High-Throughput Profiling of Small Molecules using Mass Spectrometry

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High-throughput profiling of small molecules using mass spectrometry
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Chapter 1

General Introduction

GENERAL INTRODUCTION

Phenotyping based on profiling of small molecules

Phenotyping based on molecular profiling is a type of screening which investigates the phenotype of a biological system such as cells, tissues or whole organisms. The phenotype is a description of the total of physical characteristics of a biological system including its morphology, development, but also its biochemical properties. Profiling the phenotype is interesting since phenotypic effects can be induced by various (biological) perturbations such as a disease, drug interventions and genetic alterations. Phenotyping based on the profiling of small molecules such as metabolites can provide important insights into e.g. disease and the effect of drugs, as well as a systems understanding of the ability to adapt and cope with a perturbation[1].

For the profiling of small molecules there are different techniques available. There is often an inverse relation between assay comprehensiveness (analyte coverage) and the throughput of the assay (see Figure 1). Whereas a single analyte high-throughput screen (HTS), such as microscopy assays, enzyme-linked immunosorbent essays (ELISA) or fluorescence-activated cell sorting (FACS) can screen over a million compounds per week, comprehensive mass spectrometry (MS)-based assays which can potentially screen for hundreds of analytes, are generally much slower [2]. As a consequence, comprehensive profiling assays have the potential to provide more in-depth information about the phenotype of a specific biological system than a single read-out biochemical assay. Therefore, in choosing the appropriate profiling technology, the balance between assay throughput and analytical comprehensiveness should be considered carefully with probably in many cases preferably maximal depth at high sample throughput.

Many conventional HTS approaches rely on fluorescent- or radio-labeling since these labels can be rapidly detected with high sensitivity [3]. Label-free strategies, including MS, have gained wide interest, since they can be applied to a broader range of assays and target classes. Moreover, MS-based screening is a comprehensive, selective and sensitive analytical tool which is not susceptible to the limitations imposed by labelling and coupling enzymes[4]. As a consequence, profiling of small molecules by MS has the potential to be a very powerfull technique.

Mass Spectrometry

MS is a key analytical tool for detecting and identifying small (bio) molecules. Therefore in many bio-analytical applications MS has become a powerful quantitative and qualitative analytical technique. A MS comprises i) an ion source, where gas-phase ions (positively- or negatively charged) are formed, ii) a mass analyser for separation of ions based on their mass-to-charge ratio (m/z) and iii) a detector, where arriving ions are detected. A variety of different MS instruments are available, including high resolution (HR) MS such as fourier transform ion cyclotron resonance (FTICR), Orbitrap FT and time-of-flight (TOF) and low resolution MS such as ion traps and triple quadrupole systems. Among several available ionization techniques such as electron impact (EI), desorption electrospray ionization (DESI) and matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are predominantly used. MALDI and DESI techniques have recently attracted interest in the field of imaging MS (IMS). IMS involves comprehensive profiling of biological molecules by directly 'scanning' the analytes from a cell or tissue surface, without sample homogenization or extraction, thus maintaining the spatial

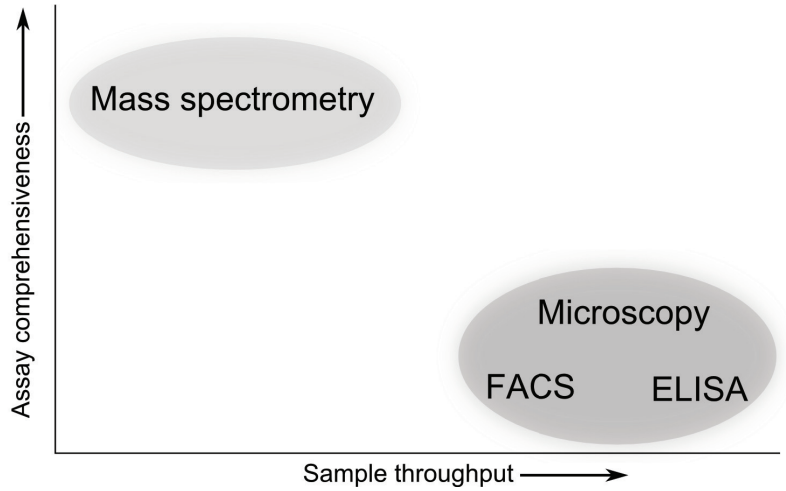

Figure 1: Schematic picture representing the comprehensiveness of the small molecule assay versus the sample throughput of the assay. Assays with potential hundreds of read-out variables such as MS are often much slower than other single variable read-out assays, such as microscopy assays, enzyme-linked immunosorbent assays (ELISA) or fluorescence-activated cell sorting (FACS).

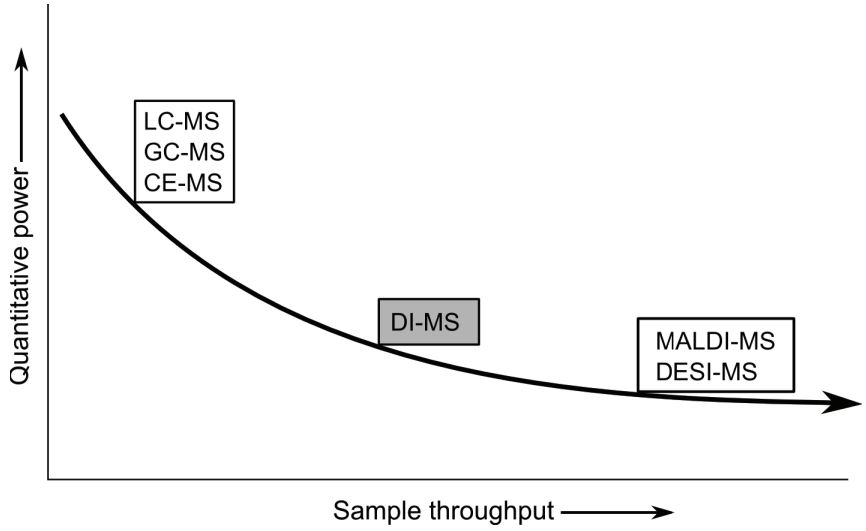

Figure 2: Schematic picture depicting the inherent trade-off between the quantitative power of a MS-based platform and its sample throughput.

distribution patterns of the analytes. IMS techniques can in principle be very fast, especially when only one spot per sample needs to be analyzed. Moreover, when employing e.g. DESI, limited or even no sample pretreatment is necessary. As a consequence, IMS has become a rapid and valuable tool across many fields, yet its major challenge is to acquire quantitative data regarding the surface concentrations of the analytes[5].

The developments in (HR)MS instruments have drastically increased ion selectivity for obtaining high resolution and moreover, enabled the detection of minute amounts of analyte (femtograms)[6]. However, one of the disadvantages of a MS is its limited dynamic range which is at best five orders of magnitude. More importantly, when using ESI, potential analyte competition during ionisation in the ion source, called ion suppression, imposes challenges in order to obtain quantitative results[7]. The same is true for most other ionization techniques. As a consequence, prior to MS detection, several separation techniques are often employed such as liquid chromatography (LC)-MS, gas chromatography (GC)-MS or capillary electrophoresis (CE)-MS in order to reduce the amount of potential co-eluting ions in the ion source and therefore reduce potential ion suppression effects. These hyphenated platforms can provide quantitative data, however at the expense of sample throughput (Figure 2).

Direct infusion-MS (DI-MS) is a fast and comprehensive analytical method in which a sample is introduced into the MS without prior separation, possibly after sample pretreatment. In DI-MS, samples can be introduced into the MS in several ways: (i) manually, by means of a syringe pump, or (ii) automated. Flow injection analysis-MS is a commonly used automated DI-MS approach which involves the injection of an analyte plug (typically a few microliters) into a flowing solvent stream by employing e.g. a LC autosampler and pump, omitting a chromatographic column[8]. Additionally, automated nanoESI robots are available which can directly introduce a sample into the MS through a small nanoESI microchip-nozzle (typically 5 μm), in a 'carryover-free' manner[9]. However, although DI-MS can be fast and enables high-throughput analysis, it is often prone to ion suppression due to co-eluting ions such as (endogenous) salts and it is often not possible to differentiate between isomers. By employing nanoelectrospray (nanoESI) instead of conventional ESI, ionization efficiency can be increased and ion suppression can be reduced [10]. As a consequence, effective sample pretreatment is a key step prior to DI-MS analysis in order to obtain quantitative data[6]. When samples are effectively purified and the extracted analyte is within the dynamic range of the MS, analytical performance of DI-MS should be comparable with hyphenated MS platforms. As discussed, MALDI and DESI-MS are in principle faster per sample point than DI-MS as they can faster acquire MS spectra per sample. When e.g. using chip-based nanoESI-DI-MS, after every sample the nozzle has to be changed in order to prevent carry over, which will usually take a few seconds.

Sample pretreatment

A typical MS-based analytical workflow comprises sampling, sample pretreatment and MS analysis. Many MS-based methods require proper sample pretreatment procedures in order to overcome the mismatch of the sample with the analytical method. The sample pretreatment procedure should allow the reproducible enrichment/extraction of the analytes so that the concentrations are within the linear range of the MS. Moreover, depending on the analytical MS-platform, interfering molecules such as proteins and salts should be removed in order to maintain the integrity of the system and to reduce ion suppression and poor detection sensitivity. Furthermore, sample pretreatment is required to release the analytes of interest from the sample matrix and transfer the analytes into a medium or form compatible for the

subsequent analysis with the analytical instrument. However, MS-based methods have the drawback that they often require tedious protocols for sample pretreatment that decrease the throughput of the assay. Therefore, to achieve high-throughput automated and integrated analytical systems have to be developed which include the sample pretreatment procedure. Although these goals can partly be met by traditional methods, new concepts are required which simplify sample pretreatment and maintain robustness and a high-throughput capacity. Additionally, when sample volume is limited, miniaturization of the sampling as well as the sample pretreatment procedure is needed which can also potentially lead to the concept of massive parallelization.

Metabolomics

The metabolome is the whole set of small molecules (typically <1000 Da) in a given biological sample. It can provide a functional readout of cellular biochemistry and therefore has the strongest correlation with the phenotype[1]. Therefore, metabolomics offers a promising phenotyping platform which has the potential to e.g. discover biomarkers for the diagnosis and prognosis of diseases and the prediction of the efficacy of drug interventions[11] [12]. Metabolites are a highly diverse range of molecules with vast differences in chemical structure, physicochemical properties and dynamic range (up to nine decades[13][14]). As a consequence, metabolomics may be the most challenging phenotyping omics science. Whereas genomics and transcriptomics can amplify DNA sequences using polymerase chain reaction (PCR), in metabolomics there is no effective method which can 'amplify' all low-abundant metabolites in an unbiased manner.

The metabolome can be considered as to the largest part a regulated dynamic phenotype in which changes in metabolite levels can e.g. reflect a transition from a healthy to a diseased state or the resilience of a system, in the context of systems homeostasis and allostasis. For the discovery of metabolic profiles to predict disease progression or the effect of interventions at an individual level, performing longitudinal studies is key in monitoring individuals which may result in personalized biology and eventually personalized medicine. For this strategy, following individuals over years will require high-throughput metabolomics approaches[1]. In addition, due to the vast biological variability of individuals, many individual samples may be analysed in a metabolomics study in order to ensure statistically meaningful results. As a consequence, such metabolomics studies should be performed preferably in a HT manner. In addition, each study should not only be carried out to analyse more samples within a period of time, but also to quantify as many relevant metabolites as possible in a single analysis, for which MS is in principle very suitable[15].

Aim and outline of the thesis

The aim of this thesis is to develop new methods for the mass spectrometry (MS)-based high-throughput profiling of small molecules. Direct infusion (DI)-MS can potentially provide high-content and high-throughput analyses, but effective and rapid sample pretreatment procedures are needed in order to obtain enough quantitative performance, i.e. precision and accuracy, in the mass spectrometric analysis while maintaining assay throughput. Moreover, when the analysis of small sample volumes is required, new miniaturized concepts in sampling

as well as sample pretreatment are needed. In addition, miniaturization of the experimental setup can play an important aspect with regards to the potential of massive parallelization and, consequently, can tremendously increase sample throughput.

In **Chapter 2** an overview of recent developments in sample pretreatment procedures for MS-based metabolomics is given. Deproteinization, removal of interfering molecules, liquid-liquid extraction (LLE), solid-phase extraction (SPE), electromigration-based extraction methods and possibly emerging sample pretreatment techniques for metabolomics are described and discussed. Advantages and limitations of these techniques for metabolomics are given, and aspects such as potential for automation and high-throughput analysis are evaluated

In **Chapter 3** the potential of nanoESI-DI-High Resolution-MS for the metabolic phenotyping of early zebrafish embryogenesis is investigated. It is studied whether efficient but limited sample pretreatment is sufficient to obtain metabolic profiles to distinguish early developmental stages during zebrafish embryos embryogenesis. Reproducible and high-quality MS data is generated by implementing an automated data-processing tool, which includes data clean-up steps and a dedicated normalization-optimization algorithm. Principle component analysis reveals that periods of 1 hour time shifts post fertilization can be differentiated from each other.

In **Chapter 4**, a new and fast and selective electromigration-based sample enrichment and purification technique called 3-phase electroextraction (3-phase EE) is presented and coupled to nanoESI-DI-MS. The electromigration of analytes from an aqueous sample through an immiscible organic filter phase into a small-volume aqueous acceptor phase is demonstrated. It is shown that selectivity of 3-phase EE can be tuned by changing the composition of the organic filter phase which also prevents proteins from migrating into the acceptor phase. Proof of principle towards online 3-phase EE-nanoESI-DI-MS is demonstrated and thus the compatibility of 3-phase EE with HTS is shown.

