min to 100 µL/min to ensure complete separation of dTTP from ATP, which was significantly higher in concentration than de others (**Figure 9**). With these minor adjustments, all 8 dNTPs/NTPs could be separated and identified with certainty when the retention times were compared to those of the standards. Because of the reduced flow rate, the analysis time was approximately 70 min per sample, but due to the isocratic conditions there were no additional time needed for conditioning the system. A chromatogram showing the separation of all 8 dNTPs/NTPs in a cell sample can be seen in **Figure 9**.

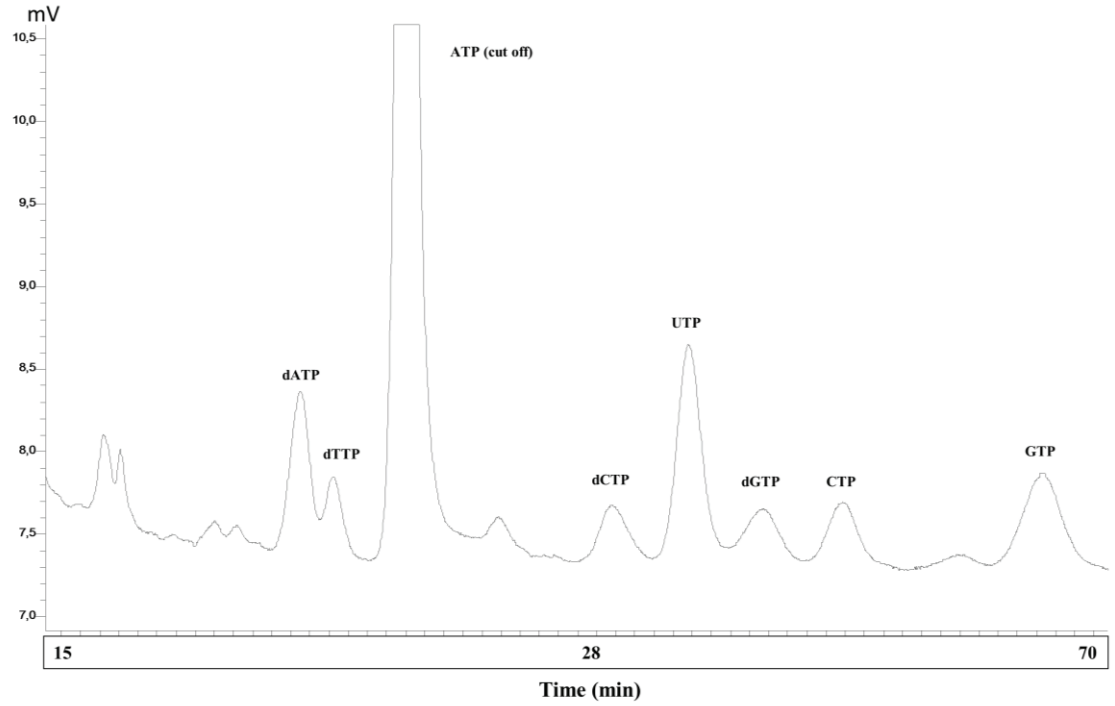


*Figure 9. Separation of the 8 dNTP/NTPs in an e.coli cell sample on two ZIC-pHILIC columns at 15 ° C with a flow rate of 100 µL/min. Isocratic elution was performed with ACN/$(NH_4)_2CO_3$ (pH 8.9, 100 mM) (70/30, v/v). Chromatogram recording began 15.0 min after injection (Adapted from **Paper I**).*

Decosterd et al. gradient separated all 8 NTPs/dNTPs in 95 min, but with a total analysis cycle time of 160 min, using an ion-pairing method [109]. Machon et al. were able to extract and determine 16 nucleotides (mono and triphosphates) in cell samples within 20 min, with a total run-time of 37 min, using a PGC column [103], and another highly accurate LC-MS method using a PGC column takes 50 min, with a total cycle time of 68 min [90], but both these methods require an MS to distinguish all the compounds.

Several published methods are unable to separate ATP and dGTP [91, 104, 112, 122]. The difficulty of achieving a satisfactory chromatographic separation is often increased for real samples, since the concentration of ATP in cells is about 1000 times greater than the concentration of dGTP [90]. ATP and dGPT have the same molar mass and the same MS/MS transition [90], and hence the lack of chromatographic separation is not necessarily resolved by the use of MS or MS/MS. Cohen et al. were able to distinguish ATP and dGTP with the use of an

ion pairing agent [90], since the two adducts formed in positive mode gave different fragmentation. However, negative ionisation mode is the most effective for several of the dNTPs/NTPs [101], so a method which can switch between negative and positive ionisation mode during analysis would be necessary. Co-elution of the abundant ATP with dTTP in cell samples has also been reported [123]. In **Paper I**, ATP was well resolved from neighbouring dNTPs/NTPs with the use of ZIC-*p*HILIC columns, making the determination of all 8 dNTPs/NTPs possible with the use of a simple UV detector.

3.1.4 Concluding remarks

The method developed in **Paper I** was based on simple LC-UV instrumentation, proving that it is possible to determine low concentrations of dNTPs/NTPs without the need of MS detection, though a fairly large sample volume (extracts from 150 mL cell culture) and a comprehensive sample preparation procedure was needed.

Looking at papers published on nucleotide determination in the last 5 years, almost all methods are based on MS detection. Even though several of the nucleotides should be separated by chromatography, e.g. ATP/dGTP, which exhibit the same *m/z* value and ion transition, and CTP/UTP which have molar masses differing by only one Da and having the same fragmentation pattern [101], chromatographic separation of all the nucleotides are not necessary when MS is used for detection. This is because *m/z* values and fragmentation patterns can be used for identification of the analytes, in addition to the retention times. The improvement in sensitivity when using MS also opens the possibility for simplifying the sample preparation substantially [94], and reducing the time and manual labour needed. Although the method described in **Paper I** was based on UV detection, HILIC is highly compatible with MS, especially in the ESI mode, due to the more efficient desolvation of highly organic mobile phases [18, 22, 124]. In **Paper III**, a validated method, where HILIC was used in combination with MS detection, is described. Thus, if lower LODs are needed, it should be possible to use MS detection with the method developed in **Paper I**.

3.2 Off-line filtration and RP - determination of drugs

Tuberculosis caused by *Mycobacterium tuberculosis* (*M.tb*) is a potentially fatal disease that kills an estimate of 1.7 million people each year [125, 126]. The standard treatment involves a combination of four antibiotics, given for at least six months [73]. This is lengthy and expensive, often leads to incomplete treatment, and allows time for the bacteria to acquire resistance. Hence, there has been a global increase of multidrug-resistant tuberculosis [126], which is defined as resistance to the two most effective anti-tuberculosis drugs; isoniazid and rifampicin (RIF) [73]. RIF is one of the first line anti-tuberculosis drugs [127], and exerts its activity by targeting the bacterial DNA dependent RNA polymerase [128] and thus suppressing the initiation of RNA synthesis, leading to cell death. The progression of antibiotic resistance of *M.tb* has now moved on to extensively drug-resistant and even totally drug-resistant forms [129], hence new strategies are needed to effectively treat tuberculosis. Recent studies have shown promising results when antibiotics are combined with bacterial efflux pump inhibitors, like thioridazine (TZ), since this can improve the antibacterial effect of existing antibiotics against *M.tb* [73, 129].

TZ is a small, water insoluble molecule, while RIF is a larger and more complex molecule, slightly soluble in water (pH dependent), and their structures can be seen in **Figure 10**.

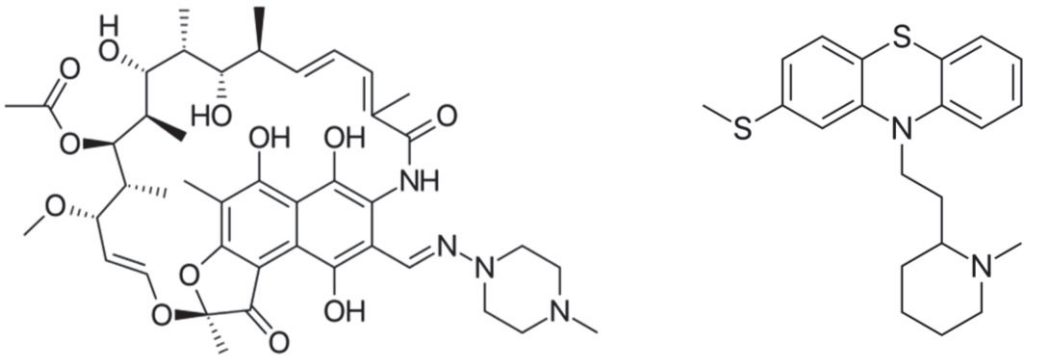


Figure 10. Molecular structures of RIF (822.9 Da) and TZ (370.6 Da).

A tuberculosis infection starts in the lungs where *M.tb* first infects resident alveolar macrophages and subsequently newly recruited macrophages [130]. Since the macrophages are the cells where the bacteria reside, a method for determination of RIF and TZ in macrophages is needed to study the synergetic properties of RIF and TZ for addressing drug resistance in tuberculosis [129]. LC-MS has been widely used for determination of RIF in biofluids such as plasma, dried blood spots and CSF ([127, 128, 131-135], and has also been used to determine TZ in blood and plasma [136, 137]. However, few methods have been published for determination of RIF or TZ in cell samples [128, 131]. An LC-MS based method has been used for the simultaneous quantification of RIF, TZ and several other drugs in cell lysate [138], but the method was not validated, no internal standards were used and no chromatograms were shown. The goal in **Paper II** was hence to develop a simple and robust LC-MS method for accurate, precise and simultaneous determination of RIF and TZ, in limited amounts of cell lysate (macrophages). The method was also intended for studying drug release from PLGA nanoparticles.

3.2.1 Development of LC-MS method for rifampicin and thioridazine

In preliminary experiments, the use of HILIC was investigated, but RIF and TZ had no retention on the ZIC-HILIC column, using the same mobile phase as in **Paper III** (70% ACN/30% 100 mM ammonium acetate, pH 3). Method development with HILIC can be a challenge, and since an initial experiment with an RP column gave good retention and separation of RIF and TZ, RP LC was chosen.

Since the goal was to develop a simple method, and since the levels of RIF and TZ in the cell lysate were expected to be in the higher ng/mL range, a 1 mm ID micro LC column was assessed. Difficulties associated with miniaturized systems, e.g. low loadability and poor robustness, were thus avoided. A typical RP mobile phase consisting of 0.1 % formic acid (FA) in 60 % ACN and 40 % H_2O was used, and chromatographic separation of RIF, TZ and their internal standards rifapentine and trifluoperazine was achieved within 4 minutes, with a column temperature of 40 ° C. Extracted ion chromatograms of RIF, TZ and the two internal standards are shown in **Figure 11.**

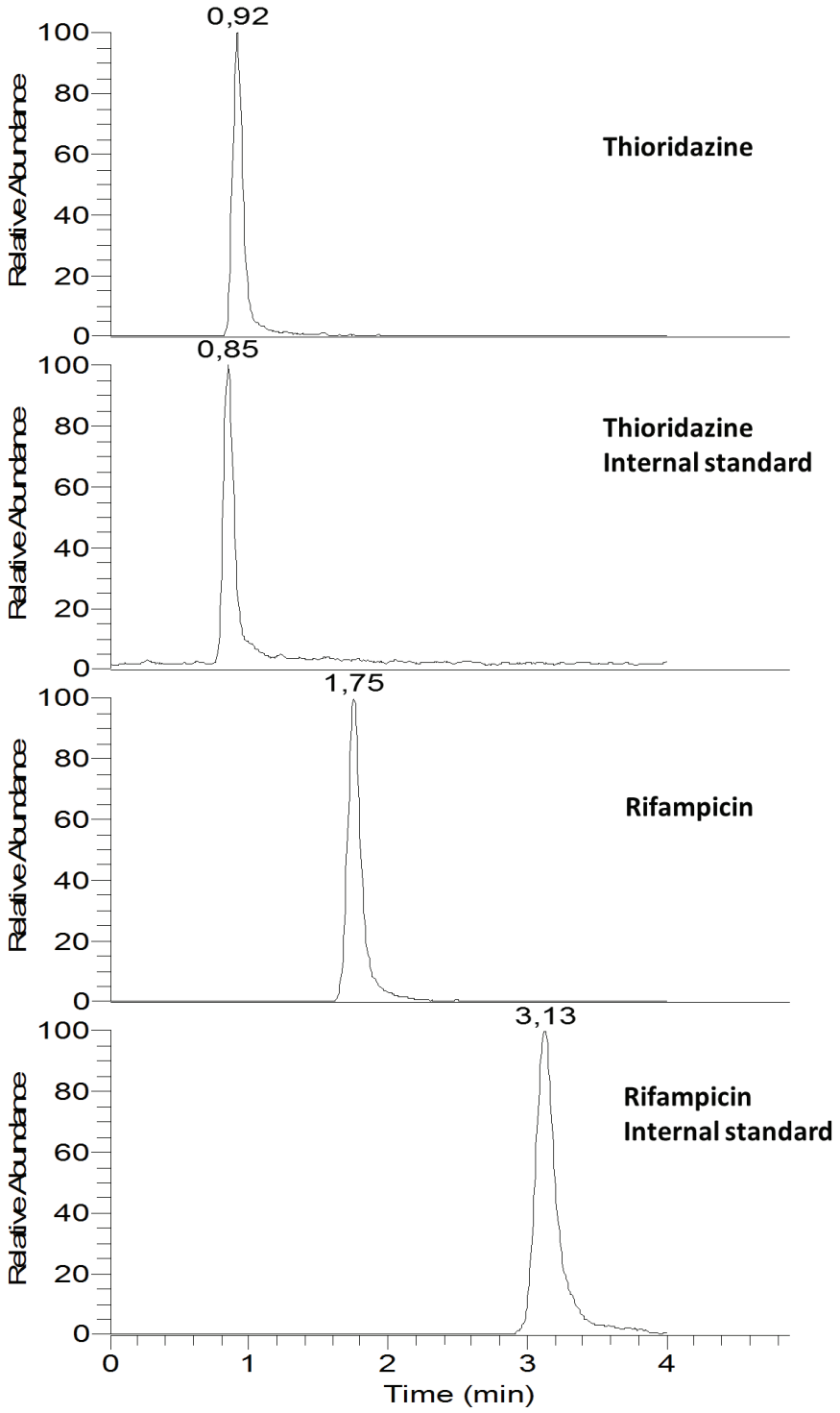


*Figure 11. Chromatograms showing RIF, TZ and the two internal standards in spiked cell lysate. Isocratic separation was achieved on an RP column (50 x 1 mm ID, 1.9 µm) with a flow rate of 0.2 mL/min. The mobile phase was 300 µM ascorbic acid in 0.1 % FA, 60 % H_2O and 40 % ACN. The column temperature was set to 40 ° C. (Adapted from **Paper II**).*

Due to the isocratic mobile phase conditions used, no column reconditioning between injections was needed. The loading volume was 0.5 µL, but could be increased to 5 µL without extra peak broadening, if lower LODs were needed. The analytes were determined using a TSQ Vantage triple quadrupole-MS equipped with a heated ESI source operated in positive mode.