In **Chapter 5** a proof of principle of a new miniaturized LLE technique in a 384-well plate is presented, based on gas pressure mixing followed by passive phase separation. This fully-automated approach is integrated online into a nanoESI-DI-MS robot. It is shown that this high-throughput platform is suitable for screening drugs from human plasma with similar or better analytical performance compared to a conventional LLE procedure. Finally, the micro LLE method applied to dried blood spots is demonstrated.

In **Chapter 6**, a miniaturized sampling technique based on microneedle sampling is developed in order to monitor drug uptake in the (small-volume) yolk of zebrafish larvae using MS analysis. It is demonstrated that conventional whole zebrafish embryo lysis is not suitable for measuring drug uptake, due to potential adherence of drugs to the skin. The *in vivo* uptake MS-data is correlated to the results of *in vitro* and *in vivo* high-throughput drug screening platforms and it was shown that this new approach can give relevant information for interpretation of drug efficacy data.

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Chapter 2

Recent Developments in Sample Pretreatment Techniques for Mass Spectrometry-based Metabolomics

ABSTRACT

Metabolomics is one of the most rapidly evolving of the omics sciences. For phenotyping individuals, it offers a promising platform that is needed for breakthroughs in biomedical and pharmacological research. A proper sample-pretreatment procedure is required for metabolomics studies to overcome the mismatch of the sample with the analytical method. Within the analytical workflow for metabolomics studies, sample pretreatment is important in order to obtain the meaningful, high-quality quantitative data needed to minimize inconsistencies between laboratories. In this review, we provide an overview of state-of-the-art sample-pretreatment techniques for mass spectrometry-based metabolomics. We pay attention to deproteinization, removal of interfering molecules, liquid-liquid extraction, solid-phase extraction, electromigration-based extraction methods and possibly emerging sample-pretreatment techniques for metabolomics. We give the advantages and the limitations of these techniques for metabolomics, and consider aspects such as automation and high-throughput analysis. Finally, we provide some conclusions and perspectives.

Based on

R.J. Raterink, P. W. Lindenburg, R. J. Vreeken, R. Ramautar and T. Hankemeier, *"Recent developments in sample pretreatment techniques for mass spectrometry-based metabolomics"*

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INTRODUCTION

Metabolites are key regulators of system homeostasis and provide a functional readout of cellular biochemistry. Metabolomics can be defined as a comprehensive analytical approach for the study of the complete set of metabolites (metabolome), present in a biological system[1]. Interest in metabolomics is rapidly expanding in various research fields, such as cancer research[2], drug discovery and development[3], toxicological, biomedical and clinical research and nutritional research. The major aim of metabolomics is to obtain an answer or insight to a biological question. An analytical workflow comprised of experimental design, sampling, sample pretreatment, data acquisition and data analysis is generally used to address a biological question by a metabolomics approach (Figure 1).

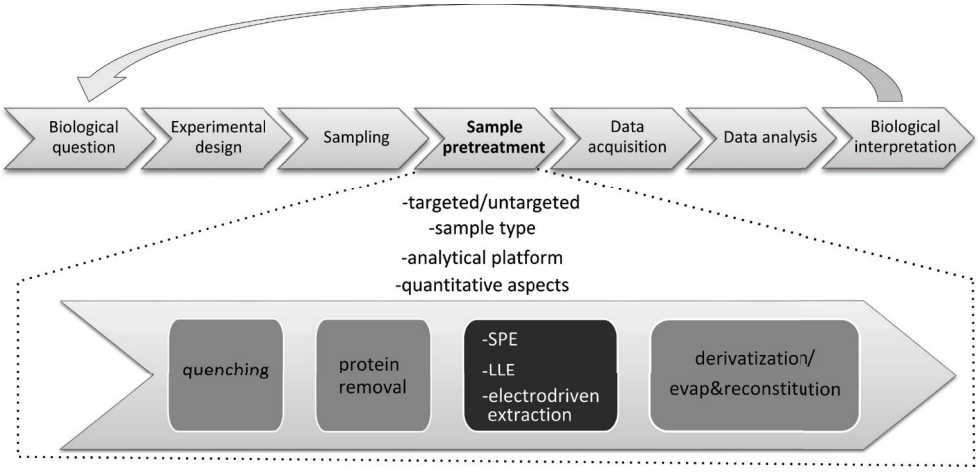

Figure 1: Schematic diagram of a typical analytical workflow for metabolomics studies.

Within the analytical workflow for metabolomics studies, sample pretreatment is a key step as it has a major influence on both the overall coverage and quality of the obtained metabolic profiles and biological interpretation of the data. As such, it may be considered the most error-prone and time-consuming step of metabolomics studies and is maybe the major source of inconsistencies between laboratories[4].

Although technical advances have been made in the fields of bioanalysis and metabolomics in terms of automation and high-throughput analysis, a significant part of the sample pretreatment procedure is still carried out manually[5]. The main challenges of sample pretreatment in metabolomics is the great diversity of chemical structures and physicochemical properties as well as vast differences in dynamic range (up to 9 decades) of the metabolites present in a biological sample[6],[7] and at the same time the lack of universal analytical comprehensive techniques with a sufficient dynamic range and physicochemical coverage. To ensure that the measured metabolic profiles are representative, the sample pretreatment procedures used should allow the reproducible enrichment of all metabolites of interest, and remove those compounds that interfere with the subsequent analytical method in order to preserve the integrity of the analytical system. Moreover, MS analysis can be disturbed by interfering, co-eluting matrix components in the samples resulting in ion suppression effects, especially when using electrospray ionization (ESI)[8]. Sample pretreatment is also required to release metabolites from the sample matrix and transport the analytes to a medium compatible with the analytical instrument. Therefore, there is an urgent need for the development of

reproducible, standardized and quantitative sample pretreatment approaches for biological samples critical for large-scale metabolomics studies and for minimizing inter-laboratory inconsistencies[9].

The purpose of this review is to present the state-of-the-art sample pretreatment methodologies for MS-based metabolomics, not focusing on lipodomics as this is covered including sample pretreatment in another review article by Fiehn *et al*. in this issue of Trends in Analytical Chemistry. In addition, some of the new emerging sample pretreatment techniques, which have not yet been fully evaluated for metabolomics studies, will also be covered. Recent developments in sample pretreatment techniques such as protein removal, liquid-liquid extraction (LLE), solid-phase extraction (SPE) and electromigration-based extraction will be highlighted, and aspects such as automation and high-throughput analysis will be considered. Subsequently, sample pretreatment issues relevant for obtaining reliable quantitative metabolomics data are shortly discussed. Finally, some general conclusions and perspectives are given. As plants represent a particular complex case as plant cell walls are difficult to break and they comprise special biochemical classes, sample pretreatment procedures for plant metabolomics studies are not discussed in this review.

SAMPLE PRETREATMENT FOR METABOLOMICS: CRITICAL ASPECTS

Sample pretreatment is an essential part of a metabolomics study as it has a major effect on the metabolite coverage and the quality of the results obtained. Prior to sample pretreatment, experimental design and pre-analytical steps such as sample collection/storage and stabilization need to be carefully examined and chosen in each metabolomics study (Figure 1); however, these aspects are not covered in this review, but are discussed elsewhere [10]–[14]. Sample pretreatment strategies used in metabolomics prior to separation and detection can include ultrafiltration, protein precipitation, LLE, SPE, derivatization, and evaporation followed by reconstitution[15]. Sample pretreatment finally results in a fraction of a sample that can be analyzed by a subsequent analysis platform. In this section important aspects with regard to the choice of a sample pretreatment technique for metabolomics are considered. First, the difference between targeted and untargeted metabolomics is outlined, followed by a discussion concerning the influence of the sample type and the subsequent analysis platform on the sample pretreatment strategy.

General aspects: targeted versus untargeted metabolomics

In metabolomics, two different approaches may be distinguished, i.e. the targeted and the untargeted detection of metabolites. The targeted approach is focused on the quantitative analysis of preselected metabolites in a biological sample. Targeted metabolomics approaches are commonly driven by a specific biochemical hypothesis, typically focusing on one or more related pathways of interest[16][17]. The untargeted approach involves the profiling of biological samples without having a priori knowledge on the nature and the identity of the measured metabolites. The goal is to obtain qualitative and (semi)-quantitative information, which can be used to compare patterns or fingerprints of metabolites that change in response to normal and abnormal biological processes, genetic alterations and to external stimuli, for example drug exposure[18]. As a consequence, untargeted metabolomics can be used to reveal the involvement of metabolic pathways that may not have been predicted and is therefore often hypothesis-generating or hypothesis-refining.

With regard to sample pretreatment, the analytical requirements for untargeted and targeted

metabolomics studies may be quite different. For untargeted metabolomics studies, the biological sample should preferably be analyzed with minimal pretreatment in order to prevent the potential loss of metabolites, or at least, within a certain wide biochemical window, metabolites should not be removed during pretreatment. The sample pretreatment steps can be quite straightforward, separating low molecular weight (LMW) compounds from proteins and/or lipids using deproteinization and/or delipidation techniques[15]. Therefore in untargeted metabolomics studies of biofluids, non-selective sample pretreatment methods such as "dilute-and-shoot" and solvent protein precipitation are often used, since they enable broad metabolite coverage and high-throughput analysis[18]. Untargeted metabolomics approaches can also be applied to a fractionated sample obtained after e.g., a LLE and/or SPE step.

In contrast to untargeted metabolomics studies, development and optimization of sample pretreatment procedures for targeted metabolomics are often relatively straightforward, as it is known a priori on which metabolite class(es) to focus. Sample pretreatment includes the separation of metabolites from proteins, often followed by LLE and/or off-line/online SPE for the selective isolation and enrichment of the target compounds, and removal of interfering matrix components.

Evaporation and reconstitution is often the final step during sample pretreatment in order to concentrate the analytes by reconstitution in a smaller volume and/or convert the solvent to an analysis-compatible solvent. During such a step, attention should be paid to analyte solubility, and potential oxidation of analytes such as thiol-containing molecules[19].

Sample type

The sample pretreatment strategy also largely depends on the nature of the sample to be analyzed. The protein content of biological samples is an important aspect to consider, as most subsequent analysis methods are not compatible with protein-rich samples such as, plasma, serum, cells and tissue. In contrast, urine, particularly from healthy human individuals, contains relatively low protein amounts, and centrifugation followed by dilution with water is often all that is required prior to analysis[18].

Metabolomics has at first mainly been applied to biofluids and cell samples, but in recent years also tissue samples or biopsies are becoming more studied with metabolomics[20]. For cell samples the quenching procedure including harvesting and washing is critical[21], however we do not cover that in this review. For tissue samples, sampling and homogenization without degradation of metabolites is critical and needs to be added to the sample pretreatment workflow as a first step. However, opposed to biofluids, limited research of (automated) sample pretreatment procedures has been performed for tissue metabolomics[15][22]. Several approaches for sample homogenization such as the manual disaggregation of cold tissue with scissors or a manual homogenizer, mechanical disruption using a bead homogenizer and a cryogenically cooled pestle and mortar on frozen tissue, have been used and are described elsewhere[23], but are beyond the scope of this review. Overall, the choice of solvent seem to have more influence on metabolite coverage than the sample homogenization method[24].

Dried blood spots (DBS) is a sample type which has been used for the screening of inborn errors of metabolism for decades now, and recently DBS samples received increased interest for metabolomics studies[25]. In DBS, blood or biofluid samples are collected on special types of absorbent paper which are then dried, and as such ready for subsequent extraction and analysis. This simple and inexpensive method is easy to automate and is able to store only a few microliters of sample. In addition, it offers a convenient way of storage and

transportation at ambient conditions. Therefore, the use of DBS may have advantages for large-scale epidemiological studies where the pretreatment of blood and the subsequent storage and transport poses logistical challenges. So far only a few studies on untargeted metabolomics have been performed with the use of DBS. Analyte coverage for DBS seem to be comparable with solvent precipitation of whole blood and plasma[25]–[27]. Moreover, the result of stability studies seem to indicate that stability for most metabolites does not seem to be critical when analysis is done within a week of collection[25]. Lately, instrumental manufacturers have developed automated systems which can handle, extract and introduce the DBS extract into the analytical system, thus enabling the use of DBS for large-scale high-throughput profiling studies[28].However, some challenges related to DBS need to be examined, such as the influence of the hematocrit level and the non-uniform distribution of the blood spot on the sampling area[29].

Saliva is another readily available biofluid that may contain metabolites of interest for diagnosis and prognosis of diseases. Nevertheless, there are only a few reports concerning metabolomics. The major challenge in saliva metabolomics is the relatively low abundance of metabolites[30]–[32]. Therefore, in this field there is a need for more sensitive analytical tools which may include sample pretreatment methods that allow analyte enrichment.

Subsequent analytical platform

One major aim of metabolomics is to obtain a comprehensive view of the metabolites present in a biological sample. As no single analytical technique covers the entire spectrum of the human metabolome, various complementary analytical platforms (e.g. gas chromatography-mass spectrometry (GC-MS), liquid chromatography-MS (LC-MS), capillary electrophoresis-MS (CE-MS) and/or direct infusion-MS (DI-MS) should be employed in order to improve metabolite coverage and identification power[33]–[36]. Each of these analytical platforms has different requirements with regard to sample pretreatment. In general, deproteinization is required for each analytical platform, as the presence of proteins can seriously influence precision, accuracy and instrument component lifetime (e.g. LC and GC columns). Moreover, proteins can induce severe matrix effects, may cause clogging of nanoESI emitters and can modify the inner surface of capillaries in CE.