3.2.2 Oxidation of rifampicin

Initially, the repeatability of the RIF peak areas was poor, and an additional compound eluting prior to RIF was observed. Further experiments revealed a slow decrease in peak area for RIF, while the peak area of the additional compound increased with time. Preliminary studies had shown that RIF was unstable in solution, as RIF oxidizes to rifampicin quinone [139]. The degradation was found to be time, pH and temperature dependent, and RIF was most stable in a non-acidic environment and at low temperatures. In addition to degradation during storage and sample preparation, it was assumed that RIF could be oxidized during ionisation in the ESI source [140]. The issue of on-line oxidation in LC-MS had been thoroughly investigated in the study of neurotransmitters described in **Paper III**, where adding ascorbic acid as an anti-oxidant [141] to the mobile phase was sufficient to avoid oxidation. By adding ascorbic acid to the mobile phase, oxidation of RIF in the electrospray source was also avoided (**Paper II)**. To prevent oxidation during storage and sample preparation, ascorbic acid was added to all stock solutions [139], and also used during cell lysing. Aliquots of standard and internal standard solution mixtures were kept in the freezer until the day of analysis, and new stock solutions were made regularly.

3.2.3 Sample preparation for rifampicin and thioridazine in cell samples

In **Paper II**, cell pellets from mouse bone marrow derived macrophages were thawed and transferred to an eppendorf vial, before centrifugation. The supernatant was removed and the cells were re-suspended in 600 µM ascorbic acid, 0.1 % FA

in H_2O. The cells were lysed with 3 freeze/thaw cycles (30 min at -80º C/5 min at 37º C).

Before RIF and TZ could be determined in the cell samples, proteins had to be removed and the drug-protein bindings had to be disrupted to obtain an acceptable recovery of the drugs. For small molecule determination, centrifugal filters have been used as a clean-up step prior to LLE [68] or PPT [69]. The possibility of using centrifugal filters as a stand-alone sample preparation procedure for RIF and TZ was investigated in **Paper II,** since this would, despite the use of off-line sample preparation, increase the throughput and ensure simplicity of the method. Another reason for choosing centrifugal filters was the intention of using the method for measuring drug release from nanoparticles in biological samples.

Prior to sample application, the centrifugation filters were washed with 600 µM ascorbic acid and 0.1 % FA in H_2O. This was done to: (i) remove glycerol from the filter, which can interfere with the LC-MS analysis, and (ii) to avoid oxidation of RIF during the filtration. When aqueous standard solutions of RIF and TZ were applied to the filters and centrifuged, the analytes eluted with high recovery (90-95%), but when spiked cell lysate (100 000 cells) were applied, the recovery was poor, probably because of strong drug-protein bindings. Preliminary studies had shown the need for an additional post-loading filter wash with organic solvent to ensure sufficient recovery of RIF and TZ from cell lysate, and similar effects have been seen by others [71]. Since the method also was intended for studying drug release from nanoparticles, which may be partly dissolved by organic solvents, high amount of ACN had to be avoided. However, the recovery decreased substantially when the ACN content was below 30 % in the washing solution, and a solvent containing 40 % ACN was needed to obtain satisfactory recovery (58-65%). Hence, 40% ACN was added to the filter before an additional centrifugation step was performed. Including the pre-wash step, the total centrifugation time was 32 minutes, and the filtrate could be analysed directly by the LC-MS system without further sample preparation.

The LC-MS method in **Paper II** was developed with the aim of quantifying RIF and TZ in limited amounts of cell samples. The sample preparation procedures used for plasma samples in e.g. [131, 133, 135] were not applicable since plasma and cells are quite different matrices [131]. Oswald et al. measured RIF and other related compounds in cell samples with LC-MS after a LLE sample preparation, but the LLE procedure was laborious and the method was intended for larger samples (20 times more cells per sample). A simple PPT with ACN was used by Hartkoorn et al. before determination of RIF in peripheral blood mononuclear cells, using LC-MS [128]. Even though the method was fully validated with satisfying results, performing only a PPT will result in a complex sample, which can contain particles. The application was limited to samples from 8 patients, but if a larger study were to be conducted, there is a risk of clogging the system. The sample preparation with centrifugal filters, used in **Paper II,** both filtrated the cell samples and removed the proteins/disrupted the drug-protein bindings. Hence, no additional PPT was needed. A solution identical to the LC-MS mobile phase was used in the post-loading filter wash, thus, the filtrate was suitable for direct injection into the LC-MS system and could be injected directly, resulting in a simple and time efficient method. To further simplify the method, parts of the procedure can easily be automated. On-line coupling is so far not an option for centrifugal filters, however an off-line PPT will anyhow be necessary for cell samples.

3.2.4 Method validation

The method was validated according to US FDA guidelines [88], and validation solutions were made by spiking RIF and TZ to cell lysate. By comparing chromatograms of blank cell samples to that of samples spiked with RIF and TZ, the selectivity of the LC-MS/MS method was confirmed. The method was validated with regard to precision (within-day and between-day repeatability), linearity (aqueous standards and cell samples), recovery and stability with satisfactory results, summarized in **Table 3.**

*Table 3. Validation data for measuring TZ and RIF in cell lysate. Level L = 5 ng/mL TZ, 50 ng/mL RIF, level M = 25 ng/mL TZ, 250 ng/mL RIF and level H = 75 ng/mL TZ, 750 ng/mL RIF. All solutions contained 50 ng/mL TZ internal standard and 500 ng/mL RIF internal standard (Adapted from **Paper II**).*

	Within-day repeatability (n = 3)			**Between-day repeatability** (n = 5)		
	Mean A/Ais	***SD***	***RSD %***	***Mean A/Ais***	***SD***	***RSD %***
Thioridazine						
L	0.20	0.01	5	0.17	0.02	12
M	1.0	0.1	9	0.9	0.2	17
H	2.8	0.3	11	2.6	0.4	16
Rifampicin						
L	0.09	0.01	6	0.11	0.02	17
M	0.490	0.002	1	0.6	0.1	17
H	1.47	0.01	1	1.8	0.3	17

	Linear range ng/mL	**R^2 Cell lysate (n = 6)**	**R^2 STD (n = 5)**	**Recovery %**	**Stability**		
					25° C	**4° C**	**- 20° C**
Thioridazine	5 - 150	0.9979	0.9981	66	For 48 h, not 1 week	For at least 1 week	For at least 1 week
Rifampicin	50 - 1500	0.9994	0.9998	58	Not for 24 h	For 48h, not 1 week	For at least 1 week

Although the precision was satisfactory (+/- 15% RSD), it would most likely be better if SIL internal standards, which are commercially available, were used instead of chemical analogues. However, the chemical analogues chosen were separated from RIF and TZ chromatographically, and could hence be used with simpler non-MS based systems as well.

Possible matrix effects were investigated by using the post-column infusion technique. No ion suppression/enhancement in the first 10 min of the chromatogram was observed (**Paper II**).

3.2.5 Application – separating nanoparticles, drug and proteins

To encapsulate the drug inside nanoparticles is an alternative to traditional drug delivery (e.g. orally or intra venous), and the goal is to selectively target the diseased cells [125]. The slow release of drugs from nanoparticles in vivo is associated with lowered toxicity and a more effective therapy [73, 142]. PLGA

nanoparticles constitute one of the most successfully developed biodegradable polymers [125], and have been extensively used in experiments aimed at delivering antibiotics against *M.tb* in animal models. Centrifugation filters have previously been used for separating PLGA nanoparticles and drugs for release profiling in simple aqueous solutions [73-75], since isolating released drug prior to measurements avoids contamination from nanoparticles which are still loaded with drugs. Hence, the possibility to use the FDA validated method for measuring RIF and TZ in cell lysate (**Paper II**), to measure drug release from nanoparticles in biological samples (cell lysate) was explored.

Since a washing step with 40 % ACN was necessary to obtain a satisfying recovery from the filter, possible degradation of nanoparticles in 10-40 % ACN or MeOH was examined by comparing nanoparticle release of drug in cell lysate with that of lysate spiked with non-encapsulated drug, and samples were collected over a 24 H period. The organic solvent wash step (40 % ACN) dissolved the filter trapped nanoparticles, and hence it was concluded that the validated method for RIF and TZ determination was not applicable for nanoparticle release studies. Solutions with less amounts of ACN and MeOH were tested, but the nanoparticles were still partially dissolved. Aqueous $ZnSO_4$, which is an alternative to organic solvents for PPT [143] was also assessed, however a >10-fold increase in MS noise and a very poor repeatability was observed. Washing with TCA [143] was not considered since previous experiments had shown that RIF degraded in strong acids (**Figure 12**).

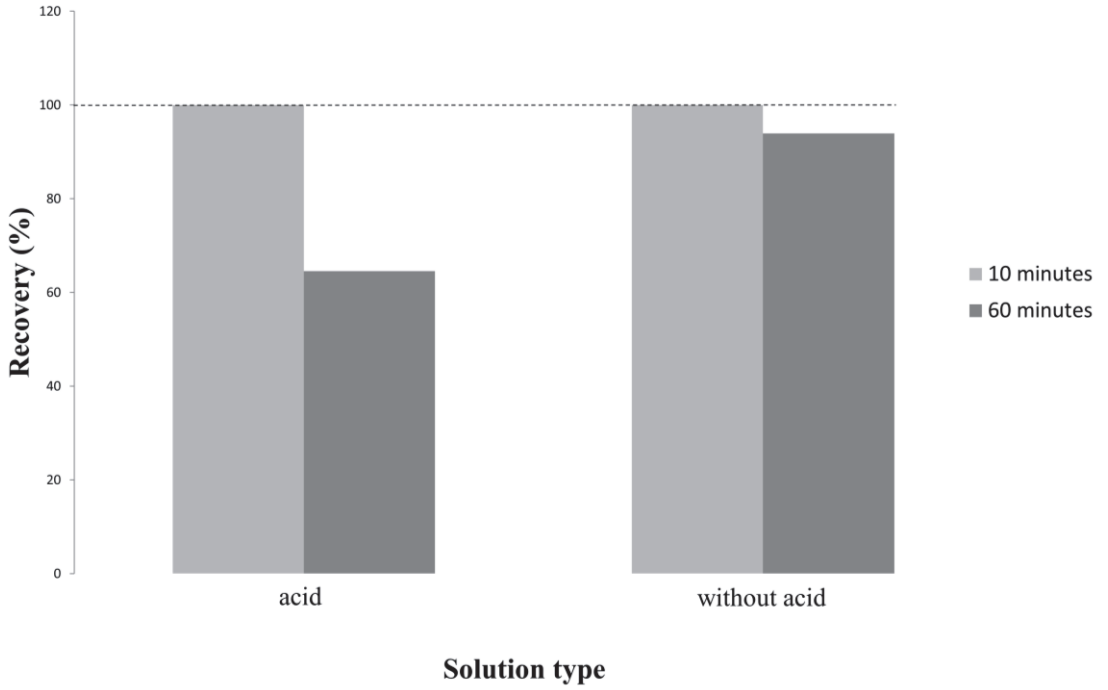


*Figure 12. Degradation of RIF in acidic (left) vs non-acidic (right) solutions after 10 and 60 min at 25° C (Adapted from supplementary data **Paper II**).*

3.2.6 Concluding remarks

The centrifugation filters were well suited for sample clean-up prior to LC-MS measurements of RIF and TZ in cell lysates, and centrifugal filters have also been successfully used for separating drugs and nanoparticles in simple aqueous solutions. However, they were found to be less applicable for separating RIF and TZ from nanoparticles and drug binding proteins in cell lysate, as common drug-protein binding disruptors such as organic solvents can dissolve nanoparticles, highly acidic ones can degrade RIF, and salts can generate noise in the MS. To reduce the noise, an additional desalting-step with SPE could have been included, but that would have made the sample preparation much more time consuming and laborious, and the simplicity and robustness of the method would probably have been compromised.

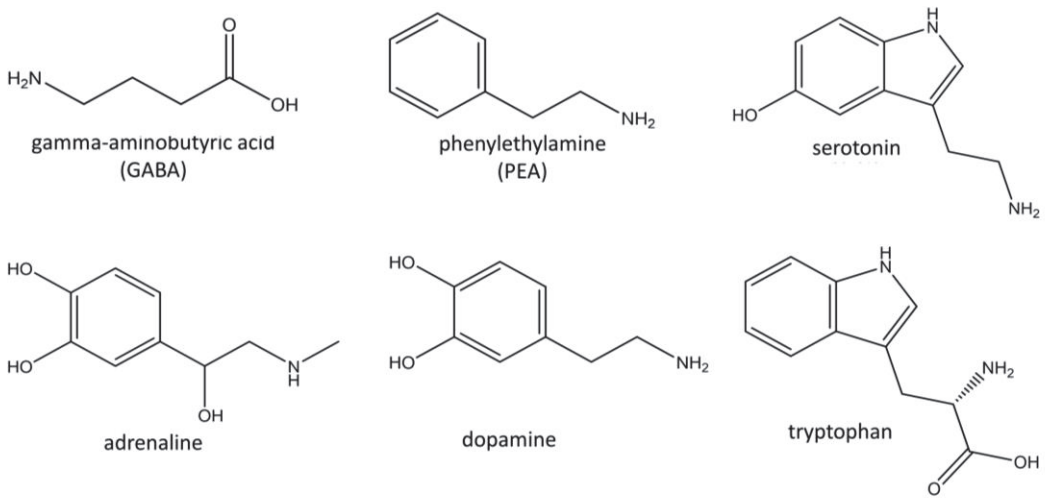
3.3 On-line SPE and HILIC – determination of neurotransmitters

Neurotransmitters (NTs) are metabolites used for communication between neurons in the brain, and they are widely distributed in the central nervous system (CNS) and the peripheral body fluids of mammals [144]. Their biological role is essential for many physiological functions and deviations from normal levels have been related to several diseases and dysfunctions of the CNS [16, 144-146]. In humans, analysis of CSF gives the most direct measure of central NT levels, but neuroimaging approaches are promising alternatives due to their less invasive nature. However, is it much simpler and less expensive to determine NTs in blood, saliva or other peripheral fluids. Measuring levels of peripheral NTs gives valuable information for several purposes, but since many NTs have a very limited ability to cross the blood-brain-barrier, the relationship between central and peripheral levels of NTs are not always clear [147]. Reliable and sensitive analytical methods are hence required to establish the roles of peripheral NTs.

NTs are small molecules like acetylcholine, the *monoamine* dopamine, and the *amino acid* gamma-aminobutyric acid (GABA) [148], but NTs can also be larger *neuropeptides* like oxytocin [149]. NTs are thought to have a well-balanced interplay in the brain [145, 148], and multi-category detection of NTs, neuromodulators, together with precursors and metabolites of NTs, permits monitoring of interactions between NTs [146, 149]. But since NTs can have very different physicochemical properties, multi-category determination can be a challenge [148, 149]. NTs are often highly polar compounds, with little or no retention on RP stationary phases, without the use of an ion-pair agent, and the use of HILIC coupled with MS has become a popular alternative for NT determination [16, 35, 146]. Several LC-MS methods [144-146, 148, 150-154] have been reported for polar NTs, but many studies only focus on NTs from one category e.g. monoamines or cathecolamines, and are hence not suited for multi-category determination. Other disadvantages of several NT studies are the need for extensive manual sample preparation and large sample amounts. The latter makes

these methods not suitable for analysis of limited, precious samples from e.g. infants and blood banks.

The goal in **Paper III** was hence to develop an LC-MS platform, using HILIC and a high resolution MS, for multi-category determination of NTs in small volumes of whole blood. *GABA*; a neuroactive amino acid, *phenyl ethyl amine* (PEA); a neuromodulator, *dopamine, serotonin and adrenaline*; neuroactive monoamines, and *tryptophan*; the precursor for serotonin, were chosen as model compounds for the LC-MS platform, and their structures and physicochemical properties can be seen in **Figure 13**.



	GABA	PEA	Dopamine	Serotonin	Adrenaline	Tryptophan
M_m	103.12	121.18	153.18	176.22	183.20	204.23
LogP	- 0.64	1.44	0.046	0.55	-0.54	0.70
pK_a (base)	11.3	9.9	10.1	9.5	9.2	9.7
pK_a (acid)	4.4					2.3

*Figure 13. Molecular structures and molar mass (M_m), octanol-water partition coefficient (log P) and pK_a values of the six NT model compounds (Adapted from **Paper III**).*

3.3.1 Development of HILIC-MS method for neurotransmitters

Since several of the NTs were expected to be present at very low concentrations in peripheral body fluids, the platform was based on a cap LC-MS system. The nature of the analytes impacts the type of retention mechanism in the employed HILIC system [34], and hence the choice of HILIC column is of great importance. In several studies where different HILIC stationary phases have been investigated for NT determination, Zwitterionic materials have shown good performance [146, 148], providing optimal retention behaviour of small, polar analytes such as NTs. Zwitterionic HILIC columns provide sufficient retention for both cationic, anionic and neutral compounds [16] and is a suitable choice for multi-category determination. A ZIC-HILIC and a ZIC-cHILIC column were both evaluated for the LC-MS platform. The ZIC-cHILIC column was employed in the final method, due to 25 % better efficiency and asymmetry factor for the six model compounds. The mobile phase was optimized by evaluating the influence of salt type and concentration (ionic strength), amount of organic modifier and temperature [16]. Preliminary experiments were conducted with ammonium acetate, but even with high concentrations of salt, the retention was too strong, leading to poor peak shapes of all NTs and full retention of dopamine and adrenaline. More symmetrical peaks were obtained with ammonium formate. Higher elution strength of the formate ion, compared to acetate has also been reported by others [16, 155]. A mobile phase with 85 % ACN and a total ionic strength of 30 mM ammonium formate gave satisfying efficiency and resolution of the six NTs. High organic content in the mobile phase makes the salts prefer to stay in the water-rich layer on the stationary phase, whereas higher salt concentrations drive more solvated salt ions into the water-rich layer. The increase in volume or hydrophilicity of the water-rich layer results in stronger retention of the solutes and more narrow peaks. However, the sensitivity of ESI-MS decreases with higher concentrations of salts, due to ion suppression [145]. In a study by Danaceau et al., a total ionic strength of 30 mM ammonium formate was found to be a good compromise between efficiency and sensitivity [35]. However, considerable ion suppression was observed with 20 mM ammonium formate in another HILIC study [155], proving

the importance of optimizing the ionic strength in HILIC. For the LC-MS platform (**Paper III**), reducing the ionic strength resulted in severe band broadening and poor repeatability, and no significant increase in sensitivity. This is consistent with the theory that increasing mobile phase ionic strength can disrupt secondary interactions between polar compounds and the stationary phase, resulting in improved chromatography [35]. The ZIC-HILIC stationary phase should remain unaffected by pH since the nominal net charge is zero, but the pH will affect the ionization of the NTs [16]. To prevent degradation of the NTs, a pH of 3 in the aqueous part of the mobile phase was chosen to ensure acidic conditions, and the effect of pH on chromatography was not investigated. Temperature is the least significant parameter to affect HILIC separation [16], even though temperature could be used for fine tuning the separations in **Paper I**. No significant effect on efficiency or retention of the NTs was observed when the temperature was varied, and 30 ° C was chosen based on other studies of NTs [35, 146]. When a brand new ZIC-HILIC column was taken into use, the dopamine and adrenaline peaks disappeared with a mobile phase consisting of 85 % ACN. This also happened when the used column was regenerated by washing. It was hence assumed that the used column had been saturated such that secondary interactions were not that dominant anymore, making it possible to elute the highly polar compounds with only 15 % aqueous buffer. When the aqueous content was increased to 30 % (and 70 % ACN), the chromatography was the same on the used and the new column and hence a mobile phase consisting of 70 % ACN and a total ionic strength of 30 mM was used in the final method in **Paper III**.

3.3.2 Oxidation of neurotransmitters

Several of the NTs are known to easily oxidize, hence, the chromatography was carried out under acidic conditions, and HCl was added to the stock solutions of the NTs [35] (**Paper III**). In the preliminary studies performed with an ion trap MS with ESI grounded emitter, this was sufficient to avoid oxidation. When the need for better resolution and sensitivity arose, the method was moved to an Orbitrap MS which has the ESI emitter at a positive/negative potential relative to ground, severe oxidation of dopamine and adrenaline (80-90 %) was observed. By

increasing the flow rate, the oxidation decreased significantly, and it was assumed that the oxidation happened on-line during the analysis. It was also assumed that the ESI source was involved in the oxidation, since the configuration of the ESI was quite different between the two mass spectrometers. The ESI voltage is applied on the emitter in the Orbitrap MS, and to protect the operator and the upstream equipment from being exposed to high voltage, a grounded contact is often placed upstream of the emitter electrode. But then a second upstream circuit is introduced, and electrochemical reactions can occur [156], especially when analytes are easily oxidized, the mobile phase has a high conductivity and the flow rate is relatively low, like in the study described in **Paper III**. To avoid oxidation, ascorbic acid was added to the mobile phase to act as an antioxidant [141]. No more oxidation was observed as long as the mobile phases were made fresh regularly.

3.3.3 Sample preparation for neurotransmitters in blood

Whole blood contains large amounts of proteins, which have to be removed prior to NT determination, hence the sample preparation used in **Paper III** started with an off-line PPT. Not all precipitation methods or reagents are suitable for separating proteins from small compounds like metabolites. Heat, salt and acid usually trap these analytes in the protein aggregate leading to co-precipitation together with the proteins, resulting in a low recovery [47]. Hence, in **Paper III**, ice cold ACN with 0.1% FA was used to precipitate the proteins. The use of SALLE [56], as an alternative method, was investigated in preliminary experiments, but the recovery was unsatisfactory. A pH of 12 was tested to deprotonate the NTs, thus making them less hydrophilic [56], but the recovery did not improve. Several of the NTs are known to be unstable at high pH, and further high pH investigations were hence not conducted.

Since whole blood only contains trace amounts of several of the NTs, on-line SPE was chosen to enrich the NTs from the PPT supernatant prior to determination. PGC has successfully been combined with HILIC in an on-line SPE-LC-MS

method for trace determination of polar compounds in water samples [155], but PGC was not able to retain the NTs. With IEX SPEs (strong cation exchange (SCX) and weak cation exchange (WCX)) all model NTs were retained, but to avoid severe band broadening the loading solvent had to contain 30 % H_2O. A water plug was then eluted from the SPE and onto the analytical column, resulting in loss of separation. Combining two principles in enrichment and separation in an on-line SPE-LC system does not seem to be a significant issue when employing larger bore SPE-LC systems [155], but becomes a factor with miniaturized system, where system void volumes can play a larger role in system performance [121]. The combination of HILIC-HILIC for SPE-LC has been successfully used in proteomics and glycoproteomics [23, 121], and hence, a ZIC-HILIC SPE column was investigated. The HILIC SPE was able to retain all the NTs with a 100 % ACN loading solvent and a two minute SPE loading time, and the NTs were successfully eluted from the SPE onto the analytical cap LC column with the LC mobile phase. The NTs were fully retained when injected in ACN/H_2O (70/30), and hence a ratio of 1 + 7.5 (blood + ACN w/0.1% FA) was used in the PPT. This allowed the PPT supernatant of the blood samples to be directly injected, without any evaporation/reconstitution step.

In cap LC injection volumes are limited to 5-10 µL, but when using on-line SPE, a much larger injection volume can be used. In the study described in **Paper III**, at least 100 µL (max injection volume of the auto-sampler) of sample could be loaded onto the SPE without analyte breakthrough occurring, and the large injection volume lowered the detection limits, without compromising chromatography. On-line SPE also greatly reduces manual steps in sample preparation, which can be a central source of error in analysis [7, 39]. However, on-line SPE can be prone to clogging when handling complex samples [157]. To avoid clogging, the AFFL feature [84] was employed, which filtrates the samples on-line before they reach the SPE column. Due to the back-flushing of the filter between injections, hundreds of whole blood samples could be analysed without pressure build-up in the LC system.

3.3.4 Method validation

The LC-MS platform was validated according to the FDA guidelines [88], and validation solutions were made by spiking whole blood with the six NTs. Determination of cLOD was limited by the lack of blood without NTs (blank matrix), however, an estimate was made by extrapolation. The method was validated with regard to precision (within-day and between-day), linearity (aqueous standards and blood), recovery and stability with satisfactory results, summarized in **Table 4** (except stability).

*Table 4. Validation data for measuring NTs in whole blood. See supplementary information for **Paper III** for details on the concentration levels used in validation and calibration samples (Adapted from **Paper III**). *No result for dopamine was obtained at the concentration level L, due to S/N < 10.*

	Within-day repeatability (n = 6)			Between-day repeatability (n = 5)		
	Mean A/Ais	*SD*	*RSD %*	*Mean A/Ais*	*SD*	*RSD %*
GABA						
L	7.0	0.3	4	6.9	0.5	7
M	14.5	0.6	4	14	1	7
H	88	4	5	92.6	7.1	8
PEA						
L	0.6	0.5	8	0.53	0.06	12
M	3.2	0.2	5	3.8	0.4	11
H	31	1	4	35	3	9
Dopamine						
L*	-	-	-	-	-	-
M	0.10	0.09	9	0.09	0.08	9
H	0.60	0.04	8	0.556	0.009	2
Serotonin						
L	1.58	0.06	4	1.71	0.04	3
M	8.4	0.4	5	8.9	0.6	7
H	77	1	2	81	4	5
Adrenaline						
L	0.12	0.02	13	0.09	0.02	16
M	0.45	0.06	13	0.11	0.02	17
H	0.92	0.05	5	0.8	0.1	14
Tryptophan						
L	16	1	7	17	1	7
M	29	2	6	29	4	13
H	167	11	7	174	15	8

	Linear range nM	R^2 Blood (n = 6)	R^2 STD (n=3)	cLOD* (nM)	mLOD* (pmol)	Recovery %
GABA	5-5000	0.998	0.998	0.2	0.02	42
PEA	5-5000	0.999	0.999	1	0.1	62
Dopamine	0.05-50	0.997	0.998	1	0.1	33
Serotonin	5-5000	0.999	0.999	1	0.1	91
Adrenaline	0.05-50	0.992	0.990	0.2	0.02	57
Tryptophan	250-250000	0.99	0.998	30	3	52

The stability was satisfying for all the NTs in the different situations during sample handling and analysis.

The matrix effects were investigated by using the post-extraction addition technique, and large matrix effects from the whole blood were revealed. Matrix effects, and the variations in extraction recovery (31-91 %), were corrected for using SIL internal standards of all six NTs, added prior to sample preparation. Carryover was not an issue for any of the NTs for the levels used in the validation.