In GC-MS, sample pretreatment involves a number of steps generally including deproteinization, lyophilization and chemical derivatization. Chemical derivatization is often required to decrease the boiling point of many endogenous metabolites. There are a multitude of different chemical derivatization reagents used, although a two-stage process of oximation followed by trimethylsilylation is mostly applied[37]. However, the derivatization process may add more error-prone complex steps in the sample pretreatment method. Furthermore in general, derivatization can impose added requirements to the sample pretreatment procedure in order to clean up compounds that can interfere with the derivatization reactions.

In DI-MS, "dilute and shoot" after deproteinization is the most common approach, including optimization of the dilution factor in order to minimize ion suppression and detector saturation effects. Additionally, prior to DI-MS, a desalting step should be included, because salts can significantly contribute to ion suppression effects[38]. However, to date no general straightforward method is available which separates (endogenous) inorganic ions from the wide range of polar and often ionic metabolites.

SAMPLE PRETREATMENT TECHNIQUES

In this section recent developments in deproteinization techniques, LLE and SPE and emerging

electromigration-driven sample extraction will be discussed. Furthermore, throughout this section delipidation techniques will be discussed.

Protein removal

In metabolomics, the most commonly used methods for protein removal are organic solvent-based protein precipitation (PPT) followed by centrifugation, or membrane-based techniques such as ultrafiltration[4]. It is preferable to use an organic solvent for PPT, since protein denaturation using heat or inorganic acids have been shown to result in lower metabolite coverage[39] and the organic solvent also disrupts binding between many metabolites and proteins. Actually, the latter is also true for many metabolites using inorganic acids, and some inorganic acids extracts metabolites more efficient than organic solvents. However, organic solvent PPT is able to extract both hydrophilic and hydrophobic compounds. Therefore, further clean-up of the extracted samples is often required as the supernatant still contains many components such as lipids which may disturb subsequent MS analysis (i.e. ion suppression) and may reduce lifetime of e.g. LC columns. As a consequence, the combination of protein and lipid removal is often used as an effective strategy to improve metabolite coverage and reproducibility. Moreover, metabolite losses may occur due to co-precipitation with proteins and/or poor solubility in the selected extraction solvent[40].

Over the past few years, the performance of various PPT procedures for metabolomics have been evaluated in terms of protein removal efficiency, metabolite coverage, and precision[20], [37], [39], [41]–[44]. The diverging practices used in these studies reveal that there is still no general consensus on the best PPT procedure that should be used for metabolomics. However, some trends are apparent: precipitation with acetonitrile or acetone seems to perform better in terms of protein removal, whereas PPT with methanol, ethanol or a mixture of both results in improved metabolic coverage and method precision. Therefore, the latter solvents are more often applied[28][15].

In conventional solvent PPT however, it is costly to include centrifugation into robotic automation solutions. Recent innovations have become available overcoming the need of centrifugation after PPT. Surface-functionalized magnetic beads have been recently suggested for sample pretreatment in several fields, addressing large and small molecules. König *et al.* reported a serum protein removal method based on surface-modified MagSi magnetic beads. Proteins were denatured using a proprietary reagent followed by the immobilization of the protein-adsorbing magnetic beads, by applying a magnetic field[45]. Neither centrifugation nor application of pressure is required, but only a time-controlled application of a magnetic field making this technique promising for automated high-throughput applications.

Also recently, solutions have become available for automated solvent PPT in a well-plate format such as the Sirocco™ (Waters), Impact™ (Phenomenex) or HyperSep™ (Thermoscientific). Using these PPT plates the solvent precipitated sample is filtered through a well plate by using a vacuum or pressure. Cao *et al.* demonstrated the automated removal of proteins in 50 µL dog plasma followed by LC-MS analysis, using the Sirocco™ plate[46]. These available solutions could be promising for metabolomics studies, but have not been demonstrated yet. In section 3.3 other recently available well plates are discussed which simultaneously can remove proteins and lipids from a complex sample.

Other protein removing treatments have been developed that do not require a PPT or ultrafiltration step. Turbulent flow chromatography (TFC) is such a promising online technique which enables the direct injection of crude biological samples onto a column packed with large particles (25-50 µm). These large particles can have an additional level of selectivity

using stationary phase functionality. After the sample is injected into a turbo flow column the high flow-rate of 1.5-5.0 mL/min generates turbulent flow conditions inside the column. Small analyte molecules are retained via diffusion into the pores, while proteins are washed to waste. In 2010, Michopoulos *et al.* demonstrated the use of TFC for the protein removal in a metabolomics study of plasma, followed by LC-MS analysis. A comparison between TFC and methanol-based PPT was made and resulted in similar numbers of molecular features (2900), with a somewhat poorer repeatability for TFC. TFC reduced the concentration of phospholipids 10-60 fold, probably because the protein binding compounds (such as certain lipids) were washed away[47]. The principle of TFC has shown potential for the direct analysis of crude serum samples in automated, high-throughput metabolomics studies, however at the price of very high flow-rates and substantial consumption of solvents.

Liquid-Liquid-Extraction

LLE enables the extraction of metabolites into two fractions (aqueous and organic phase) that separately contain polar and apolar compounds and which can be then independently analyzed. In LLE it is challenging to extract polar analytes into an organic phase, therefore in metabolomics LLE is mostly used for sample cleanup purposes, predominantly for the removal of lipids. According to Folch *et al.*[48] and Bligh and Dyer[49], chloroform extraction can be used to recover all major lipid classes, but the use of dichloromethane has been suggested as a less toxic alternative[50]. Recently Matyash *et al.* reported that substituting chloroform with methyl tert-butyl ether (MTBE) also recovered the major lipid classes with the same or better recovery, but with reduced health risks[51]. Moreover, MTBE has a more advantageous phase configuration in which the MTBE phase is at the top and where the precipitated proteins are at the bottom (instead of between the phases using chloroform-based LLE), therefore simplifying online coupling. For these reasons, in the last years several papers have been published comprising LLE with MTBE for metabolomics studies. Chen *et al.* presented an untargeted approach to simultaneously perform metabolomics as well as lipidomics from a small piece of tissue sample (10 mg) based on a MTBE LLE. After the MTBE-based extraction, a mix of the resulting polar and apolar fraction was pooled, evaporated and reconstituted and analyzed with ultra performance (UP)LC-MS. This approach was comparable or superior in yield (3429 vs. 2641 features) and reproducibility (0.3-9.9% vs. 0.5-15.4%) to a standard methanol extraction for the profiling of polar and apolar metabolites[52].

Recently the group of Barbas and co-workers developed a MTBE-based extraction method by performing the whole sample preparation and analysis within and from a single LC-vial, which they coined as in vial dual extraction (IVDE)[53]. The upper MTBE and lower methanol-water phases were successively injected onto an reversed-phase (RP)LC-MS system directly from the vial by the adjustment of the instrument needle height in two separate runs. This way, pretreatment time and analytical variation was reduced. To date, this approach was tested for the direct LC-MS analysis of 20 µL plasma resulting in over 4500 reproducible features, as well as DBS samples for untargeted metabolomics[54], see Figure 2, and also for the application of a biological case [55].

In 2012 Saric *et al.* optimized a single LLE by varying solvent mixtures for comprehensive untargeted metabolic profiling of the aqueous phase from the Fasciola hepatica using a multiplatform approach comprised of RPLC-MS, Hydrophilic Interaction Liquid Chromatography (HILIC)-MS and CE-MS. A choloroform:methanol:water mixture with proportion of 15:59:26 (v:v) delivered the best compromise for all five analytical methods in terms of the number of extracted metabolites and resulted in 142 uniquely identified

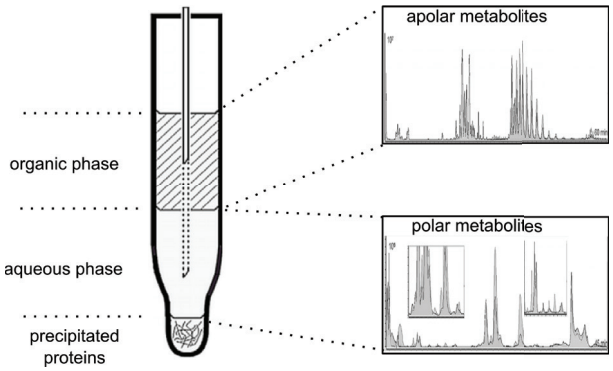

Figure 2: Schematic illustration of the IVDE method comprising the (from bottom to top) precipitated proteins, aqueous and organic MTBE layer. Both organic and aqueous layer are injected on a LC separately for apolar and polar metabolite analysis respectively. Figure adapted with permission from [55].

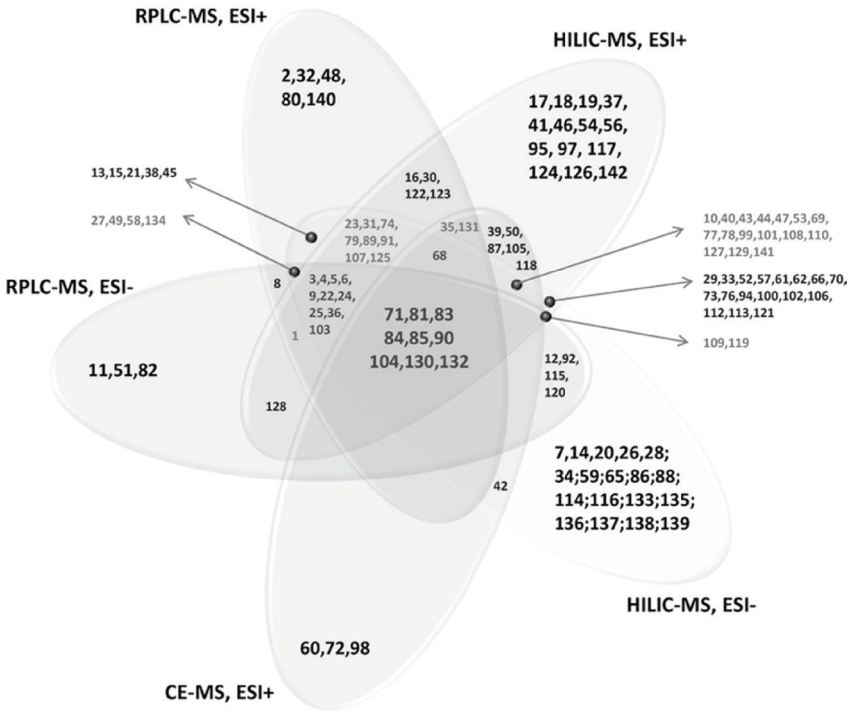

Figure 3: Venn diagram depicting 142 metabolites identified across five analytical platforms after using a single optimized LLE procedure[56].

metabolites, see Figure 3[56].

LLE can also be used for cleaning up samples in order to obtain efficient labeling in metabolomics. Recently, Peng *et al*. reported an improved method for detecting organic acids based on LLE followed by differential isotope p-dimethylaminophenacyl (DmPA) labeling of the acidic metabolites[57]. It is shown that this strategy offers superior performance (3-fold increase in putative detected metabolites) over the method of direct labeling of metabolites in biofluids such as human urine.

Still, conventional LLE as described above has drawbacks being time-consuming and difficult to automate (e.g. in a multi-well format), due to the necessary mixing and often required centrifugation steps.

New LLE approaches have emerged in overcoming automation difficulties, as discussed below. Additionally, these approaches may use less organic solvent than conventional LLE.

Supported Liquid Extraction (SLE), or solid-supported liquid extraction, is an emerging LLE-based technique for metabolite analysis in which an aqueous sample phase is absorbed into a chemically inert, porous, high-surface area diatomaceous earth support. The organic phase is passed through the cartridge and a highly efficient extraction is obtained, therefore bypassing cumbersome mixing and centrifugation steps (Figure 4). As a consequence, the SLE is easier to automate and uses less organic solvent than conventional LLE[58]. Several commercial 96-well plate SLE are available, compatible with automated high-throughput analysis. Although, to date, the analysis of drugs in biofluids is one of the most popular application of SLE, it may also be promising for high-throughput sample clean-up in metabolomics[59][60].

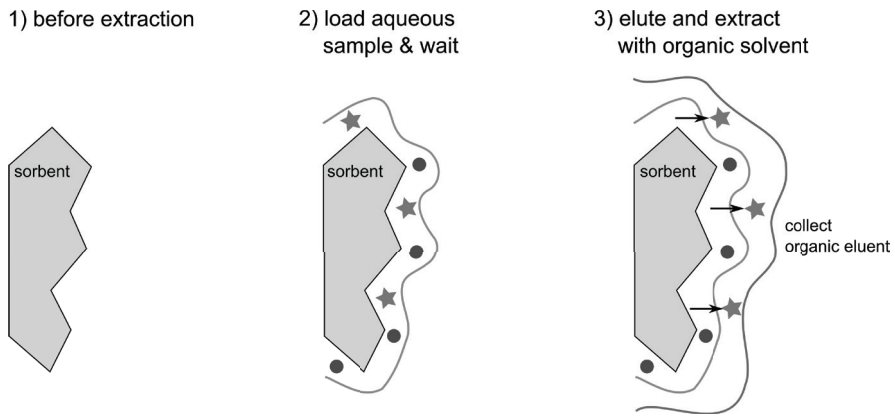

Figure 4: Schematic picture of a typical SLE procedure, modified from[84].