3.3.5 Application – neurotransmitters in whole blood

The validated platform's ability to identify/quantify NTs in only 100 µL of whole blood was also demonstrated in **Paper III**. Since the calibration solutions were made by spiking blood which already contained the NTs of interest, the endogenous levels of NTs were calculated using the regression equation from the calibration curve, corrected for the endogenous levels (**Figure 14**).

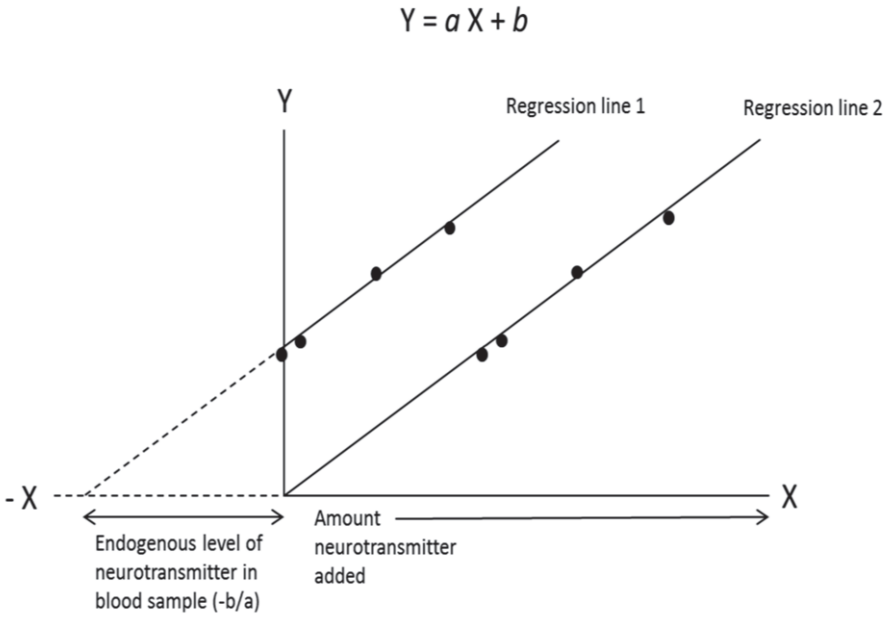


*Figure 14. Illustration of the method used for correcting for the endogenous levels of NTs in blood. Regression line 1 is the original line, and regression line 2 is the corrected one (Supplementary data **Paper III**).*

Due to the platform's sensitivity and versatility, trace levels of the larger neuropeptides oxytocin and vasopressin could be detected as well. The platform provided good and similar chromatography of monoamines, amino acids and peptides (**Figure 15**), suggesting that AFFL-SPE-cap LC-MS is a suitable platform for comprehensive NT determinations in small samples of peripheral body fluids.

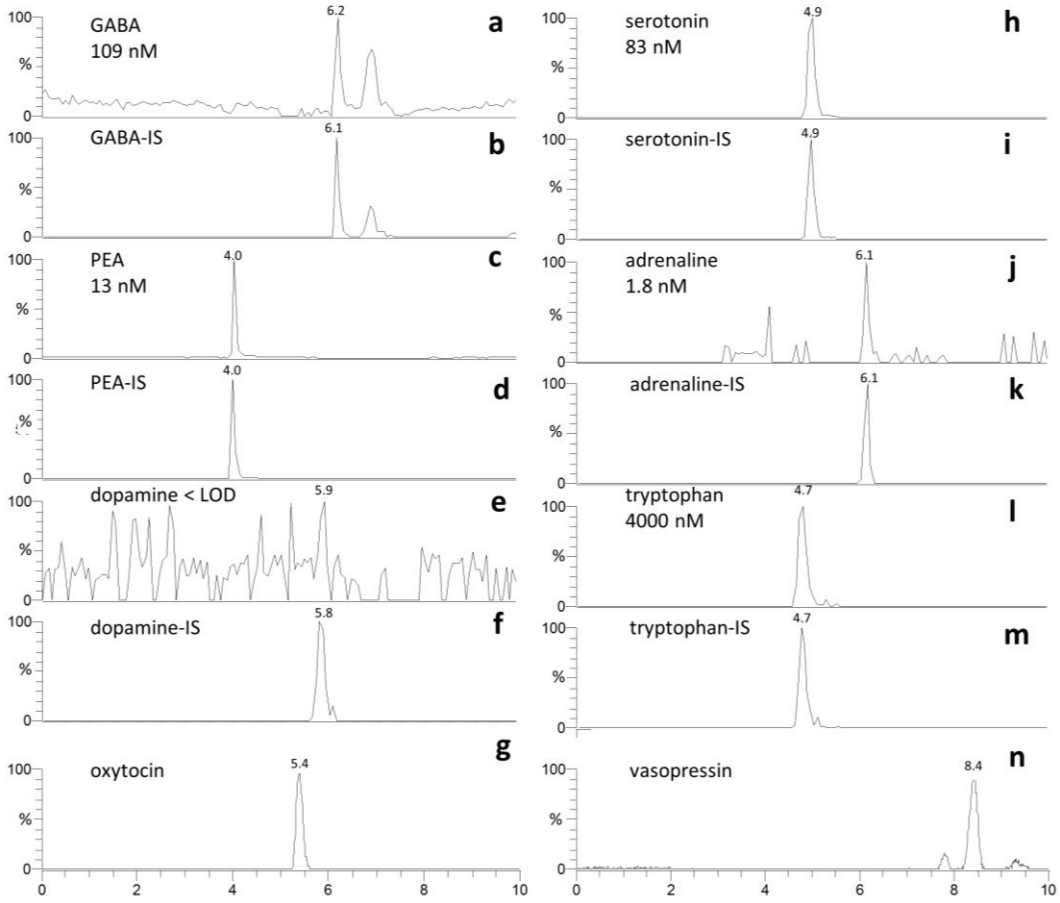


*Figure 15. Chromatograms showing the endogenous NTs identified/measured in a whole blood sample. The NT peak is compared with the SIL internal standard peak and retention times are included. All chromatograms were obtained from the same recording. Dopamine was present below the cLOD and could not be determined in the sample (**Paper III**).*

Dopamine was present below the cLOD, and could not be determined in the whole blood samples, but this was expected due to dopamine's rapid metabolism. When including the two-min SPE loading time, all NTs were on-line extracted, chromatographed and detected in 11 min. A one-min post-run SPE re-equilibration time was sufficient, so the platforms total analysis time cycle was 12 min per sample. A one-step PPT had to be conducted prior to injection onto the SPE, but this step could probably be automated in a 96-well formate to save time and manual labour. For comparison, an RP LC method was able to separate/identify four NTs in cell extract with an analysis time of 6 min [152], while an analysis time of 3 min were sufficient for simultaneous determination of 8 amino acids and two NTs with a HILIC method [158]. However, in the former method no sample preparation was performed, resulting in high cLODs, and if their method were to be used for whole blood samples, enrichment of the NTs would be necessary. In the latter method, an ultrafiltration step, taking 30 min, was needed in addition to the PPT step, severely increasing the total analysis time.

3.3.6 Neuropeptides and drugs

Since the LC-MS platform developed in **Paper III**, had shown ability to determine oxytocin and vasopressin in blood, the possibility of incorporating neuropeptides into the validated method was investigated. The chromatography was satisfying for oxytocin, vasopressin, leucine enkephalin, methionine enkephalin, endomorphin I, endomorphin II and substance P, but a 20-30 % carryover was found for all the peptides. After dissembling the system, cleaning all parts and changing the SPE, it was revealed that the carryover came from the auto-sampler. The carryover increased when water was injected, and since peptides have a low solubility in organic solvent [159], it was suspected that the carryover was due to a solubility issue with the loading solvent (100 % ACN). When 3 % H_2O was added to the loading solvent, the carryover decreased, but the peak areas were non-repeatable. With 5 % water in the loading solvent the peak areas of the peptides decreased, indicating breakthrough due to insufficient retention of the peptides on

the HILIC SPE. Hence, it was concluded that peptides` lack of solubility in organic solvents made the NT platform unsuited for determination of neuropeptides.

HILIC can be appropriate for drug determinations as well [160]. Morphine, and the morphine-antagonist naltrexone, could be successfully determined in spiked whole blood samples showing the potential of the NT platform to simultaneously monitor therapeutics affecting NT activity and the NTs.

3.3.7 Concluding remarks

Although a ZIC-cHILIC column was chosen for the LC-MS platform, the more common ZIC-HILIC column performed nearly as well, but with some differences in selectivity [161]. ZIC-HILIC stationary phases were used for both on-line SPE and LC columns, however fine tuning of HILIC separations can be given an additional dimension by investigating all four combinations; ZIC-HILIC/ZIC-HILIC, ZIC-HILIC/ZIC-cHILIC, ZIC-cHILIC/ZIC-HILIC and ZIC-cHILIC/ZIC-cHILIC, and optimizing the methods based on the compounds of interest and the matrix assessed.

One example is noradrenaline, which was included in the preliminary studies performed on the ZIC-HILIC column. Due to selectivity differences between the two columns an interference from the matrix (whole blood) had the same retention time as noradrenaline, and made the quantification of this compound impossible in real samples using the ZIC-cHILIC column. If noradrenaline were to be determined as well, the method developed in **Paper III** could easily be transferred to a ZIC-HILIC column, and being able to switch between the two column types can give fine tuning of HILIC-separations yet another dimension.

3.4 On-line SPE and RP – determination of oxytocin

Of the neuropeptides, oxytocin (**Figure 16**) is especially interesting due to its association with a plethora of social behaviours and neurological deceases [159, 162-165]. In humans, oxytocin levels have been related to e.g. anxiety [166], autism [167] and schizophrenia [168]. Several studies have reported a coordinated release of central and peripheral oxytocin [164, 169] , and that measurements of peripheral levels can provide a minimally-invasive indicator of central state [166]. Enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) are often used to monitor oxytocin in peripheral body-fluids, but the selectivity of these methods are criticized [163, 170]. A few LC-MS methods have been developed for determination of oxytocin in e.g. plasma [159], microdialysate [162] and an intravenous-solution [165], but they provide unsatisfactory sensitivity and/or varying results. Since neuropeptides could not be determined with the validated NT platform developed in **Paper III**, the goal in **Paper IV** was to develop an alternative LC-MS based method for determination of oxytocin in blood.

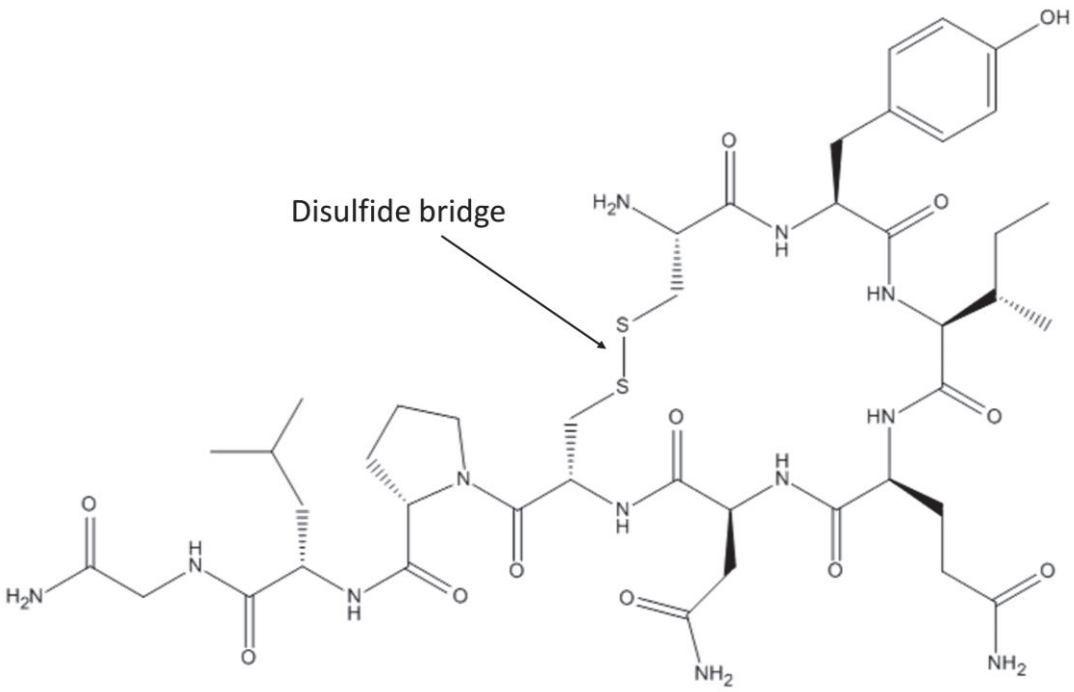


Figure 16. The molecular structure of oxytocin. The disulfide bridge (DSB) between the two cysteine amino acids are marked.

3.4.1 Development of a nano LC-MS method for oxytocin

As before mentioned, an advantage of using HILIC with ESI-MS is the high content of ACN in the mobile phase, providing good electrospray and desolvation efficiency [15]. The ESI-MS sensitivity was compared for oxytocin infused in the HILIC mobile phase and in a standard RP mobile phase (0.1 % FA in 50 % ACN and 50 % H_2O). The latter resulted in a 50 % better sensitivity, and since preliminary experiments had shown sufficient retention of neuropeptides on an RP column, RP was chosen for the method. The endogenous levels of oxytocin in human plasma were expected to be very low [159], hence a nano LC system was assessed to obtain sufficient sensitivity. A triple quadrupole-MS was used, since this MS is considered more suited for targeted determination than the Orbitrap-MS used in **Paper III**. A silica monolithic RP column gave satisfactory retention and efficiency with a 20 min gradient of 20-80 % mobile phase B, where mobile phase A was 0.1% FA in H_2O, and mobile phase B was 0.1% FA in ACN. For aqueous standards the method had a cLOD of 1 pg/mL (18 fg on-column) for oxytocin, which was assumed to be sufficient for determination of endogenous oxytocin.

3.4.2 Sample preparation for oxytocin in plasma

To remove proteins from the plasma, a PPT was conducted, and 80 % ACN gave a satisfactory recovery of oxytocin. Preliminary experiments were performed with a standard nano LC-MS set-up, consisting of an on-line SPE column and a nano LC column, both made of a silica monolithic RP material [171]. Nano LC-MS is rarely used for largescale blood/serum/plasma sample analysis, in part due to its limited robustness, and indeed injecting the protein precipitated plasma clogged the columns. By implementing the AFFL system used in **Paper III**, this weakness was overcome, and only a minor increase in the back pressure between the first and the hundredth plasma injection was observed. To avoid breakthrough of oxytocin on the RP-SPE used in **Paper IV**, the supernatant from the PPT had to be evaporated to dryness and reconstituted in H_2O with 0.1 % FA before injection.

Despite the method's low cLOD, and ability to handle large volume injections of plasma (10 µL), endogenous oxytocin was not detected in human plasma. Two alternative sample preparation procedures for oxytocin, utilizing off-line SPE, were examined. None of the procedures resulted in detection of endogenous oxytocin, even though one of them had a start volume of 1.4 mL plasma [159] and the other one was the SPE procedure recommended in an oxytocin ELISA kit[2].

When a PPT spiked plasma sample was repeatedly injected it was revealed that the signal dropped and levelled off after 40 min. Oxytocin was stable in the solvents used during and after PPT (**Figure 17**), and did not stick to tubes and vials. It was hence speculated if the recovery profile depicted a slow rebinding to protein remains.

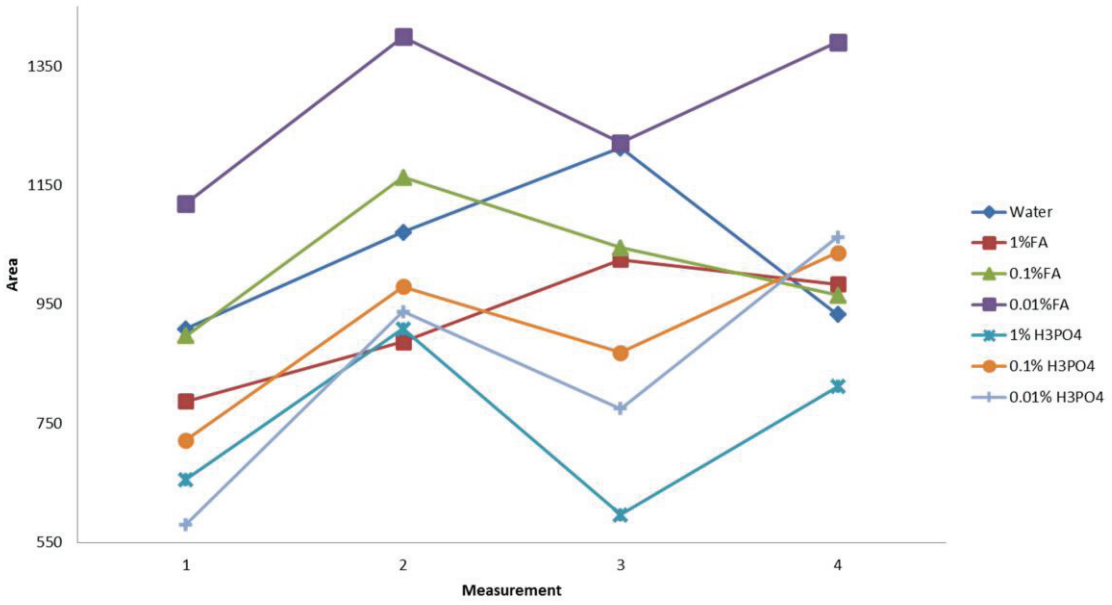


*Figure 17. Stability of oxytocin spiked in water, FA (0.01-1 %) and H_3PO_4 (0.01-1 %). Measurement 1 was conducted 10 min after addition of acid, measurement 2 after 2 hours, measurement 3 after 3 hours and measurement 4 after 5 hours. No significant decrease in peak area was observed in any of the acidic solutions in 5 hours (Supplementary data **Paper IV**).*

[2] Oxytocin ELISA kit. Catalog # ADI-901-153A (ENZO)

Protein binding of oxytocin

Experiments with plasma spiked with oxytocin revealed that the recovery declined almost linearly as a function of time if the samples were stored at room temperature prior to PPT, and it was hypothesized that strong protein binding was preventing detection of endogenous oxytocin due to co-precipitation during PPT. Tight plasma binding is not uncommon for biomarkers [172]. The DSB of oxytocin (**Figure 16**) can engage in complexes [173], and likely with serum albumin, which contains multiple DSBs. A R/A [174] step, prior to PPT, was implemented since this will irreversibly break DSBs and hence disrupt plasma protein binding. The R/A procedure resulted in a complete and stable derivatization of oxytocin, and when analysing unspiked R/A treated plasma samples, endogenous oxytocin was found to be present at high levels. A R/A step was hence included in the sample preparation procedure, prior to the PPT.

3.4.3 Application – oxytocin in plasma and human cord serum

In **Paper IV**, the nano LC-MS method's ability to quantify oxytocin was shown by analysing pooled human plasma and human cord serum obtained from commercial sources. Only 100 µL of plasma/serum was needed, and a SIL internal standard was added prior to the R/A step. The oxytocin levels observed were several orders higher compared to that obtained with an off-line SPE step followed by either ELISA or RIA [175], but more in agreement with an approach involving an isolation of redox sequestered fractions in plasma [176].

3.4.4 Concluding remarks

The R/A step was necessary to break the binding between plasma proteins and oxytocin. Without this step, the vast majority of oxytocin is discarded with plasma proteins in the PPT step, leaving only a small amount of free oxytocin to be measured. Free oxytocin was also found to bind to protein remains after the PPT. It is currently recommended [170] to measure only the free fraction, but this

fraction can be drastically changed by e.g. a person's age or by drugs that displace oxytocin from proteins [177]. A third of the human samples analysed by Zhang et al. did not contain detectable levels of oxytocin [159], indicating large variations when measuring the free fraction of oxytocin. Inconsistencies like that were also experienced when only the free fraction of oxytocin was measured with the NT platform developed in **Paper III**. Total oxytocin may be a better biomarker than the free fraction of oxytocin, however, the biological activity of the bound fraction is less clear, and further studies should be performed to investigate this.

3.5 FINAL DISCUSSION

In this study **HILIC**, as an alternative to **RPLC**, and it has been shown that HILIC has strong retention capability for highly polar compounds. It has also been shown that HILIC has other benefits compared to RPLC, e.g. a highly volatile mobile phase which provides good electrospray and desolvation efficiency. Another benefit is that samples containing high organic solvent concentrations can be directly injected into the LC system without evaporation and reconstitution steps.

Despite significant advances in the development of efficient analytical instruments, LC analyses of biological samples are restricted by the **sample preparation** of the highly complex matrices. Hence, a suitable sample preparation is needed prior to chromatographic analysis. An ideal sample preparation methodology should be fast, accurate and precise, and the possibility of automation is a great benefit due to the increased through-put and less manual handling. One way to improve the efficiency and accuracy of the analytical procedure is **on-line coupling** of the sample preparation techniques with the LC system. In this study, the use of on-line SPE made it possible to load large sample volumes onto miniaturized columns (cap/nano) avoiding loss of detection sensitivity even though these columns allow only small injection volumes. Although on-line SPE-LC can be a very effective tool, pressure build-up and column clogging are well-known issues. Due to the use of **the AFFL feature**, which filtrates the sample on-

line, hundreds of precipitated whole blood and plasma samples could be injected without problems in this study.

Ultrafiltration, which has been used extensively for the study of macromolecules, has in recent years gained popularity in the study of small molecules as well. In this study, ultrafiltration was successfully used as a stand-alone sample preparation procedure for determination of anti-tuberculosis drugs in cell lysate, resulting in a simple, but efficient method with a minimal consumption of sample compared to more traditional **off-line approaches**.

The true level of plasma oxytocin is a much debated question, reflected by the thousands of papers published on the subject. The free levels of oxytocin are often reported as extremely low and often under the LOD of the analytical method utilized. The total oxytocin, which was measured in this study, may be a better biomarker than the free fraction of oxytocin, giving a higher and more correct answer. However, the biological activity of the bound fraction is less clear, and further studies should be performed to investigate this.

In this thesis the ability of different high performance analytical tools for determination of metabolites/small molecules in complex biosamples have been shown. In **Paper AI**, centrifugal ultrafiltration followed by HILIC-MS was used in a simple and rapid method for determination of the highly polar metabolite PAPS in cell lines, showing yet another example of how these tools can be combined to allow simple handling of complex samples.

Several of the methodologies used in this thesis are sophisticated and in need of complex instrumentation, but they are addressing important issues in bioanalysis of metabolites/small molecules which are not easily satisfied by traditional analytical tools. Fortunately, the use of on-line sample preparation and downscaling in LC is much more common nowadays, and instrumentation is commercially available which increases the ease of laboratory implementation.

4. CONCLUSION

This thesis demonstrates novel analytical methodologies for the measurement of metabolites and other small molecules in complex biosamples.

The successful separation of the highly polar triphosphate nucleotides shows why HILIC is a very useful methodology for separating hydrophilic compounds, especially those who lack retention on RP stationary phases. All the parameters that can be adjusted in a HILIC method e.g. temperature and buffer concentration, constitute a diverse toolkit for fine tuning the separations, and makes up for the somewhat more advanced method development compared to RPLC. Since RPLC and HILIC are complementary separation principles, the combination of these, either off-line or on-line, is of great value in e.g. metabolomics where the compounds can have very different chemical and physical properties. Since HILIC columns are commercially available in most sizes, a suitable column can be found to be implemented into existing instrumentations and procedures.

For LC-MS determination of anti-tuberculosis drugs in cell lysate, centrifugal filtration could be used as an efficient off-line sample preparation technique. The combination of protein precipitation and filtration makes it a simple, yet robust method, which deals with the issue of drug-protein binding. The methodology only requires a centrifuge and is definitely a simple and efficient alternative to SPE or LLE for small molecules.

For quantitative determination of neurotransmitters in whole blood on-line sample preparation techniques were successfully combined with LC-MS. The on-line SPE made it possible to inject large sample volumes onto a capillary column, hence, low abundant neurotransmitters could be determined. By removing disturbing compounds, the AFFL methodology made it possible to inject hundreds of whole blood samples without clogging the system. The combination of on-line sample preparation and filtration minimizes the manual handling, which leads to a time efficient methodology with lesser sources of error. Although some additional instrumentation is needed, all parts are commercially available and the gain in

sensitivity and decrease in manual handling makes up for the extra work setting up the instrumentation.

By including an additional reduction/alkylation step in the sample preparation the neuropeptide oxytocin could be successfully quantified in serum and plasma using on-line SPE, the AFFL feature and nano LC. It was suspected that oxytocin binds strongly to plasma proteins, and when the protein binding was eliminated, startlingly high amount of oxytocin was revealed. The oxytocin method is a good example of how high performance analytical tools can be used to solve important issues in bioanalysis of small molecules.

5. REFERENCES

1. A. Roux, D. Lison, C. Junot, and J.-F. Heilier. **Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: A review**. *Clinical Biochemistry*, 44 (**2011**) 119-135
2. J.L. Spratlin, N.J. Serkova, and S.G. Eckhardt. **Clinical Applications of Metabolomics in Oncology: A Review**. *Clinical Cancer Research*, 15 (**2009**) 431-440
3. J.K. Nicholson, J. Connelly, J.C. Lindon, and E. Holmes. **Metabonomics: a platform for studying drug toxicity and gene function**. *Nature Review Drug Discovery*, 1 (**2002**) 153-161
4. P.L. Kole, G. Venkatesh, J. Kotecha, and R. Sheshala. **Recent advances in sample preparation techniques for effective bioanalytical methods**. *Biomedical Chromatography*, 25 (**2011**) 199-217
5. H. Pearson. **Meet the human metabolome**. *Nature*, 446 (**2007**) 8-8
6. D. Vuckovic. **Current trends and challenges in sample preparation for global metabolomics using liquid chromatography–mass spectrometry**. *Analytical and Bioanalytical Chemistry*, 403 (**2012**) 1523-1548
7. H.G. Gika, G.A. Theodoridis, R.S. Plumb, and I.D. Wilson. **Current practice of liquid chromatography–mass spectrometry in metabolomics and metabonomics**. *Journal of Pharmaceutical and Biomedical Analysis*, 87 (**2014**) 12-25
8. D.S. Wishart, T. Jewison, A.C. Guo, R. Greiner and A. Scalbert. **HMDB 3.0—The Human Metabolome Database in 2013**. *Nucleic Acids Research* (**2012**)
9. D.-Q. Tang, L. Zou, X.-X. Yin, and C.N. Ong. **HILIC-MS for metabolomics: An attractive and complementary approach to RPLC-MS**. *Mass Spectrometry Reviews* (**2015**)
10. W. Lu, B.D. Bennett, and J.D. Rabinowitz. **Analytical strategies for LC–MS-based targeted metabolomics**. *Journal of Chromatography B*, 871 (**2008**) 236-242
11. W.B. Dunn and D.I. Ellis. **Metabolomics: Current analytical platforms and methodologies**. *TrAC Trends in Analytical Chemistry*, 24 (**2005**) 285-294
12. Y. Guo and S. Gaiki. **Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography**. *Journal of Chromatography A*, 1074 (**2005**) 71-80
13. P. Hemström and K. Irgum. **Hydrophilic interaction chromatography**. *Journal of Separation Science*, 29 (**2006**) 1784-1821
14. A.J. Alpert. **Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds**. *Journal of Chromatography A*, 499 (**1990**) 177-196
15. A. Periat, I.S. Krull, and D. Guillarme. **Applications of hydrophilic interaction chromatography to amino acids, peptides, and proteins**. *Journal of Separation Science*, 38 (**2015**) 357-367
16. R.-I. Chirita, C. West, A.-L. Finaru, and C. Elfakir. **Approach to hydrophilic interaction chromatography column selection: Application to neurotransmitters analysis**. *Journal of Chromatography A*, 1217 (**2010**) 3091-3104
17. P. Jandera, T. Hájek, V. Škeříková, and J. Soukup. **Dual hydrophilic interaction-RP retention mechanism on polar columns: Structural correlations and implementation for 2-D separations on a single column**. *Journal of Separation Science*, 33 (**2010**) 841-852
18. D.V. McCalley. **Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography**. *Journal of Chromatography A*, 1217 (**2010**) 3408-3417
19. N.P. Dinh, T. Jonsson, and K. Irgum. **Probing the interaction mode in hydrophilic interaction chromatography**. *Journal of Chromatography A*, 1218 (**2011**) 5880-5891
20. M.E.A. Ibrahim, Y. Liu, and C.A. Lucy. **A simple graphical representation of selectivity in hydrophilic interaction liquid chromatography**. *Journal of Chromatography A*, 1260 (**2012**) 126-131
21. Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, and N. Tanaka. **Chromatographic characterization of hydrophilic interaction liquid chromatography stationary phases: Hydrophilicity, charge effects, structural selectivity, and separation efficiency**. *Journal of Chromatography A*, 1218 (**2011**) 5903-5919
22. B. Buszewski and S. Noga. **Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique**. *Analytical and Bioanalytical Chemistry*, 402 (**2011**) 231-247