Miniaturization of LLE is another trend such as liquid-phase microextraction (LPME) techniques [61]. In LPME, extraction generally takes place from an aqueous sample phase into a small amount of a water-immiscible organic acceptor phase, and reducing the acceptor-to-donor ratio. Even though this miniaturized approach might be promising in metabolomics studies where limited sample amounts are available, no recent metabolomics applications of LPME techniques have been reported so far.

Another development in LLE is the integration of ultra-sound in sample pretreatment. The application of ultra-sound in LLE can significantly shorten the time required for extraction

of metabolites which increases sample throughput. So far, the potential of this approach has been only demonstrated for a few metabolomics studies [62].

Solid Phase Extraction

SPE is an effective sample pretreatment method for the removal of interfering substances and for the enrichment of analytes. A variety of extraction sorbents is available for SPE; examples are: reversed-phase materials including phenyl groups or polymeric material, weak and strong ion exchange materials including amino groups, mixed mode materials covering for example reversed- phase and weak ion exchange and stationary phases for HILIC separations. Therefore, SPE can address more specific molecular characteristics of target analytes and allows the design of protocols which can be more selective than LLE[5]. However, due to sorbent selectivity, obtaining high metabolite coverage may be challenging, especially in untargeted metabolomics. Therefore, within targeted metabolomics, the combination of SPE and LC systems using reversed-phase sorbents are most often employed for clean-up (desalting and delipidation) and enrichment of relatively hydrophobic analytes. This approach can also be used for analysis of more polar compounds if employing alternative phases (e.g. C_{30}) or ion-pairing reagents[63]. However complementary principles may be more appropriate, such as mixed-mode or HILIC phases. RP SPE-and LC columns are used in most online SPE-LC systems and are now widely commercially available in various dimensions[64].

Recently, Yang *et al.* reported a combined untargeted MTBE-based LLE and SPE (NH_2) method to improve the coverage of the metabolome in plasma caused by effective delipidation. [65]. They separated the sample into five fractions including aqueous species, lipids, fatty acids, neutral lipids and hydrophobic lipids prior to RPLC-MS and HILIC-MS analysis, and detected over 3806 versus 1851 molecular features using methanol extraction only (Figure 5). Improved reproducibility was shown with CV's below 15% for the combined LLE-SPE method, compared to 30% using the methanol method. However, this LLE-SPE method is time-consuming and therefore may be less suitable for high-throughput applications.

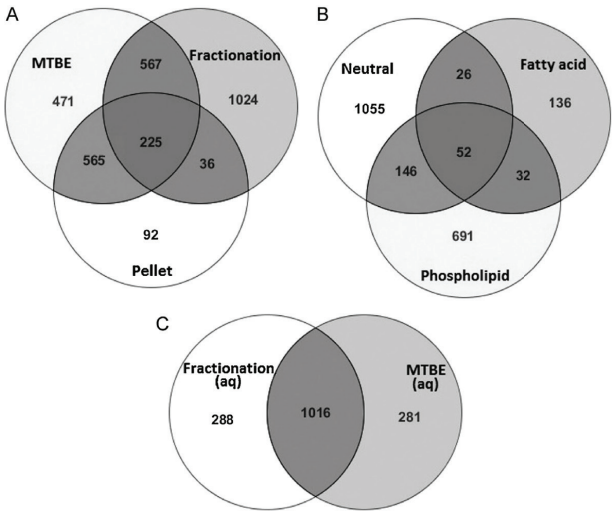

Figure 5: Venn diagrams showing the comparison of MTBE extraction vs MTBE-SPE fractionation method. Extraction was performed and resulting samples were analyzed using positive mode LC-MS by using RPLC (lipids) or HILIC (aqueous). (A) Comparison of lipid fraction, fractionated lipid fractions and pellet fraction; (B) comparison of fractionated fractions; (C) comparison of aqueous fractions. Figure reprinted with permission from[65].

Fully automated SPE has clear advantages over conventional LLE and can outperform manual protocols in terms of reproducibility and sample throughput. Off-line automated SPE sample pretreatment using robotics is available, as well as SPE platforms coupled on-line to MS such as the Agilent Rapidfire system which is a robotic high-throughput SPE-MS system or the Spark Symbiosis™ system which combines SPE and LC in parallel for high sample throughput. These systems could be suitable for rapidly tailoring an efficient targeted metabolite extraction protocol, by employing the various SPE materials that are currently available and optimizing organic solvents, pH or ion strength.

As discussed, delipidation of the sample has gained increased interest, since it can reduce ion suppression effects and can increase column lifetime. Recently, several commercial available lipid depletion plates have become available such as the Hybrid SPE™ (Sigma Aldrich), Ostro™ (Waters), Captiva™ ND (Agilent) and the Phree™ (Phenomenex) 96 well plates which can remove lipids as well as proteins from a sample in a single step. These recent innovations are effective for removing phospholipids and compatible with automation and high sample throughput, but their protocols dilute samples and do not easily allow sample concentration[66].

In SPE recent promising innovations regarding miniaturization, sorbent material and sorbent functionality has appeared in literature and is discussed below.

Also in SPE there is a trend towards miniaturization. One such approach is solid-phase microextraction (SPME) in which a fiber is coated with a thin layer of sorbent material. SPME can integrate sampling, extraction, concentration and sample introduction into a single step[61]. For more details the reader is referred to the review article by Pawliszyn *et al*. in this issue of Trends in Analytical Chemistry. Microextraction by packed sorbent (MEPS) is a more recent miniaturized SPE approach that integrates the sorbent cartridge in a microliter syringe which can be used in e.g. an autosampler. Sample and solvent volume has been scaled down to the microliter level and is therefore very compatible with volume-limited samples. Several MEPS sorbents are available, including reversed- and normal phase, HILIC, mixed mode and ion exchange functionalities[67]. This technique could be very promising in online automated, high-throughput metabolomics studies.

The use of alternative SPE sorbent chemistry based on aptamers could also bring potential to highly selective targeted metabolomics approaches, for example by employing RNA oligomers that have high affinity to small molecule targets including ATP and several amino acids[68]. Another interesting alternative sorbent is the use of immobilized ionic liquids in sample pretreatment for the extraction of polar compounds[69]. However, the potential of these alternative sorbents for metabolomics has not been demonstrated yet.

The use of monolithic material in SPE columns has gained increased interest. This material has several advantages over the use of particle sorbents including low back pressure, high efficiency and a low dead volume[70]. Recently, Abe *et al.* used a spin column, which was packed with a monolithic silica disk, for the extraction of drugs from human urine. In this spin column, the monolithic silica works as a frit, the surface to volume ratio is larger than the use of silica particles and it requires a small volume of extraction solvent [71]. As a consequence this material could be promising for metabolomics, but this has not been demonstrated yet. However, sample loading, washing and elution were accomplished by centrifugation of the spin column, which makes it less suitable for robotic automation.

Carbon nanotubes (CNT) is another SPE sorbent material which also demonstrated to have great potential. CNT are characterized by highly specific surface area, high mechanical strength, chemical stability and strong adsorbance capacity. By modifying the surface of CNT using carboxyl groups, the extraction of highly polar analytes is enabled[72]. As a consequence

CNT could be promising for metabolomics, however it has not been demonstrated yet.

Electromigration-based Extraction Techniques

Electromigration-based extraction utilizes an electric field to induce selective migration of charged compounds. A major part of the metabolome can be targeted with this approach, as many metabolites are charged in solution or can be made charged by adjusting the pH. Electromigration-based sample extraction has received increased attention in the past 5 years[73][74]. In this section, we outline recent developments in two techniques that may be promising for metabolite analysis: electromembrane extraction (EME) and electroextraction (EE). EME is based on an the application of an electric field between a donor phase and an acceptor phase which are separated by a membrane filled with an organic solvent in its pores[75]. Via the membrane, which consists of a polymeric material, analytes are extracted from a sample to an acceptor compartment. The electric field enhances the extraction rate of the analytes and as a consequence it is fast and typically enriches analytes (expressed in concentration units) by one order of magnitude. Strieglerová *et al.* demonstrated the potential of EME for the targeted extraction of endogenous amino acids from human plasma and urine[76]. Here, a simple EME device was coupled with capillary electrophoresis with capacitively coupled contactless conductivity detection. In total, 12 endogenous physiological amino acids were analyzed in human plasma, whole blood, serum and urine with peak area RSDs ranging from 1 to 13%. However, not all endogenous 20 physiological amino acids were recovered[76]. Further optimization of the composition of the liquid phase of the membrane may result in an EME protocol that is capable of extracting a wider physicochemical range of analytes in one run.

EE is an electromigration-based sample enrichment technique in which analytes are concentrated from a large donor phase, through one or more liquid-liquid interfaces, into a small acceptor phase[77]. In the most simple set-up, the donor phase is an organic solvent, which may contain some water to dissolve ions as has been done for ethyl acetate, and the adjacent acceptor phase is an aqueous phase; both phases should be immiscible. It has been shown that EE allows the fast enrichment of metabolites and is promising for high-throughput applications and analysis of low-abundant metabolites [78][79]. The driving force of EE is an electric field that is applied over the liquid-liquid system: in the donor phase a very high field strength is present. When the metabolites enter the aqueous acceptor phase, they enter a very low electric field, their migration speed diminishes and they are enriched in a small volume.

In 2012, Lindenburg *et al.* presented EE of acylcarnitines from human urine. EE took place in a wide-bore PTFE capillary, was coupled online with LC-MS via a switching valve and was automated. Within a few minutes, acylcarnitines at the nM level in urine were enriched up to 1000 times with excellent repeatability (below 12%) in comparison with conventional LC-MS injection, see Figure 6 [78].

Recently, Raterink *et al.* developed a new EE strategy consisting of three liquid phases, so-called 3-phase EE. The extraction in 3-phase EE takes place from the aqueous donor phase, via an organic phase that acts as a filter, into a hanging acceptor droplet. The selectivity of 3-phase EE can be altered by changing the composition of the organic filter phase. After the 3-phase EE procedure was completed, the acceptor droplet was transferred to nanoESI-DI-MS. The 3-phase EE–nanoESI-DI-MS was successfully applied to the targeted analysis of acylcarnitines spiked to human plasma with a good linear response over 2 orders of magnitude and repeatability better than 15%, and could be fully automated. Importantly, it was demonstrated that proteins were excluded from extraction, indicating that 3-phase EE can cope with difficult matrices and requires no prior PPT[79].

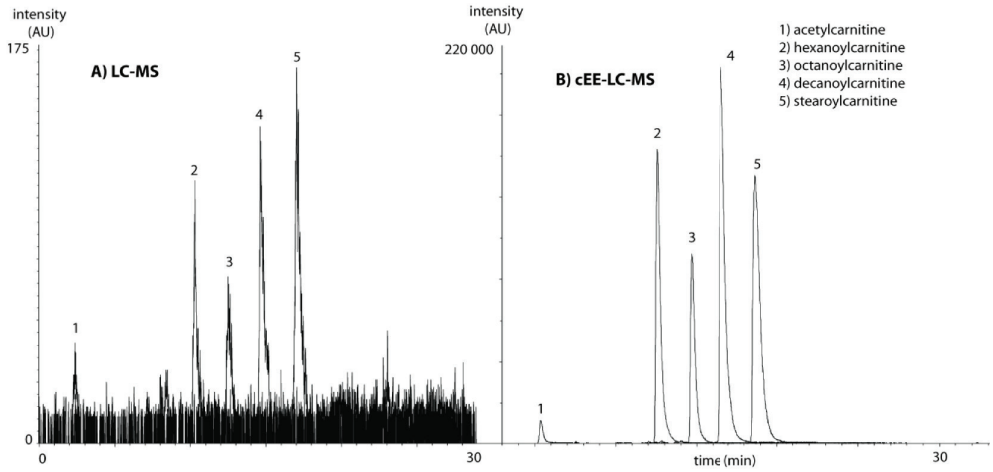

Figure 6: Comparison of (A) LC-MS obtained for a 0.1 µL injection of urine and (B) cEE-LC-MS of 100 µL urine; urine was spiked with acylcarnitines. Figure reprinted with permission from[78].

Until now, it has been shown that electromigration-driven sample extraction methods are in principle promising for metabolomics. Its approach can offer speed, selectivity and excellent potential for both miniaturization and high-throughput analysis. Therefore, we expect that electromigration-based sample extraction will be used as complementary tools for metabolomics studies in the upcoming years. However, an important aspect to study is the applicability of these methods to a wider range of metabolites.

QUANTITATION AND VALIDATION

The systematic evaluation and validation of an extraction procedure for a given biological sample matrix is necessary in order to obtain reliable and meaningful metabolomics data. In this section, attention is paid to some issues that should be considered during sample extraction to obtain reliable quantitative metabolite data.