23. S. Di Palma, P.J. Boersema, A.J.R. Heck, and S. Mohammed. **Zwitterionic Hydrophilic Interaction Liquid Chromatography (ZIC-HILIC and ZIC-cHILIC) Provide High Resolution Separation and Increase Sensitivity in Proteome Analysis**. *Analytical Chemistry*, 83 (**2011**) 3440-3447
24. W. Jiang, G. Fischer, Y. Girmay, and K. Irgum. **Zwitterionic stationary phase with covalently bonded phosphorylcholine type polymer grafts and its applicability to separation of peptides in the hydrophilic interaction liquid chromatography mode**. *Journal of Chromatography A*, 1127 (**2006**) 82-91
25. A. Lafaye, J. Labarre, J.-C. Tabet, E. Ezan, and C. Junot. **Liquid chromatography-mass spectrometry and 15N metabolic labeling for quantitative metabolic profiling**. *Analytical Chemistry*, 77 (**2005**) 2026-2033
26. E. Eckert, H. Drexler, and T. Göen. **Determination of six hydroxyalkyl mercapturic acids in human urine using hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC–ESI-MS/MS)**. *Journal of Chromatography B*, 878 (**2010**) 2506-2514
27. A. Conventz, A. Musiol, C. Brodowsky, P. Dewes, T. Kraus and T. Schettgen. **Simultaneous determination of 3-nitrotyrosine, tyrosine, hydroxyproline and proline in exhaled breath condensate by hydrophilic interaction liquid chromatography/electrospray ionization tandem mass spectrometry**. *Journal of Chromatography B*, 860 (**2007**) 78-85
28. P.Y. Han, P.N. Shaw, and C.M. Kirkpatrick. **Determination of para-aminohippuric acid (PAH) in human plasma and urine by liquid chromatography–tandem mass spectrometry**. *Journal of Chromatography B*, 877 (**2009**) 3215-3220
29. T. Langrock, P. Czihal, and R. Hoffmann. **Amino acid analysis by hydrophilic interaction chromatography coupled on-line to electrospray ionization mass spectrometry**. *Amino Acids*, 30 (**2006**) 291-297
30. P.N. Demacker, A.M. Beijers, H. van Daal, J.P. Donnelly, N.M. Blijlevens, and J.M. van den Ouweland. **Plasma citrulline measurement using UPLC tandem mass-spectrometry to determine small intestinal enterocyte pathology**. *Journal of Chromatography B*, 877 (**2009**) 387-392
31. M. Schwalbe-Herrmann, J. Willmann, and D. Leibfritz. **Separation of phospholipid classes by hydrophilic interaction chromatography detected by electrospray ionization mass spectrometry**. *Journal of Chromatography A*, 1217 (**2010**) 5179-5183
32. R. Tuytten, F. Lemiere, W.V. Dongen, E. Esmans, and H. Slegers. **Short capillary ion-pair high-performance liquid chromatography coupled to electrospray (tandem) mass spectrometry for the simultaneous analysis of nucleoside mono-, di-and triphosphates**. *Rapid Communications in Mass Spectrometry*, 16 (**2002**) 1205-1215
33. Y. Huang, Y. Tian, Z. Zhang, and C. Peng. **A HILIC–MS/MS method for the simultaneous determination of seven organic acids in rat urine as biomarkers of exposure to realgar**. *Journal of Chromatography B*, 905 (**2012**) 37-42
34. R. Sardella, S. Scorzoni, C. Conte, A. Lisanti, F. Ianni, and B. Natalini. **Novel orthogonal liquid chromatography methods to dose neurotransmitters involved in Parkinson's disease**. *Journal of Pharmaceutical and Biomedical Analysis*, 98 (**2014**) 253-259
35. J.P. Danaceau, E.E. Chambers, and K.J. Fountain. **Hydrophilic interaction chromatography (HILIC) for LC-MS/MS analysis of monoamine neurotransmitters**. *Bioanalysis*, 4 (**2012**) 783-794
36. Y. Wang, R. Lehmann, X. Lu, X. Zhao, and G. Xu. **Novel, fully automatic hydrophilic interaction/reversed-phase column-switching high-performance liquid chromatographic system for the complementary analysis of polar and apolar compounds in complex samples**. *Journal of Chromatography A*, 1204 (**2008**) 28-34
37. J.P.C. Vissers. **Recent developments in microcolumn liquid chromatography**. *Journal of Chromatography A*, 856 (**1999**) 117-143
38. Y. Saito, K. Jinno, and T. Greibrokk. **Capillary columns in liquid chromatography: between conventional columns and microchips**. *Journal of Separation Science*, 27 (**2004**) 1379-1390
39. M. Rogeberg, H. Malerod, H. Roberg-Larsen, C. Aass, and S.R. Wilson. **On-line solid phase extraction–liquid chromatography, with emphasis on modern bioanalysis and miniaturized systems**. *Journal of Pharmaceutical and Biomedical Analysis*, 87 (**2014**) 120-129
40. J.P.C. Vissers, H.A. Claessens, and C.A. Cramers. **Microcolumn liquid chromatography: instrumentation, detection and applications**. *Journal of Chromatography A*, 779 (**1997**) 1-28

41. S.R. Wilson, T. Vehus, H.S. Berg, and E. Lundanes. **Nano-LC in proteomics: recent advances and approaches**. *Bioanalysis*, 7 (**2015**) 1799-1815
42. X. Lu, X. Zhao, C. Bai, C. Zhao, G. Lu, and G. Xu. **LC–MS-based metabonomics analysis**. *Journal of Chromatography B*, 866 (**2008**) 64-76
43. S.R. Wilson, H. Malerød, A. Holm, P. Molander, E. Lundanes, and T. Greibrøkk. **On-line SPE—Nano-LC—Nanospray-MS for Rapid and Sensitive Determination of Perfluorooctanoic Acid and Perfluorooctane Sulfonate in River Water**. *Journal of Chromatographic Science*, 45 (**2007**) 146-152
44. J.P.C. Vissers, A.H. de Ru, M. Ursem, and J.-P. Chervet. **Optimised injection techniques for micro and capillary liquid chromatography**. *Journal of Chromatography A*, 746 (**1996**) 1-7
45. J. Pan, C. Zhang, Z. Zhang, and G. Li. **Review of online coupling of sample preparation techniques with liquid chromatography**. *Analytica Chimica Acta*, 815 (**2014**) 1-15
46. F.M. Musteata and J. Pawliszyn. **Bioanalytical applications of solid-phase microextraction**. *TrAC Trends in Analytical Chemistry*, 26 (**2007**) 36-45
47. M.S. Chang, Q. Ji, J. Zhang, and T.A. El-Shourbagy. **Historical review of sample preparation for chromatographic bioanalysis: pros and cons**. *Drug Development Research*, 68 (**2007**) 107-133
48. J. Płotka-Wasylka, N. Szczepańska, M. de la Guardia, and J. Namieśnik. **Modern trends in solid phase extraction: New sorbent media**. *TrAC Trends in Analytical Chemistry*, 77 (**2016**) 23-43
49. L. Chen, et al. **On-line coupling of solid-phase extraction to liquid chromatography—a review**. *Journal of Chromatographic Science*, 47 (**2009**) 614-623
50. S.J. Bruce, I. Tavazzi, V. Parisod, S. Rezzi, S. Kochhar, and P.A. Guy. **Investigation of Human Blood Plasma Sample Preparation for Performing Metabolomics Using Ultrahigh Performance Liquid Chromatography/Mass Spectrometry**. *Analytical Chemistry*, 81 (**2009**) 3285-3296
51. C.F. Poole. **New trends in solid-phase extraction**. *TrAC Trends in Analytical Chemistry*, 22 (**2003**) 362-373
52. J. Zhang, H. Wu, E. Kim, and T.A. El-Shourbagy. **Salting-out assisted liquid/liquid extraction with acetonitrile: a new high throughput sample preparation technique for good laboratory practice bioanalysis using liquid chromatography–mass spectrometry**. *Biomedical Chromatography*, 23 (**2009**) 419-425
53. I.M. Valente, L.M. Gonçalves, and J.A. Rodrigues. **Another glimpse over the salting-out assisted liquid–liquid extraction in acetonitrile/water mixtures**. *Journal of Chromatography A*, 1308 (**2013**) 58-62
54. M. Yoshida, A. Akane, M. Nishikawa, T. Watabiki, and H. Tsuchihashi. **Extraction of Thiamylal in Serum Using Hydrophilic Acetonitrile with Subzero-Temperature and Salting-Out Methods**. *Analytical Chemistry*, 76 (**2004**) 4672-4675
55. H. Wu, J. Zhang, K. Norem, and T.A. El-Shourbagy. **Simultaneous determination of a hydrophobic drug candidate and its metabolite in human plasma with salting-out assisted liquid/liquid extraction using a mass spectrometry friendly salt**. *Journal of Pharmaceutical and Biomedical Analysis*, 48 (**2008**) 1243-1248
56. B.T. Røen, S.R. Sellevåg, and E. Lundanes. **Quantification of nerve agent biomarkers in human serum and urine**. *Analytical Chemistry*, 86 (**2014**) 11833-11840
57. J.-H. Chen, B.-C. Liau, T.-T. Jong, and C.-M.J. Chang. **Extraction and purification of flavanone glycosides and kaemferol glycosides from defatted Camellia oleifera seeds by salting-out using hydrophilic isopropanol**. *Separation and Purification Technology*, 67 (**2009**) 31-37
58. T.F. Jenkins, P.H. Miyares, K.F. Myers, E.F. McCormick, and A.B. Strong. **Comparison of solid phase extraction with salting-out solvent extraction for preconcentration of nitroaromatic and nitramine explosives from water**. *Analytica Chimica Acta*, 289 (**1994**) 69-78
59. M. Anastassiades, S.J. Lehotay, D. Štajnbaher, and F.J. Schenck. **Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce**. *Journal of AOAC international*, 86 (**2003**) 412-431
60. M.S. Young and K.M. Jenkins. **Oasis® WCX: A Novel Mixed-Mode SPE Sorbent for LC-MS Determination of Paraquat and Other Quaternary Ammonium Compounds**. *LC GC NORTH AMERICA*, 22 (**2004**) 44-45
61. P. Ivanov Dobrev and M. Kamínek. **Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction**. *Journal of Chromatography A*, 950 (**2002**) 21-29

62. N. Karlonas, A. Padarauskas, A. Ramanavicius, and A. Ramanaviciene. **Mixed-mode SPE for a multi-residue analysis of benzodiazepines in whole blood using rapid GC with negative-ion chemical ionization MS**. *Journal of Separation Science*, 36 (**2013**) 1437-1445
63. I. Ferrer and D. Barceló. **Validation of new solid-phase extraction materials for the selective enrichment of organic contaminants from environmental samples**. *TrAC Trends in Analytical Chemistry*, 18 (**1999**) 180-192
64. S. Rodriguez-Mozaz, M.J. Lopez de Alda, and D. Barceló. **Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography–mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water**. *Journal of Chromatography A*, 1152 (**2007**) 97-115
65. C.A. Daykin, P.J. Foxall, S.C. Connor, J.C. Lindon, and J.K. Nicholson. **The comparison of plasma deproteinization methods for the detection of low-molecular-weight metabolites by 1 H nuclear magnetic resonance spectroscopy**. *Analytical Biochemistry*, 304 (**2002**) 220-230
66. D.W. Greening and R.J. Simpson. **A centrifugal ultrafiltration strategy for isolating the low-molecular weight (≤ 25K) component of human plasma proteome**. *Journal of proteomics*, 73 (**2010**) 637-648
67. O. Chertov, J.T. Simpson, A. Biragyn, T.P. Conrads, T.D. Veenstra, and R.J. Fisher. **Enrichment of low-molecular-weight proteins from biofluids for biomarker discovery**. *Expert Review of Proteomics*, 2 (**2005**) 139-145
68. T. Reyns, M. Cherlet, S. De Baere, P. De Backer, and S. Croubels. **Rapid method for the quantification of amoxicillin and its major metabolites in pig tissues by liquid chromatography-tandem mass spectrometry with emphasis on stability issues**. *Journal of Chromatography B*, 861 (**2008**) 108-116
69. L. Pont, F. Benavente, J. Barbosa, and V. Sanz-Nebot. **An update for human blood plasma pretreatment for optimized recovery of low-molecular-mass peptides prior to CE–MS and SPE–CE–MS**. *Journal of Separation Science*, 36 (**2013**) 3896-3902
70. K. Inoue, S. Nitta, T. Hino, and H. Oka. **LC-MS/MS and centrifugal ultrafiltration method for the determination of novobiocin in chicken, fish tissues, milk and human serum**. *Journal of Chromatography B*, 877 (**2009**) 461-464
71. M. Sergi, C. Montesano, S. Napoletano, D. Pizzoni, C. Manetti, F. Colistro, R. Curini, D. Compagnone. **Analysis of bile acids profile in human serum by ultrafiltration clean-up and LC-MS/MS**. *Chromatographia*, 75 (**2012**) 479-489
72. K. Kishida. **Simplified extraction of tetracycline antibiotics from milk using a centrifugal ultrafiltration device**. *Food Chemistry*, 126 (**2011**) 687-690
73. C.B. Vibe, F. Fenaroli, D. Pires, S.R. Wilson, R. Kalluru, G. Griffiths and J. Hildahl. **Thioridazine in PLGA nanoparticles reduces toxicity and improves rifampicin therapy against mycobacterial infection in zebrafish**. *Nanotoxicology* (**2015**) 1-9
74. S. Wohlfart, A.S. Khalansky, M. Glatzel and J. Kreuter. **Efficient chemotherapy of rat glioblastoma using doxorubicin-loaded PLGA nanoparticles with different stabilizers**. *PloS One*, 6 (**2011**) e19121
75. N. Jimenez, J. Galan, A. Vallet, M. Egea, and M. Garcia. **Methyl trypsin loaded poly (D, L-lactide-coglycolide) nanoparticles for contact lens care**. *Journal of Pharmaceutical Sciences*, 99 (**2010**) 1414-1426
76. H.M. Georgiou, G.E. Rice, and M.S. Baker. **Proteomic analysis of human plasma: failure of centrifugal ultrafiltration to remove albumin and other high molecular weight proteins**. *Proteomics*, 1 (**2001**) 1503-1506
77. D.W. Greening and R.J. Simpson. **Low-molecular weight plasma proteome analysis using centrifugal ultrafiltration**. *Serum/Plasma Proteomics: Methods and Protocols* (**2011**) 109-124
78. D. Barceló and M.-C. Hennion. **On-line sample handling strategies for the trace-level determination of pesticides and their degradation products in environmental waters**. *Analytica Chimica Acta*, 318 (**1995**) 1-41
79. J.F.K. Huber, R. Van Der Linden, E. Ecker, and M. Oreans. **Column swithiching in high-pressure liquid chromatography**. *Journal of Chromatography A*, 83 (**1973**) 267-277
80. R.J. Dolphin, F.W. Willmott, A.D. Mills, and L.P.J. Hoogeveen. **Column switching techniques in the liquid chromatographic analysis of organochlorine pesticides in milk**. *Journal of Chromatography A*, 122 (**1976**) 259-268

81. D.H. Freeman. **Review: ultraselectivity through column switching and mode sequencing in liquid chromatography**. *Analytical Chemistry*, 53 (**1981**) 2-5
82. J.-L. Veuthey, S. Souverain, and S. Rudaz. **Column-switching procedures for the fast analysis of drugs in biologic samples**. *Therapeutic Drug Monitoring*, 26 (**2004**) 161-166
83. P. Campíns-Falcó, R. Herráez-Hernández, and A. Sevillano-Cabeza. **Column-switching techniques for high-performance liquid chromatography of drugs in biological samples**. *Journal of Chromatography B: Biomedical Sciences and Applications*, 619 (**1993**) 177-190
84. K.O. Svendsen, H.R. Larsen, S.A. Pedersen, I. Brenna, E. Lundanes, and S.R. Wilson. **Automatic filtration and filter flush for robust online solid-phase extraction liquid chromatography**. *Journal of Separation Science*, 34 (**2011**) 3020-3022
85. H. Roberg-Larsen, M.F. Strand, A. Grimsmo, P.A. Olsen, J.L. Dembinski, F. Rise, E. Lundanes, T. Greibrokk, S. Krauss and S.R. Wilson. **High sensitivity measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction-liquid chromatography–mass spectrometry**. *Journal of Chromatography A*, 1255 (**2012**) 291-297
86. H. Roberg-Larsen, K. Lund, T. Vehus, N. Solberg, C. Vesterdal, D. Misaghian, P.A. Olsen, S. Krauss, S.R. Wilson and E. Lundanes. **Highly automated nano-LC/MS-based approach for thousand cell-scale quantification of side chain-hydroxylated oxysterols**. *Journal of Lipid Research*, 55 (**2014**) 1531-1536
87. O. González, M.E. Blanco, G. Iriarte, L. Bartolomé, M.I. Maguregui, and R.M. Alonso. **Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect**. *Journal of Chromatography A*, 1353 (**2014**) 10-27
88. **Guidance for industry: Bioanalytical Method Validation**. 2001; Available from: www.fda.gov/cder/guidance/index.htm.
89. S. Kollipara, G. Bende, N. Agarwal, B. Varshney, and J. Paliwal. **International guidelines for bioanalytical method validation: a comparison and discussion on current scenario**. *Chromatographia*, 73 (**2011**) 201-217
90. S. Cohen, M. Megherbi, L.P. Jordheim, C. Dumontet and J. Guitton. **Simultaneous analysis of eight nucleoside triphosphates in cell lines by liquid chromatography coupled with tandem mass spectrometry**. *Journal of Chromatography B*, 877 (**2009**) 3831-3840
91. G. Henneré, F. Becher, A. Pruvost, C. Goujard, J. Grassi, and H. Benech. **Liquid chromatography–tandem mass spectrometry assays for intracellular deoxyribonucleotide triphosphate competitors of nucleoside antiretrovirals**. *Journal of Chromatography B*, 789 (**2003**) 273-281
92. S. Hooshfar, B. Basiri, and M.G. Bartlett. **Development of a surrogate matrix for cerebral spinal fluid for liquid chromatography/mass spectrometry based analytical methods**. *Rapid Communications in Mass Spectrometry*, 30 (**2016**) 854-858
93. L. Liu, Z. Cui, Y. Deng, B. Dean, C.E. Hop, and X. Liang. **Surrogate analyte approach for quantitation of endogenous NAD+ in human acidified blood samples using liquid chromatography coupled with electrospray ionization tandem mass spectrometry**. *Journal of Chromatography B*, 1011 (**2016**) 69-76
94. T. Kamčeva, T. Bjånes, A. Svardal, B. Riedel, J. Schjøtt, and T. Eide. **Liquid chromatography/tandem mass spectrometry method for simultaneous quantification of eight endogenous nucleotides and the intracellular gemcitabine metabolite dFdCTP in human peripheral blood mononuclear cells**. *Journal of Chromatography B*, 1001 (**2015**) 212-220
95. S. White, B. Ingelse, B. Barroso, N. Gray, C. Sykora, P. Vinck and Martina Wein. **European Bioanalysis Forum: recommendation for dealing with internal standard variability**. *Bioanalysis*, 6 (**2014**) 2767-2774
96. A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, and Y. Michotte. **Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects**. *Journal of Chromatography B*, 877 (**2009**) 2198-2207
97. P.J. Taylor. **Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry**. *Clinical Biochemistry*, 38 (**2005**) 328-334
98. I. Marchi, et al. **Characterization and classification of matrix effects in biological samples analyses**. *Journal of Chromatography A*, 1217 (**2010**) 4071-4078

99. O. González, M. van Vliet, C.W. Damen, F.M. van der Kloet, R.J. Vreeken, and T. Hankemeier. **Matrix Effect Compensation in Small-Molecule Profiling for an LC–TOF Platform Using Multicomponent Postcolumn Infusion**. *Analytical Chemistry*, 87 (**2015**) 5921-5929
100. T.M. Annesley. **Ion suppression in mass spectrometry**. *Clinical Chemistry*, 49 (**2003**) 1041-1044
101. S. Cohen, L.P. Jordheim, M. Megherbi, C. Dumontet, and J. Guitton. **Liquid chromatographic methods for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy: a review**. *Journal of Chromatography B*, 878 (**2010**) 1912-1928
102. R.M. Seifar, C. Ras, J.C. van Dam, W.M. van Gulik, J.J. Heijnen, and W.A. van Winden. **Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry**. *Analytical Biochemistry*, 388 (**2009**) 213-219
103. C. Machon, L.P. Jordheim, J.-Y. Puy, I. Lefebvre, C. Dumontet, and J. Guitton. **Fully validated assay for the quantification of endogenous nucleoside mono-and triphosphates using online extraction coupled with liquid chromatography–tandem mass spectrometry**. *Analytical and Bioanalytical Chemistry*, 406 (**2014**) 2925-2941
104. Z. Cai, F. Song, and M. Yang. **Capillary liquid chromatographic–high-resolution mass spectrometric analysis of ribonucleotides**. *Journal of Chromatography A*, 976 (**2002**) 135-143
105. M.H. Buckstein, J. He, and H. Rubin. **Characterization of Nucleotide Pools as a Function of Physiological State in Escherichia coli**. *Journal of Bacteriology*, 190 (**2008**) 718-726
106. M.L. Mole, D.L. Hunter, P. Gao, and C. Lau. **Sample Preparation and High-Performance Liquid Chromatographic Analysis of Deoxyribonucleoside Triphosphates in Individual Rat Embryos**. *Analytical Biochemistry*, 259 (**1998**) 245-252
107. S. Palmer and S. Cox. **Comparison of extraction procedures for high-performance liquid chromatographic determination of cellular deoxynucleotides**. *Journal of Chromatography A*, 667 (**1994**) 316-321
108. D.S. Shewach. **Quantitation of deoxyribonucleoside 5'-triphosphates bya sequential boronate and anion-exchange high-pressure liquid chromatographic procedure**. *Analytical Biochemistry*, 206 (**1992**) 178-182
109. L.A. Decosterd, E. Cottin, X. Chen, P.A. Coucke. **Simultaneous Determination of Deoxyribonucleoside in the Presence of Ribonucleoside Triphosphates in Human Carcinoma Cells by High-Performance Liquid Chromatography**. *Analytical Biochemistry*, 270 (**1999**) 59-68
110. H. Sakamaki, T. Uchida, L.W. Lim, and T. Takeuchi. **Evaluation of column hardware on liquid chromatography–mass spectrometry of phosphorylated compounds**. *Journal of Chromatography A*, 1381 (**2015**) 125-131
111. R. Tuytten, E. Witters, R. P. Newton and E.L. Esmans. **Stainless steel electrospray probe: A dead end for phosphorylated organic compounds?** *Journal of Chromatography A*, 1104 (**2006**) 209-221
112. T. De Vijlder, J. Boschmans, E. Witters, and F. Lemière. **Study on the loss of nucleoside mono-, di-and triphosphates and phosphorylated peptides to a metal-free LC–MS hardware**. *International Journal of Mass Spectrometry*, 304 (**2011**) 83-90
113. A. Wakamatsu, K. Morimoto, M. Shimizu, and S. Kudoh. **A severe peak tailing of phosphate compounds caused by interaction with stainless steel used for liquid chromatography and electrospray mass spectrometry**. *Journal of Separation Science*, 28 (**2005**) 1823-1830
114. Y. Asakawa, N. Tokida, C. Ozawa, M. Ishiba, O. Tagaya, and N. Asakawa. **Suppression effects of carbonate on the interaction between stainless steel and phosphate groups of phosphate compounds in high-performance liquid chromatography and electrospray ionization mass spectrometry**. *Journal of Chromatography A*, 1198–1199 (**2008**) 80-86
115. J. Urban, V. Škeříková, P. Jandera, R. Kubíčková, and M. Pospíšilová. **Preparation and characterization of polymethacrylate monolithic capillary columns with dual hydrophilic interaction reversed-phase retention mechanism for polar compounds**. *Journal of Separation Science*, 32 (**2009**) 2530-2543
116. S. Louw, F. Lynen, M. Hanna-Brown, and P. Sandra. **High-efficiency hydrophilic interaction chromatography by coupling 25cm× 4.6 mm ID× 5µm silica columns and operation at 80° C**. *Journal of Chromatography A*, 1217 (**2010**) 514-521
117. A.J. Alpert. **Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides**. *Analytical Chemistry*, 80 (**2008**) 62-76