The performance (i.e. recovery, repeatability, enrichment factors etc.) of the extraction method should be critically examined by using proper internal standards. In untargeted metabolomics, the performance of an extraction method is still often only evaluated in terms of the number of molecular features observed[15]. This strategy may provide useful information, however specific information concerning metabolite enrichment and recovery, or metabolite losses due to e.g. protein binding, is not provided. In this context, we suggest using multiple stable isotopically labeled internal standards, each representing a metabolite (sub)class, during sample extraction in order to evaluate the performance of the method[80][81]. Recently Strassburg *et al*. developed a quantitative (precision RSD ranged from 4% - 15%) and sensitive (down to nM levels) LC-MS/MS method for the profiling of approximately 100 oxylipins in human plasma by using 11 deuterated internal standards which were considered representative for the different compound classes[82]. Method validation in untargeted metabolomics is more challenging than in targeted metabolomics regarding the choice of the internal standard. Another elegant approach to tackle these challenges is the use of 13C-labeled cell extracts, which has been recently used for the quantification of 91 metabolites representing central carbon and energy metabolism[33]. For targeted metabolites, isotopically-labelled

metabolites can be used to correct for potential matrix effects (ion suppression/enhancement) and batch-to-batch effects of the corresponding endogenous metabolites.
At present, there is no consensus yet on the most suited type of sample extraction method that should be used for a certain biological sample matrix or question. Standardization and inter-laboratory comparisons are crucial in order to demonstrate the validity of a sample extraction procedure for metabolic profiling of a particular biological sample, and this would help to compare different metabolomics studies published or even accessible in data repositories. For inter-laboratory comparisons, reference material[83] should be used and preferably absolute concentrations should be provided for metabolites. Especially when reporting relative concentrations, as is often the case in untargeted metabolomics studies, it is crucial to use pooled samples and/or internal standards as quality controls and for correction of variations and possible biases in the overall analytical procedure during studies[81].

CONCLUSIONS AND OUTLOOK

Sample pretreatment is a key step in the metabolomics workflow as it has a major influence on the overall quality of the obtained metabolic profile, metabolite coverage and sample throughput. This review highlights that the development of sample pretreatment for metabolomics is still an active area of research. Although no universal sample pretreatment technique is available for the comprehensive analysis of all metabolites in a given biological sample, the combination of protein and lipid removal is often used as an effective strategy to improve metabolite coverage and reproducibility. In LLE several developments are made towards the compatibility with automation, of which supported liquid extraction is one of the most promising techniques, readily available in a well-plate format. As a consequence, deproteinization and delipidation can be performed by using LLE as well as SPE techniques in a fully automated, high-throughput manner. The recent innovations in automated offline well-plate extraction (including protein precipitation, LLE and SPE) or online extraction (including turbulent flow chromatography) have allowed fast sample cleanup and partly removed the bottlenecks associated with sample pretreatment. All these techniques are very promising for metabolomics, however the effective extraction of polar metabolites is still challenging and the separation of polar analytes from interfering salts is a hurdle for some analytical methods which still needs to be overcome. New emerging electromigration-based sample pretreatment techniques are promising and their compatibility with automation has been demonstrated. Still, more research is needed in order to exploit the full potential of these techniques for metabolomics.
To improve quantitative aspects during sample pretreatment, more research is needed in the use of internal standards, such as 13C-labeled cell extracts. Proper internal standards are crucial to correct for matrix and batch effects and to obtain insights in metabolite enrichment and recovery. In the future, sample pretreatment procedures should not only be automated and fast, but also quantitative and standardized so that metabolomics data of different studies are comparable within and between labs, which would be needed for breakthroughs in biomedical and pharmacological research.

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Chapter 3

Rapid metabolic screening of early zebrafish embryogenesis based on direct-infusion-nanoESI-FTMS

ABSTRACT

Single zebrafish eggs were rapidly profiled using high resolution nanoelectrospray direct infusion mass spectrometry with limited sample preparation and without separation. The analysis time per sample is around 1 minute. Using this approach the different developmental stages of zebrafish eggs can be characterized by their active metabolites. Five different development stages with distinct metabolic fingerprints could clearly be observed when untargeted analysis is performed and the data are plotted using principal component analysis (PCA). Using this approach early embryogenesis is followed with a time resolution of 1 hour and 102 features proved relevant. Of these, significant number of putatively identified compounds has not been reported earlier to have any association with early zebrafish embryogenesis yet. The onset of gene expression and the increase in energy requirement is reflected by the measured metabolome complementing earlier reported transcriptomics studies from a systems biology point of view. By deyolking and dechorionation eggs at two early developmental stages, we were able to observe distinct changes in localized metabolism.

Based on

R.J. Raterink*, F. M. Kloet*, J. Li, N. A. Wattel, M. J. M. Schaaf, H. P. Spaink, R. Berger, R. J. Vreeken, and T. Hankemeier, *"Rapid metabolic screening of early zebrafish embryogenesis based on direct infusion-nanoESI-FTMS", Metabolomics, vol. 9, no. 4, pp. 864–873, 2013.*

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INTRODUCTION

In the past decade the Zebrafish (*Danio Rerio*) has become a popular vertebrate model system for studying human development and disease[1]–[3]. This is because the development of the zebrafish is very similar to the embryogenesis in higher vertebrates, including humans. But unlike mammals, zebrafish develop from a fertilized egg to an adult outside the female in a transparent egg. This makes it possible to observe developing embryos from the single cell to the entire organism level[4]. The development time of the embryo is fast (after about 2 days most common vertebrate specific body features can be seen including brain, eyes, ears and all internal organs) and the number of offspring is large (100-200 eggs per mating). Moreover, because they are small, available in large numbers and maintained at low cost, zebrafish embryos are ideal as model systems in high-throughput whole organism preclinical drug screening and toxicology studies as less drug is required, a larger number of animals can be used and less ethical issues are associated[5], [6]. Therefore, the zebrafish embryo can bridge the gap between cell assays and rodent assays.

The external and rapid development as well as transparency of zebrafish embryos is ideal for observable phenotype-based screening at cellular and organ-tissue level using live imaging, for example using a complex object parametric analyzer (COPAS)[7]. Although these phenotypic changes are adequate for specific drug-induced biological responses, they don't reveal system-wide responses and are not sufficient in elucidating the mode of action or potential toxicity of drugs[6]. With the ever increasing interest in studying biological systems in a holistic manner (systems biology) the necessity for delivering qualitative and quantitative data of complete biological systems for which zebrafish offer many advantages becomes clear[8]. Metabolomics is a powerful tool within systems biology and investigates the complex interactions of the metabolism and metabolic networks[9]–[11]. One of the greatest strengths of metabolomics is the ability to capture a molecular snapshot of metabolites as reactants, intermediates or products of (enzyme-mediated) biochemical reactions. Metabolomics complements genomics, transcriptomics and proteomics for metabolites are in a unique position as they are building blocks for all other biochemical structures including proteins (amino acids), genes and transcripts (nucleotides) and cell walls (lipids) [12], [13].

So far, the number of metabolomics studies on zebrafish embryos is limited[14]–[18]. Most of these studies included chromatography which is time consuming and therefore less suitable for high-throughput metabolic screening. Recent studies outlined the power of high-resolution direct infusion mass spectrometry (HR-DI-MS) metabolomics on other complex samples[19]–[22]. Most of these studies were performed using flow injection electrospray ionization (ESI)-MS and therefore did not explore the advantages of nanoESI over normal ESI with respect to ionization efficiency and ion suppression effects[23].

In this paper we describe a rapid metabolic profiling method of zebrafish eggs, based on lysis of single zebrafish eggs and subsequent HR-nanoESI-DI-MS analysis. A clear metabolic distinction between developmental stages in early embryogenesis is described and up-and down regulation of some important primary metabolites is shown. By deyolking and dechorionating the embryos, we were able to highlight localized and time resolved metabolism.

MATERIALS AND METHODS

Chemicals and materials

Methanol was from Biosolve (Valkenswaard, The Netherlands). Water was obtained from a Millipore high purity water dispenser (Billerica, MA, USA). All solvents were HPLC grade. The labeled amino acids ("Cell Free" amino acid mix (20 AA) (U-13C, 98%+; U-15N, 98%))

were bought from Cambridge Isotope Laboratories (Andover, MA, USA) and added to the samples at a concentration of 1 µg/mL. Reserpine (also used as internal standard) was supplied by Fluka (Buchs, Switzerland) and added to the samples at a concentration of 500 ng/mL.

Whole embryo experiments
Two wild type (strain A/B) parent groups were maintained under standard zebrafish aquarium conditions. Since many cell differentiation and phenotopic processes occur within 48 hours after fertilization, the following 5 early developmental stages were chosen: the 4-cell (1 hpf), 64-cell (2 hpf), 1k (3 hpf), 50% Epiboly (5 hpf) and the 18-somites stage (18 hpf) For each of the five developmental stages 8 eggs were analyzed separately in triplicate.

Lysation protocol for the whole embryo
1) The egg was optically selected under a microscope in the relevant developmental stage and pipetted to a 1.5 mL eppendorf tube. As much as possible of the egg water was removed.
2) The egg was washed 3 times with 1 mL demineralized H_2O.
3) 100 µL (9:1, v/v methanol:water) including the internal standards (1µg/mL labeled amino acids+ 500 ng/mL reserpine) was added. Immediately the sample was snap-frozen in liquid nitrogen for 2 minutes. This step should quench metabolism and precipitate the proteins.
4) After snap-freezing the sample was sonicated for 2 minutes to lyse and to homogenize the sample and visually inspected to confirm homogeneity. In case of non-homogeneity the sample was snap-frozen and sonicated a second time.
5.)Precipitated proteins were spun down by centrifuging the lysate at 16.1 krcf at 0 °C. The supernatant (80 µL) was used for DI-MS analysis.

Deyolking and dechorionating experiments
Two developmental stages 1kcell (3 hpf) and somite (18 hpf)) of the same wild type were measured (pool of n=10) in triplicate.
Dechorionating
1) The chorion of the egg was removed mechanically by a tweezer and was put in a 1.5 mL eppendorf tube. Using this methodology only the membrane of the chorion could be analysed. The same lysation protocol as described above was used however, instead of 100 µL , now 1 mL of solvent + IS (since it was a pool of 10 egg instead of a single egg) was added and no washing was done (since it would remove parts of the sample).
Deyolking
1) After dechorionating the egg only consists of the yolk with its cells (see Figure 1). The yolk was removed by adding a Ringer solution to the egg and pipetting it up-and down a few times.
2) Subsequently, the sample was centrifuged for 5 minutes at 0.8 krcf (to segregate the cells) and the supernatant (including the yolk) was pipetted away. This way only the cells of the embryo could be analyzed[4].
3) 1 mL of IS was added to the pallet and the same lysation protocol was used as described above.
Non-fertilized egg (pool of n=10, in triplicate)
1) First the non-fertilized egg (only 3 hpf stage) was dechorionated resulting in only the yolk with its fluid. This way also the fluid in the yolk could be analyzed.
2) 1 mL of IS was added and the same lysation protocol was used as described above.
In the deyolking experiments again also whole-eggs were included as a reference.

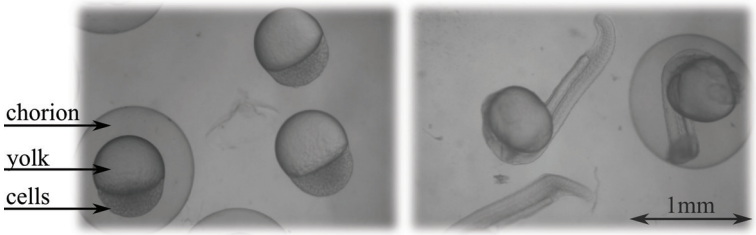

Figure 1: Photomicrograph of zebrafish embryos: (left) 1k-cell stage and (right) somite stage. In both stages some of the embryos were dechorionated in order to perform the deyolking.

MS Analysis

The analyses were performed by DI-nanoESI-MS in the positive ion mode using the automated Advion NanoMate Triversa system (type 'A' chip) coupled to a LTQ-FT Ultra (Thermo Fisher Scientific). Eppendorf 96 well plates were used on which all the samples were randomly distributed. Of each sample 5μL was infused using a pressure of 0.2 psi and an electrospray voltage of 1.48 kV in the positive ion mode. Mass spectra were recorded using three scan ranges containing 20 scans: 50-250; 250-500; 500-1000 *m/z* (in this order) at a resolution of 100.000. Separate scan ranges instead of one full scan range was chosen in order to enhance sensitivity. The MS was tuned with inlet capillary temperature of 120 °C, capillary voltage of 35V and the tube lens voltage of 50 V. For tandem MS as well as for the deyolking experiments an LTQ-Orbitrap XL (Thermo Fisher Scientific) was used with inlet capillary temperature of 120 °C, capillary voltage of 25 V and the tube lens voltage of 80 V.

Data processing

The first 10 scans (approximately 10 seconds) of every sample were averaged using XCalibur software (version 2.0.7; Thermo Fischer). These average scans were stored in separate files. In some samples no spray or no stable spray was obtained, i.e. no average scan could be made. These samples were discarded from further processing. Using a resolution of 100.000 (at 400 *m/z*) each sample typically resulted in 5 to 10 thousand unique features in the average spectrum. In contrast to hyphenated MS data, the drawback of DI-MS data is that all masses co-elute. Aligning features over samples now is solely based on accurate mass. To align the masses across the different samples we created mass-bins of very small sizes (0.0003 Da) and assigned the different masses to the nearest bin. For a mass range of 50 to 250 *m/z* this generated approximately 650.000 mass bins. All data analysis was done using Matlab (version 2011a 64bit; MathWorks). In order for the data to be analyzed in Matlab the Xcalibur averaged spectra files were converted to mzXML format (ReAdW version 4.3.1).

RESULTS AND DISCUSSION

Our primary goal was to explore the possibility of discerning developmental stages in zebrafish embryogenesis by applying HR-nanoESI-DI-MS analysis on lysed eggs. Therefore, reproducible and high-quality MS data should be generated and preferably an automated data processing tool is required. The implementation of these (automated) tools, viz., acquisition and processing strategy, forms an integral part of the proposed method. After binning, empty and almost empty features were removed. This reduced the number of features from ~650.000 to ~30.000. To reduce analytical variation as much as possible, the data was normalized by selecting the optimal internal standard for each compound from the mix of internal standards. This selection was based on the minimization of the RSD of the response of replicate measure-

ments[24]. Beckmann *et al.* [21] normalized the data by using the total ion current, however with our data this would reduce group differences which is not desirable. To enable statistical interpretation, features that showed consistency per group were selected [25]. Features were considered consistent if they were either present or absent in all samples for a group. However, as we were interested in changes between developmental stages, we allowed for, at most, one missing feature in the replicates of a particular developmental stage. This step further reduced the number of possible features to ~5.000. As we anticipate differences between the samples to be apparent in the most abundant metabolites for that sample, we focused on the top 100 features per sample reducing the number of features of interest to 200. Out of these 200 features, 102 showed significant differences in concentration between the different developmental stages. As observed in other complex samples in other research[20], the lowest mass range (50-250 *m/z*) showed a clear clustering of the different developmental stages (see Figure 3). This would indicate that at least small molecules relate to metabolic variations between the ages of the embryo.

Robustness/validation of the analytical method

Metabolomics often deals with differential analysis of fingerprints[20] to highlight biomarkers. Especially, when a large number of analytes is taken into consideration absolute quantitation is only reported in some cases. In order to be able to give some indication of the analytical rigor of the method, the RSD of repeated measurements, for example using QC samples is often reported. In the absence of QC samples , the RSD was calculated for each feature using a RSD approach based on multiple sets of replicated measurements[26] and classified in one of the six possible classes (0-0.1,0.1-0.25,0.25-0.35,0.35-0.5,0.5-1 and >1). The result of this inter-assay reproducibility for the 102 features is displayed in Figure 2. As can be seen almost half of these features showed an RSD lower than 25%.

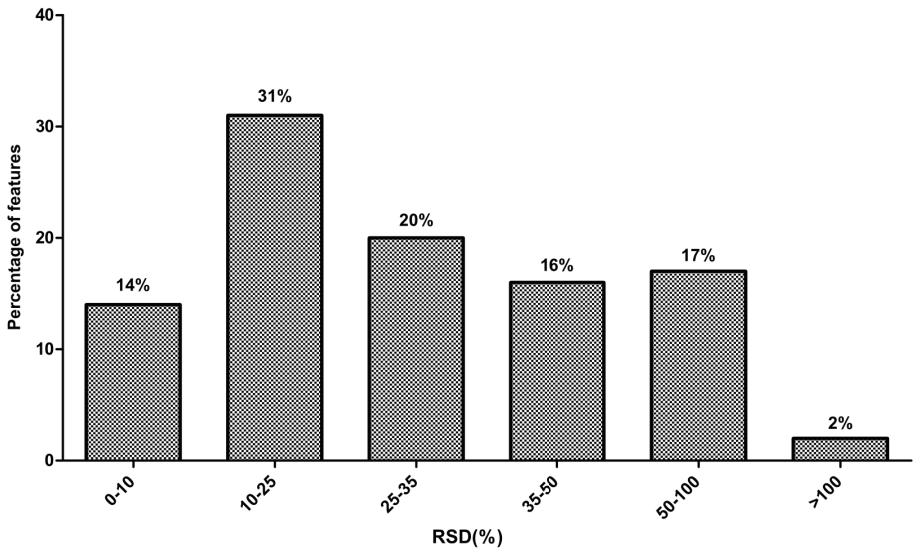

Figure 2: Histograms showing the technical reproducibility of the measurements. 45% of the 102 features has an RSD <25%.

One could argue that by focusing on the top N features too much information is being discarded. For example, tryptophan, hypoxanthine, carnosine, methionine, aspartic acid, propionylcarnitine, dimethyllysine, methyllysine, acetyllysine and serine were discarded (see Supporting Figure 1). However, including lesser abundant features invariably led to higher RSD values for these new features. This could be explained by ion suppression effects: as we measured crude, complex samples containing various compound classes and compound sizes as a result of lysis of the whole embryo. As a comparison: yeast has an estimated 1100 metabolites which is expected to be significantly less than the metabolome of a whole zebrafish embryo[13]. This together with using nanoESI lead to quite some sample-loss due to, e.g., clogging of the nanoESI emitter, which resulted in a loss of replicates. Nevertheless, by discarding the missing variables from the dataset, still 102 features were extracted which revealed the developmental stage differences (Figure 3). (FDA suggests that for LC-MS profiling an RSD of 20% is acceptable[13]).

Identification of metabolites

Out of the significant 102 features 27 separate metabolites (36 features; including all adducts) were putatively identified (see Table 1). Identification was based on (1) search of HR-MS data against HMDB (Human Metabolome project Data Base) and (2) searching MS/MS data against HMDB comparison to standards. After the first HMDB search 171 possible structures were found of which 21 were unique and 150 isomers. From those 150 remaining isomers, 6 were identified using tandem MS and subsequent database search or comparison to standards.

Table 1: RSD values of the 27 (putatively) identified features. The features that were not found in the cited references were ticked as 'new' in the last column.

Feature	M+H	M+Na	M+K	$M+NH_4$	New
Acetylcarnitine	0.33	0.29	n.d.	n.d.	
Alanine	n.d.	n.d.	0.31	n.d.	
Arginine	0.11	n.d.	n.d.	n.d.	✔
Asparagine	n.d.	n.d.	0.07	n.d.	
Aspartylphosphate/acetylaspartic acid	n.d.	n.d.	0.16	n.d.	✔
Carnitine	0.28	0.27	n.d.	n.d.	✔
Creatine	0.26	0.19	0.09	n.d.	
Dimetylarginine	0.11	n.d.	n.d.	n.d.	✔
Dopamine/vanillyamine	n.d.	n.d.	0.16	n.d.	✔
FAPy-adenine	n.d.	n.d.	0.2	n.d.	✔
Glutamine	n.d.	n.d.	0.06	n.d.	
Histidine	0.19	n.d.	0.22	n.d.	
Homoserine/threonine	n.d.	n.d.	0.34	n.d.	
Indoleacetic acid	0.05	n.d.	n.d.	n.d.	✔
Inositol cyclic phosphate	0.16	n.d.	n.d.	n.d.	
Iso/nor leucine	0.14	0.12	0.09	n.d.	
Iso valeraldehyde	n.d.	n.d.	n.d.	0.2	✔
Lysine	0.12	n.d.	n.d.	n.d.	
Phenylalanine	n.d.	0.13	0.09	n.d.	
Phosphoethanolamine	n.d.	n.d.	0.2	n.d.	✔
Proline	n.d.	n.d.	0.12	n.d.	
Quinone	n.d.	0.37	n.d.	n.d.	✔
Safrole	n.d.	0.33	n.d.	n.d.	✔
Spermine dialdehyde	0.25	n.d.	n.d.	n.d.	✔
Tyrosine	n.d.	n.d.	0.07	n.d.	
Valine	n.d.	0.2	0.13	n.d.	
Vinylacetylglycine	0.08	n.d.	n.d.	n.d.	✔

The features that were not found in the cited references were ticked as 'new' in the last column

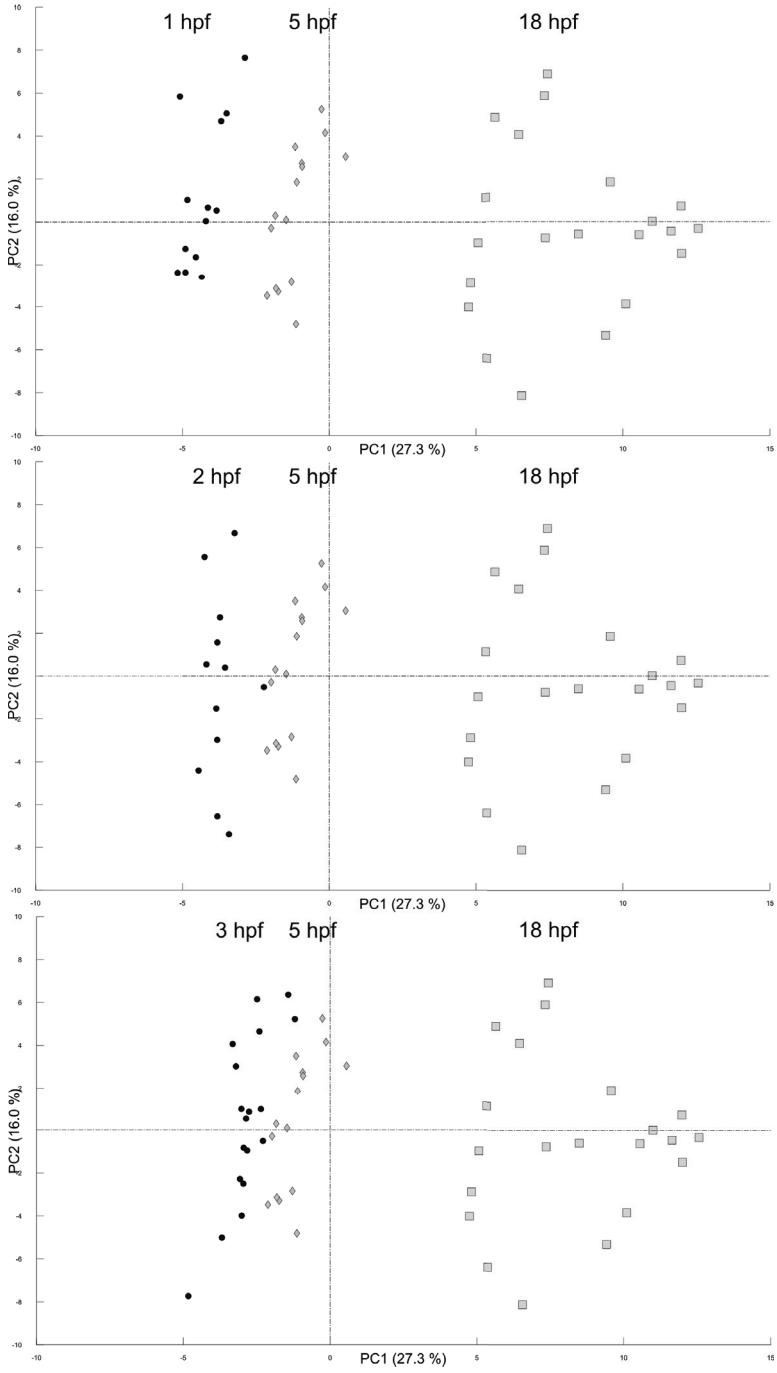

Figure 3: PCA score plots indicating a visible difference between 5 different developmental stages. For these plots only one PCA model was created using all samples and only those features with an RSD < 30%. The blocks represent the 18 hpf stage, the diamonds the 5 hpf stage. The circles represent the 1, 2 and 3 hpf respectively.

Table 1 shows all the putatively identified features, with their associated RSD values. Remarkably, the potassium adducts nearly always shows the best RSD, followed by the sodium adducts and finally the protonated molecule. As our samples were not acidified, alkali adducts were not suppressed. The reason for the pre-dominant potassium adducts is most likely due to relative high potassium concentration inside the cell as opposed to the relative high concentration of sodium outside living cells. From several of the 27 (putatively) identified features the boxplots with their up-or down regulation are shown in Figure 4. Still 63% of the 102 significant features remained unidentified. This confirms that further research is required to expand the zebrafish metabolome database in order to increase identification using DI-MS methods[16].

Biological relevance

In order to evaluate the possibility of using this technology for early fingerprinting of zebrafish embryos, different stages after fertilization were analyzed using this approach. 1, 2, 3, 5 and 18 hours post fertilization (hpf) were tested. The PCA in Figure 3 shows the somite stage (18 hpf) as the most distinctive group as well as having the most within-group variation. The fact that this stage is most distinctive from the other groups can be explained by the fact that different tissue types and organs (i.e. nervous system, skin, blood, and heart) begin to form at this stage. Since small time differences result already in differences of organ formation this will lead to biological variations in the different samples, explaining why this time point has the most variation in the metabolome measurements. Thus, as Chen *et al.* [16] remarked, the differences in the metabolome between the early embryonic stages may reflect the embryological properties of the cells. These results show that the zebrafish embryonic metabolome reflects differentiation. With our method we were able to observe a post fertilization time-trend with a time resolution of 1 hpf. The metabolic shift between 1, 2, 3 and 5 hpf can be clearly observed indicating that in early embryogenesis the metabolome changes quickly and significantly. As can be seen, the metabolome of the first three stages (1, 2 and 3 hpf) closely resemble each other. This suggests that zygotic gene transcription only begins at the onset of the midblastula transition (3 hpf)[27].

The boxplots in Figure 4 allows us to go more into depth regarding the biology during early embryogenesis. Interestingly, the increase in concentration of dimethylarginine can be a result of the enhanced methylation and acetylation metabolism of lysine and arginine which play an important role in histone activity and gene expression[28][29]. When we searched for other methylated and acetylated forms of lysine and arginine by discarding the described missings data cleaning step (resulting in a larger data subset) we also found increasing trends for dimethyllysine, methyllysine and acetyllysine (Supporting Figure 1). Although we are aware these analytes did not make it to the best 102 features, these findings correlate to the onset of gene expression at 3 hpf.