118. C.V. McNeff, B. Yan, D.R. Stoll, and R.A. Henry. **Practice and theory of high temperature liquid chromatography**. *Journal of Separation Science*, 30 (**2007**) 1672-1685
119. G. Shi, Y. Li, K. Gallagher, T. Emm, T. Olah and S. Unger. **Novel direct detection method for quantitative determination of intracellular nucleoside triphosphates using weak anion exchange liquid chromatography/tandem mass spectrometry**. *Rapid Communications in Mass Spectrometry*, 16 (**2002**) 1092-1099
120. R.L. Cordell, S.J. Hill, C.A. Ortori, and D.A. Barrett. **Quantitative profiling of nucleotides and related phosphate-containing metabolites in cultured mammalian cells by liquid chromatography tandem electrospray mass spectrometry**. *Journal of Chromatography B*, 871 (**2008**) 115-124
121. H. Malerod, M. Rogeberg, N. Tanaka, T. Greibrokk, and E. Lundanes. **Large volume injection of aqueous peptide samples on a monolithic silica based zwitterionic-hydrophilic interaction liquid chromatography system for characterization of posttranslational modifications**. *Journal of Chromatography A*, 1317 (**2013**) 129-137
122. L.R. Bushman, J.J. Kiser, J.E. Rower, B. Klein, M.L. Ray and P.L. Anderson. **Determination of nucleoside analog mono-, di-, and tri-phosphates in cellular matrix by solid phase extraction and ultra-sensitive LC–MS/MS detection**. *Journal of Pharmaceutical and Biomedical Analysis*, 56 (**2011**) 390-401
123. I. Odsbu and K. Skarstad. **A reduction in ribonucleotide reductase activity slows down the chromosome replication fork but does not change its localization**. *PloS One*, 4 (**2009**) e7617
124. K.J. Fountain, J. Xu, D.M. Diehl, and D. Morrison. **Influence of stationary phase chemistry and mobile-phase composition on retention, selectivity, and MS response in hydrophilic interaction chromatography**. *Journal of Separation Science*, 33 (**2010**) 740-751
125. R. Kalluru, F. Fenaroli, M.P. Madsen, S.R. Wilson, H. Roberg-Larsen, G.K. Khuller and G. Griffiths. **Poly (lactide-co-glycolide)-rifampicin nanoparticles efficiently clear Mycobacterium bovis BCG infection in macrophages and remain membrane-bound in phago-lysosomes**. *Journal of Cell Science*, 126 (**2013**) 3043-3054
126. S.D. Lawn and A.I. Zumla. **Tuberculosis**. *The Lancet*, 378 57-72
127. D. Vu, R.A. Koster, M.S. Bolhuis, R.V. Altena, D.R.A Uges and J.W.C. Alffenaar. **Simultaneous determination of rifampicin, clarithromycin and their metabolites in dried blood spots using LC–MS/MS**. *Talanta*, 121 (**2014**) 9-17
128. R.C. Hartkoorn, S. Khoo, D.J. Back, J.F. Tjia, A. Ardrey, S. Ashleigh and S.A. Ward. **A rapid and sensitive HPLC–MS method for the detection of plasma and cellular rifampicin**. *Journal of Chromatography B*, 857 (**2007**) 76-82
129. L. Amaral and M. Viveiros. **Why thioridazine in combination with antibiotics cures extensively drug-resistant Mycobacterium tuberculosis infections**. *International Journal of Antimicrobial Agents*, 39 (**2012**) 376-380
130. F. Fenaroli, D. Westmoreland, J. Benjamisen, T. Kolstad, F. M. Skjeldal, L. Ulanova, J. Hildahl and G. Griffiths. **Nanoparticles as drug delivery system against tuberculosis in zebrafish embryos: direct visualization and treatment**. *ACS Nano*, 8 (**2014**) 7014-7026
131. S. Oswald, J. Peters, M. Venner, and W. Siegmund. **LC–MS/MS method for the simultaneous determination of clarithromycin, rifampicin and their main metabolites in horse plasma, epithelial lining fluid and broncho-alveolar cells**. *Journal of Pharmaceutical and Biomedical Analysis*, 55 (**2011**) 194-201
132. D.H. Vu, R.A. Koster, A.M.A. Wessels, B. Greijdanus, J.W.C. Alffenaar, and D.R.A. Uges. **Troubleshooting carry-over of LC–MS/MS method for rifampicin, clarithromycin and metabolites in human plasma**. *Journal of Chromatography B*, 917–918 (**2013**) 1-4
133. A. Srivastava and S. Ward. **Development and validation of a highly sensitive LC-MS/MS assay for determination of rifampicin in human plasma: application to clinical pharmacokinetic study.** *British Journal of Clinical Pharmacology* (**2012**)
134. A. Srivastava, D. Waterhouse, A. Ardrey, and S.A. Ward. **Quantification of rifampicin in human plasma and cerebrospinal fluid by a highly sensitive and rapid liquid chromatographic–tandem mass spectrometric method**. *Journal of Pharmaceutical and Biomedical Analysis*, 70 (**2012**) 523-528
135. F. de Velde, J.-W.C. Alffenaar, A.M.A. Wessels, B. Greijdanus, and D.R. Uges. **Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by**

liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, 877 (**2009**) 1771-1777

136. D. Montenarh, M. Hopf, H.H. Maurer, P. Schmidt, and A.H. Ewald. **Development and validation of a multi-analyte LC-MS/MS approach for quantification of neuroleptics in whole blood, plasma, and serum**. *Drug Testing and Analysis* (**2015**)
137. V. Viette, D. Guillarme, R. Mylonas, Y. Mauron, M. Fathi, S. Rudaz, D. Hochstrasser and J.-L. Veuthey. **A multi-target screening analysis in human plasma using fast liquid chromatography–hybrid tandem mass spectrometry (Part I)**. *Clinical Biochemistry*, 44 (**2011**) 32-44
138. J. Sarathy, V. Dartois, T. Dick, and M. Gengenbacher. **Reduced drug uptake in phenotypically resistant nutrient-starved nonreplicating Mycobacterium tuberculosis**. *Antimicrobial Agents and Chemotherapy*, 57 (**2013**) 1648-1653
139. A.L. Allanson, M.M. Cotton, J.N.A. Tettey, and A.C. Boyter. **Determination of rifampicin in human plasma and blood spots by high performance liquid chromatography with UV detection: A potential method for therapeutic drug monitoring**. *Journal of Pharmaceutical and Biomedical Analysis*, 44 (**2007**) 963-969
140. K. Morand, G. Talbo, and M. Mann. **Oxidation of peptides during electrospray ionization**. *Rapid Communications in Mass Spectrometry*, 7 (**1993**) 738-743
141. S. Plattner, R. Erb, J.-P. Chervet, and H. Oberacher. **Ascorbic acid for homogenous redox buffering in electrospray ionization–mass spectrometry**. *Analytical and Bioanalytical Chemistry*, 404 (**2012**) 1571-1579
142. X. Xu, C.R. Sabanayagam, D.A. Harrington, M.C. Farach-Carson, and X. Jia. **A hydrogel-based tumor model for the evaluation of nanoparticle-based cancer therapeutics**. *Biomaterials*, 35 (**2014**) 3319-3330
143. C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, and R. Grant. **Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry**. *Journal of Chromatography B*, 785 (**2003**) 263-275
144. B. Wei, Q. Li, R. Fan, D. Su, X. Chen, Y. Jia and K. Bi. **Determination of monoamine and amino acid neurotransmitters and their metabolites in rat brain samples by UFLC–MS/MS for the study of the sedative-hypnotic effects observed during treatment with S. chinensis**. *Journal of Pharmaceutical and Biomedical Analysis*, 88 (**2014**) 416-422
145. X. Zhang, A. Rauch, H. Lee, H. Xiao, G. Rainer, and N.K. Logothetis. **Capillary hydrophilic interaction chromatography/mass spectrometry for simultaneous determination of multiple neurotransmitters in primate cerebral cortex**. *Rapid Communications in Mass Spectrometry*, 21 (**2007**) 3621-3628
146. S. Tufi, M. Lamoree, J. de Boer, and P. Leonards. **Simultaneous analysis of multiple neurotransmitters by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry**. *Journal of Chromatography A*, 1395 (**2015**) 79-87
147. I.D. Neumann, R. Maloumby, D.I. Beiderbeck, M. Lukas, and R. Landgraf. **Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice**. *Psychoneuroendocrinology*, 38 (**2013**) 1985-1993
148. Y.-B. Tang, F. Sun, L. Teng, W.-B. Li, L. Zhu and H.-Z. Chen. **Simultaneous determination of the repertoire of classical neurotransmitters released from embryonal carcinoma stem cells using online microdialysis coupled with hydrophilic interaction chromatography–tandem mass spectrometry**. *Analytica Chimica Acta*, 849 (**2014**) 70-79
149. M. Perry, Q. Li, and R.T. Kennedy. **Review of recent advances in analytical techniques for the determination of neurotransmitters**. *Analytica Chimica Acta*, 653 (**2009**) 1-22
150. F. Gosetti, E. Mazzucco, M.C. Gennaro, and E. Marengo. **Simultaneous determination of sixteen underivatized biogenic amines in human urine by HPLC-MS/MS**. *Analytical and Bioanalytical Chemistry*, 405 (**2013**) 907-916
151. J. Lee, B.X. Huang, Z. Yuan, and H.-Y. Kim. **Simultaneous determination of salsolinol enantiomers and dopamine in human plasma and cerebrospinal fluid by chemical derivatization coupled to chiral liquid chromatography/electrospray ionization-tandem mass spectrometry**. *Analytical Chemistry*, 79 (**2007**) 9166-9173
152. V. Carrera, E. Sabater, E. Vilanova, and M.A. Sogorb. **A simple and rapid HPLC–MS method for the simultaneous determination of epinephrine, norepinephrine, dopamine and 5-**

hydroxytryptamine: Application to the secretion of bovine chromaffin cell cultures. *Journal of Chromatography B*, 847 (**2007**) 88-94

153. E. Tareke, J.F. Bowyer, and D.R. Doerge. **Quantification of rat brain neurotransmitters and metabolites using liquid chromatography/electrospray tandem mass spectrometry and comparison with liquid chromatography/electrochemical detection**. *Rapid Communications in Mass Spectrometry*, 21 (**2007**) 3898-3904
154. Q. Gu, X. Shi, P. Yin, P. Gao, X. Lu, and G. Xu. **Analysis of catecholamines and their metabolites in adrenal gland by liquid chromatography tandem mass spectrometry**. *Analytica Chimica Acta*, 609 (**2008**) 192-200
155. B.T. Røen, S.R. Sellevåg, and E. Lundanes. **On-line solid phase extraction-liquid chromatography–mass spectrometry for trace determination of nerve agent degradation products in water samples**. *Analytica Chimica Acta*, 761 (**2013**) 109-116
156. G.J.V.B. Kertesz and Vilmos. **Using the electrochemistry of the electrospray ion source**. *Analytical Chemistry*, 79 (**2007**) 5510-5520
157. H. Røberg-Larsen. **Determination of oxysterols in cancer stem cells using on-line automated filtration and filter-flush solid phase extraction liquid chromatography tandem mass spectrometry**. (**2011**)
158. D.S. Domingues, E.J. Crevelin, L.A.B. Moraes, J.E. Cecilio Hallak, J.A. Souza Crippa, and M.E. Costa Queiroz. **Simultaneous determination of amino acids and neurotransmitters in plasma samples from schizophrenic patients by hydrophilic interaction liquid chromatography with tandem mass spectrometry**. *Journal of Separation Science*, 38 (**2015**) 780-787
159. G. Zhang, Y. Zhang, D.M. Fast, Z. Lin, and R. Steenwyk. **Ultra sensitive quantitation of endogenous oxytocin in rat and human plasma using a two-dimensional liquid chromatography–tandem mass spectrometry assay**. *Analytical Biochemistry*, 416 (**2011**) 45-52
160. Y. Hsieh. **Potential of HILIC-MS in quantitative bioanalysis of drugs and drug metabolites**. *Journal of Separation Science*, 31 (**2008**) 1481-1491
161. R. Zhang, D.G. Watson, L. Wang, G.D. Westrop, G.H. Coombs, and T. Zhang. **Evaluation of mobile phase characteristics on three zwitterionic columns in hydrophilic interaction liquid chromatography mode for liquid chromatography-high resolution mass spectrometry based untargeted metabolite profiling of Leishmania parasites**. *Journal of Chromatography A*, 1362 (**2014**) 168-179
162. O.S. Mabrouk and R.T. Kennedy. **Simultaneous oxytocin and arg-vasopressin measurements in microdialysates using capillary liquid chromatography–mass spectrometry**. *Journal of Neuroscience Methods*, 209 (**2012**) 127-133
163. M.E. McCullough, P.S. Churchland, and A.J. Mendez. **Problems with measuring peripheral oxytocin: can the data on oxytocin and human behavior be trusted?** *Neuroscience & Biobehavioral Reviews*, 37 (**2013**) 1485-1492
164. H.E. Ross and L.J. Young. **Oxytocin and the neural mechanisms regulating social cognition and affiliative behavior**. *Frontiers in Neuroendocrinology*, 30 (**2009**) 534-547
165. C.M. Karbiwnyk, K.C. Faul, S.B. Turnipseed, W.C. Andersen, and K.E. Miller. **Determination of oxytocin in a dilute IV solution by LC–MS n**. *Journal of Pharmaceutical and Biomedical Analysis*, 48 (**2008**) 672-677
166. D. Carson, S.W. Berquist, S.H. Cheshier, S. Partap, A.Y. Hardan and K.J. Parker. **Cerebrospinal fluid and plasma oxytocin concentrations are positively correlated and negatively predict anxiety in children**. *Molecular Psychiatry*, 20 (**2015**) 1085-1090
167. C. Modahl, L.A. Green, D. Fein, M. Morris, C. Feinstein and H. Levin. **Plasma oxytocin levels in autistic children**. *Biological Psychiatry*, 43 (**1998**) 270-277
168. D. Feifel, K. Macdonald, A. Nguyen, P. Cobb, H. Warlan, M. Lefebvre, J. Gonzales and A. Hadley. **Adjunctive intranasal oxytocin reduces symptoms in schizophrenia patients**. *Biological Psychiatry*, 68 (**2010**) 678-680
169. C. Wotjak, J. Ganster, G. Kohl, F. Holsboer, R. Landgraf, and M. Engelmann. **Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons**. *Neuroscience*, 85 (**1998**) 1209-1222
170. G. Leng and M. Ludwig. **Intranasal oxytocin: myths and delusions**. *Biological Psychiatry*, 79 (**2016**) 243-250

171. K. Miyamoto, T. Hara, C. Yang, T. Ikegami, H. Takubo and N. Tanaka. **High-efficiency liquid chromatographic separation utilizing long monolithic silica capillary columns**. *Analytical Chemistry*, 80 (**2008**) 8741-8750
172. A.I. Mehta, S. Ross, M.S. Lowenthal, V. Fusaro, D.A. Fishman and L.A. Liotta. **Biomarker amplification by serum carrier protein binding**. *Disease Markers*, 19 (**2003**) 1-10
173. C. Avanti, W.L.J. Hinrichs, A. Casini, A.C. Eissens, H.W. Frijlink and H.P. Permentier. **The formation of oxytocin dimers is suppressed by the zinc–aspartate–oxytocin complex**. *Journal of Pharmaceutical Sciences*, 102 (**2013**) 1734-1741
174. H. Fraenkel-Conrat, A. Mohammad, E. Ducay, and D. Mecham. **The molecular weight of lysozyme after reduction and alkylation of the disulfide bonds**. *Journal of the American Chemical Society*, 73 (**1951**) 625-627
175. J.C. Christensen, P.A. Shiyanov, J.R. Estepp, and J.J. Schlager. **Lack of association between human plasma oxytocin and interpersonal trust in a prisoner's dilemma paradigm**. *PloS One*, 9 (**2014**) e116172
176. W.L. Martin, **Measurement of oxytocin and vasopressin**, *patent application PCT/US2014/044467* (**2014**)
177. S.M. Bryson. **Clinical Pharmacokinetics: Concepts and applications**. *Journal of Clinical Pharmacy and Therapeutics*, 8 (**1983**) 87-88