The increased concentration of spermine dialdehyde could reflect the turnover of the polyamine spermine which plays a role in normal and neoplastic growth as well as the (uni) directional transport of molecules by GAP junctions which is an important process in early embryogenesis[30][31].

It can also be observed that the concentrations of several amino acids and biogenic amines are increasing during early embryogenesis. Apparently the embryos are able to release amino acids from storage proteins to provide the cells with building blocks and energy already in an early stage. Some of these reveal more than a 10-fold change going from 1 hpf to 18 hpf. In the 18 hpf stage the rise of most of the amino acids is the largest. This could be explained by the

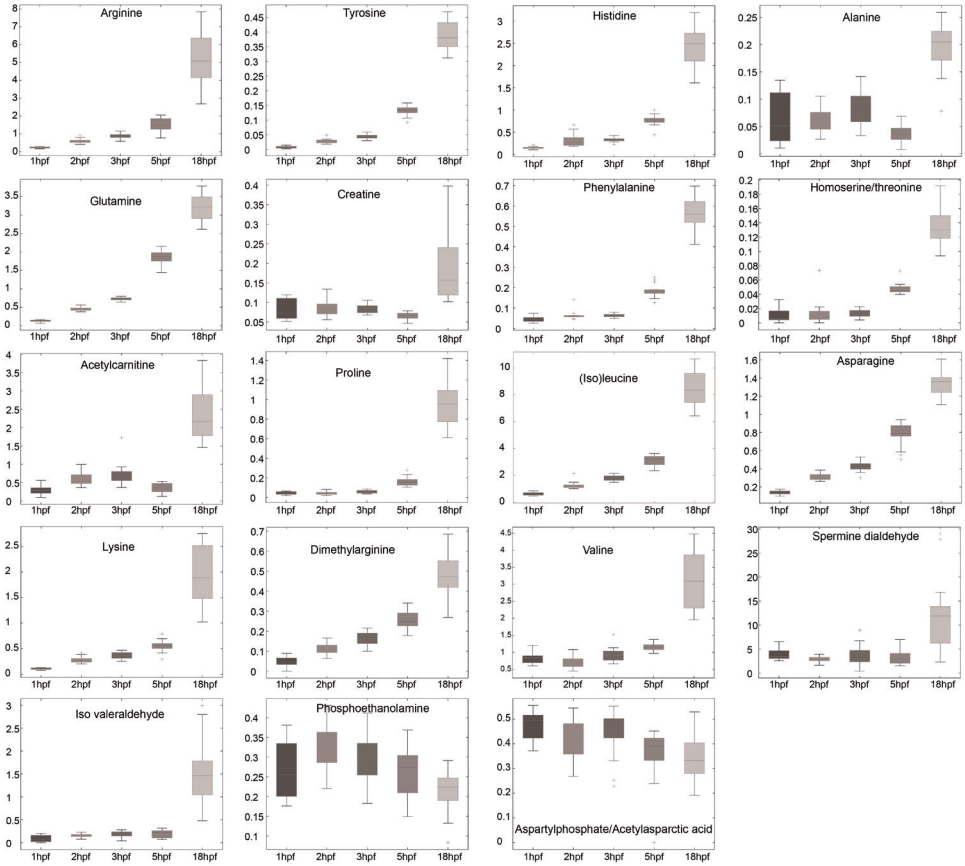

Figure 4: Boxplots of the up-and down regulation of some of the (putatively) identified metabolites through the five different developmental stages (for arg, his, actylcarnitine, lys, dimethylarg and spermine dialdehyde the $(M+H)^+$ plot is shown, for tyr, glu, creatine,phe, pro, leu, val, ala, homoserine, asparagine, phophoethanolamine, acetylasparctic acid the $(M+K)^+$ plot is shown, and for isovaleraldehyde the $(M+NH4)^+$ plot is shown. The y-axis represents the ratio of the analyte/optimal internal standard.

increase in energy requirement for evoked muscle contraction starting at around 18 hpf[32]. Isovaleraldehyde, vinylacetylglycine could reflect the degradation of branched chain amino acids like valine and (iso)leucine, both associated with the increase in energy requirement. The concentration of hypoxanthine (see supplemental figure) is decreasing which can be explained by the enhanced DNA/RNA synthesis via xanthine, as also indicated by the decrease of guanine.

Some metabolites, like acetylcarnitine and creatine, show up- as well as- down regulation within the five developmental stages. The observed trends of almost all of our identified features is supported by the observations of previous publications[15][17]. Moreover, features that are indicated as new in table 1(arginine, acetylasparctic acid, carnitine, dimethylarginine, dopamine, FAPy-adenine, indoleacetic acid, isovaleraldehyde, phosphoethanolamine, quinone, safrole, spermine dialdehyde, vinylacetylglycine) were exclusively found with our method and not in the aformentioned references. This indicates the potential of HR-DI-MS for metabolic profiling purposes.

Deyolking

To obtain more insight in the biology of early embryogenesis, a series of deyolking experiments were performed in order to zoom in on the localization of metabolism. Figure 5 shows several bar-plots of the group means of metabolites that were also (putatively) identified in the previous section. Hypoxanthine and carnitine (metabolites which were discarded using our data cleanup steps (Supporting Figure 1)) showed a down-regulation trend during embryogenesis).

The charts confirm the same developmental trends as observed earlier. Because of the limited number of replicates that was measured (3 times) the statistical power is limited but ANOVA calculations showed that no significant difference between the zygote part and the whole egg could be detected. This could indicate that most of the metabolites and most of the metabolic conversions take place in the zygote part.

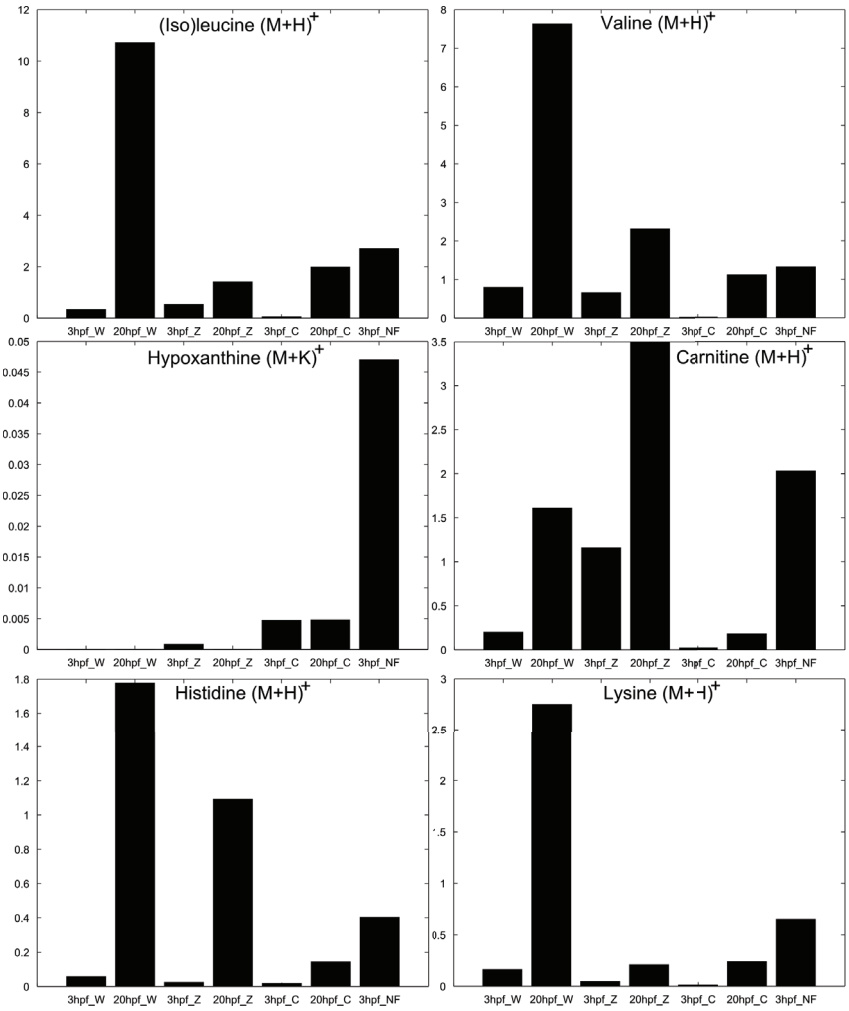

Figure 5: Bar-plots showing the mean of the different embryonic location at the age of 3 hpf and 20 hpf. W=whole embryo; C=chorion membrane; Z=zygote part; NF=dechorionated non-fertilized embryo (=yolk). The y-axis represents the ratio of the analyte/ optimal internal standard.

CONCLUDING REMARKS

Using our rapid metabolic fingerprinting method we were able to distinguish metabolic profiles of early developmental stages of zebrafish embryos. Interestingly, the onset of gene expression and the increase in energy requirement is reflected by the measured metabolome confirming that from a systems biology point of view metabolomics complements transcriptomics. After data cleanup, only those features were selected that showed consistent behavior within each developmental stage resulting in 102 features. PCA revealed that periods of 1 hour time shifts post fertilization could be differentiated from each other. In total 27 out of the 102 features were (putatively) identified. Although unambiguous identification is beyond the scope of our approach, identification on 6 of these 27 extracted features was pursued using standards and tandem MS. Several trends of the putatively identified metabolites are included and almost all of these findings are supported by previous publications. Moreover our method exclusively found several new features. By deyolking and dechorionating we showed the potential of this method to enable more in-depth studies on localization of metabolism. We conclude that HR-DI-MS is suitable for rapid metabolic profiling on zebrafish embryos. However, to improve robustness and obtain more high-quality features fast sample preparation methods are required.

ACKNOWLEDGEMENTS

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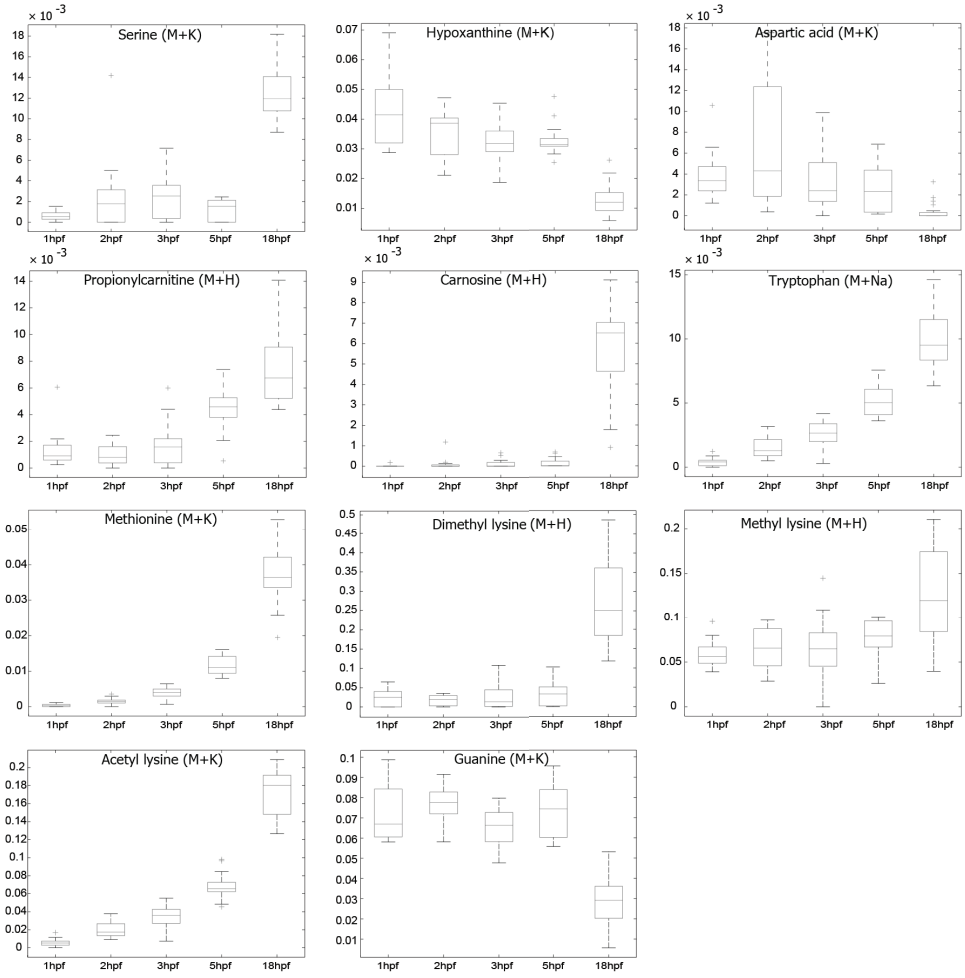
SUPPORTING INFORMATION



Supporting Figure 1: Boxplots of some of the discarded features due to our data cleanup steps.

Chapter 4

Three-phase Electroextraction: a new (online) sample purification and enrichment method for bioanalysis

ABSTRACT

The migration, and at the same time enrichment of analytes from a liquid aqueous sample donor phase, through an immiscible organic solvent layer acting as a filter phase, into a liquid aqueous acceptor phase is enabled by the application of an electric field between the donor and acceptor phase. The organic filter phase acts as a purification filter which prevents e.g. proteins from migrating into the acceptor phase. Moreover, the composition of the organic filter phase influences the selectivity of the extraction. We show that analytes can be rapidly enriched from a 50 µL donor phase at the bottom of a sample vial, via an immiscible organic filter phase, into a 2 µL acceptor phase which consists of a droplet that is hanging from a (conductive) pipette tip in the organic filter phase. Acylcarnitines spiked to human plasma as a donor phase were extracted reproducibly with good linearity and a 10-fold improved limit of detection and, importantly, resulted in a stable, protein-free nanoelectrospray signal. Finally, a proof of principle towards the on-line integration in an automated nanoelectrospray-Direct Infusion-Mass Spectrometry platform has been realized. This makes 3-phase electroextraction (3-phase EE) a novel sample purification and enrichment method, with straightforward on-line integration possibility. We envision that 3-phase EE will enable new possibilities using electrokinetic sample pretreatment for fully automated, high-throughput bioanalysis purposes.

Based on

R.J. Raterink, P. W. Lindenburg, R. J. Vreeken, and T. Hankemeier, *"Three-phase electroextraction: a new (online) sample purification and enrichment method for bioanalysis", Analytical Chemistry, vol. 85, no. 16, pp. 7762–8, 2013.*

Cover article.

INTRODUCTION

In the last decades the demand for rapid and accurate bioanalysis has increased. Effective sample preparation is an important step in the chemical analysis of biological samples. The main purposes of sample preparation are to (1) transport the analytes to a medium compatible with the analytical instrument, (2) remove interfering matrix components and (3) concentrate the analytes, i.e. increase the concentration in the analysis solution compared to the concentration in the sample. Commonly used sample preparation methods include protein precipitation, liquid-liquid extraction and solid phase extraction[1]. For special applications special modifications of these approaches have been developed, such as e.g. single droplet microextraction (SDME) in where a hanging immiscible organic droplet is inserted in an aqueous sample in order to perform liquid-liquid extractions[2]. Although not often used yet, electrically driven sample pretreatment methods are increasingly reported such as electromembrane extraction (EME), electroextraction (EE)[3] and online preconcentration techniques in capillary electrophoresis[4]. The latter, however, are not further considered in this article as for complex biosamples additional prior steps such as removal of plasma proteins is necessary. Sample preparation procedures based on electromigration are very promising, since a considerable part of the analytes in bioanalysis include compounds that are or can be charged (e.g. metabolites, xenobiotics and drugs) and thus are suitable for an electrophoretic approach. A recent three-phase elektrokinetic sample preparation method is EME[5]–[10]. In this technique, an organic solvent is held by capillary forces in the pores of a polymeric membrane. This membrane acts as a phase boundary between the aqueous donor and acceptor solutions. The analyte of interest is extracted from the aqueous donor phase on one side of the supported liquid membrane, through the organic membrane into the aqueous acceptor phase on the other side of the supported liquid membrane. This promising method has been successfully applied to the analysis of several pharmaceuticals in plasma and urine and also low-abundant peptides in human plasma[11], [12]. Another rapid electromigration-driven technique is 2-phase EE, which takes place in a two phase liquid-liquid system consisting of an aqueous and an organic phase, where an electric field causes the analytes to be extracted from the organic donor phase into the aqueous acceptor phase[13]–[15]. Ions in the organic phase are subjected to very high electric field strength due to low conductivity. As a consequence, ions in the organic phase will migrate at high velocity, to be concentrated just after the liquid-liquid interface since the electric field of the aqueous acceptor is much lower. 2-phase EE has been successfully applied to several endogenous metabolites like peptides and carnitines and also to standard solutions of leukotriens and catecholamines[16], [17].

Direct infusion-mass spectrometry (DI-MS) is a rapid, comprehensive analytical method without the use of chromatography prior to MS. By using nanoelectrospray (nanoESI) instead of conventional ESI, ionization efficiency is enhanced, only a small amount (sub)-µL of sample is needed and ion suppression is reduced or even eliminated[18]. However, nanoESI emitters (with internal diameters of typically a few µm) are susceptible to clogging due to protein precipitation, salt crystallization and dust/impurities which drives for an efficient sample preparation prior to analysis. Moreover, when a selective sample preparation procedure is chosen, ion suppression could be further reduced, since the enriched samples may contain less molecules when co-introduced in the MS. Additionally, coupling electrokinetic driven sample preparation techniques to nanoESI would be very suitable, since cations or anions are selectively migrating.

In this research paper we present 3-phase EE which enables fast migration of analytes from an aqueous donor phase through an immiscible organic filter phase into an aqueous acceptor

phase by applying an electric field between donor and acceptor, without the use of a membrane like in EME. Moreover, in this system the analytes do not have to be dissolved and diluted in the organic phase prior to extraction, opposed to 2-phase EE. By locating the acceptor phase in a conductive pipette tip, a convenient implementation of the whole sample preparation procedure is realized, enabling easy integration with downstream applications. Extraction is realized by dispensing a hanging acceptor droplet out of the conductive pipette tip into the immiscible organic filter phase and applying an electric field between both aqueous donor and acceptor phases (see Figure 1A). Compared to SDME our method is stagnant, but moreover the driving force is an applied electrical field rather than a distribution coefficient, and by that, a more complete extraction can be achieved into such a small volume. The organic phase placed on top of the donor phase prevents proteins being transported into the acceptor phase and furthermore, by changing its composition, selectivity of the extraction can be tuned. In this way it is possible to extract analytes from a relatively large donor volume into a small droplet, since only (sub)-µL volumes are needed for performing nanoESI. In this paper, we introduce and characterize the 3-phase EE process by studying the influence of extraction time and voltage, organic phase selectivity and protein elimination, and we demonstrate its potential in combination with mass spectrometry-based bioanalysis by the extraction of acylcarnitines spiked to plasma.

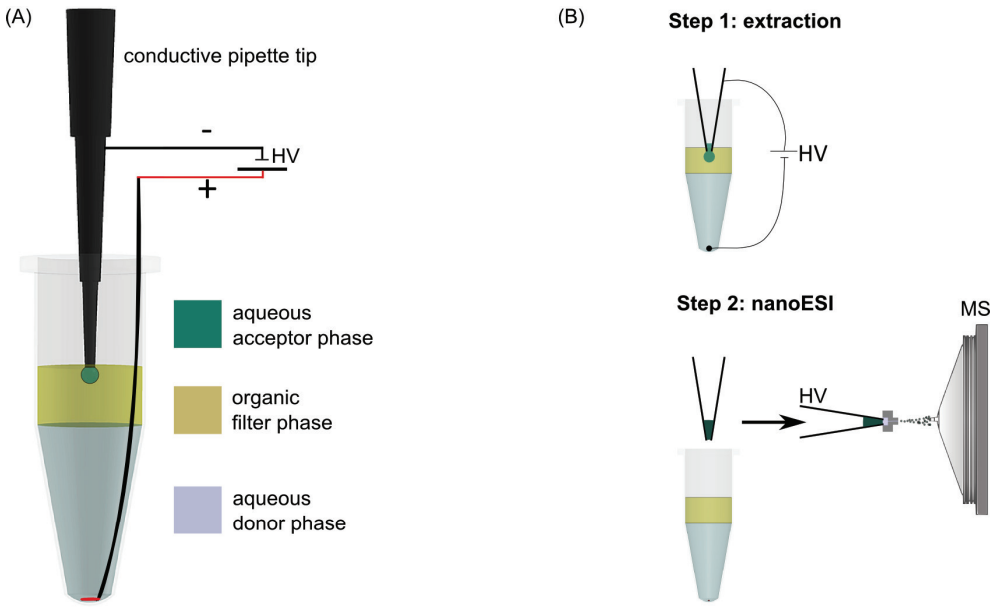


Figure 1: Schematic illustration of (A) the 3-phase EE setup and (B) 3-phase EE followed by nanoESI-DI-MS analysis

EXPERIMENTAL SECTION

Chemicals

Methanol was from Biosolve (Valkenswaard, The Netherlands). Water (dH_2O) was obtained from a Millipore high purity water dispenser (Billerica, USA). Ethylacetate (EtoAc), methylacetate (MEtoAc), bis(2-ethylhexyl)phosphate (DEHP), crystal violet, bovine serum albumine (BSA), cytochrome C (Cyt C), formic acid (FA), L-carnitine, acetyl-l-carnitine, propionyl-L-

carnitine DL-hexanoylcarnitine, DL-octanoylcarnitine, DL-decanoylcarnitine were obtained from Sigma-Aldrich (Steinheim, Germany). Deuterated butyryl-L-carnitine-d_3 was obtained from CDN Isotopes (Pointe-Claire, Canada) and was used as an internal standard. Butyryl-L-carnitine was bought from Larodan AB (Malmö, Sweden). All solvents were HPLC grade. Human plasma (EDTA) was obtained from healthy volunteers and combined in a plasma pool.

3-phase EE

All 3-phase EE results were obtained using an off-line setup (except for the experiments as described in the last paragraph). The equipment used for the off-line 3-phase EE is illustrated in Figure 1A. The DC power supply used had a voltage range from 0-2 kV, delivering a current of maximum 0.5 mA measured with a Keithley 485 picoammeter. A platinum wire (diameter = 0.28 mm) isolated with a Teflon sleeve was stripped from the end (1 mm) resulting in the electrode at the bottom of the donor phase. The pipette tip made from a conductive polymer (Advion, Ithaca, USA) was used as the counter electrode. A 10 µL syringe was connected to the pipette tip in order to aspire and dispense the acceptor phase. Off-line 3-phase EE was performed according to the following procedures; first the platinum electrode was inserted into a 500 µL eppendorf tube. The donor phase was pipetted (50 µL) into the eppendorf tube, followed by 150 µL of the immiscible organic filter phase as a layer above. The organic phase was saturated with dH_2O in order to conduct ions and to prevent the acceptor droplet from dissolving. The pipette tip, filled with 2.5 µL aqueous acceptor phase, was inserted into the immiscible organic filter phase after which 2 µL was dispensed so that a hanging droplet at the tip was created and a voltage (typically 140V) was applied for 3 minutes (or stated otherwise). After 3-phase EE, the voltage was switched off and the 2 µL acceptor droplet was aspired back into the pipette tip and transferred for analysis (Figure 1B). The organic filter phase used was a composition of EtoAC:MEtoAC (3:2) or stated otherwise in the selectivity experiments. Using this composition, the organic filter phase was immiscible with the donor and acceptor phase. In all experiments the donor phase consisted of 33% methanol (to decrease conductivity, thus increase the electric field strength) and in case of the plasma experiments also 5% FA was added to reduce plasma protein binding of the model analytes. The acceptor phase consisted of 33% methanol (to enhance ionization efficiency in ESI) and 5% FA (to anticipate on possible pH drop due to electrochemical reactions). Moreover, using these aqueous compositions, the essential condition of immiscibility with the organic filter phase was still maintained. The internal standard was spiked to the acceptor phase prior to 3-phase EE.

Mass Spectrometry

The 3-phase electroextraction analyses were performed by DI-MS in the positive mode using an automated nanoESI (Advion Triversa NanoMate, Ithaca, USA) source coupled to a LTQ-Orbitrap XL (Thermo Fisher Scientific). In the off-line setup, the 2 µL acceptor was pipetted into Eppendorf 384 well plate in order to be infused with a back pressure of 0.25 psi and an electrospray voltage of 1.55 kV in the positive mode.

The inlet capillary temperature was 120 °C, the capillary voltage and the tube lens voltage was 30 and 100 V, respectively. Mass spectra were recorded at a resolution of 30,000 and 10-20 scans were averaged in order to further process the data.

RESULTS AND DISCUSSION

The general operation of 3-phase EE is illustrated in Figure 1A. The sample (50 µL) was pip-

peted into an Eppendorf tube and 150 µL of the immiscible organic filter phase was placed on top as a second layer. For a visual proof of concept of the general operation, an experiment was conducted using a 10 µM crystal violet (cationic dye) standard in the donor phase. The result before and after 3-phase EE is shown in Figure 2. After 3 minutes the donor phase appeared depleted of crystal violet, whereas the acceptor droplet was enriched with crystal violet (see Movie 1, Supporting Information). In addition the movie shows that in the acceptor droplet the extracted crystal violet appeared to distribute homogeneously within seconds caused by diffusion and convection: this shows that a possible problem due to improper mixing of analytes and internal standards spiked to the acceptor phase prior to 3-phase EE does not occur.

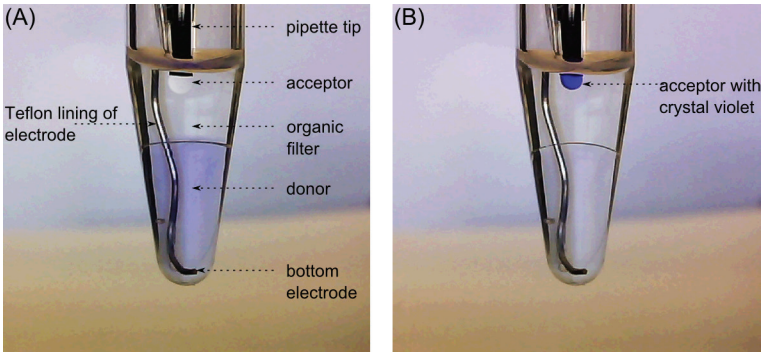


Figure 2: Video stills of crystal violet subjected to 3-phase EE at (A) t=0, no voltage applied, (B) t=3 minutes after applying the voltage.

Two processes play an important role in 3-phase EE: (1) The driving force in 3-phase EE is the applied electrical field between the donor and acceptor phase. Therefore, to enable 3-phase EE, the whole system comprising the donor, organic filter and acceptor phase should serve as an electrical circuit. Represented in a simplified manner, the electrical circuit model consists of three serial resistors representing the three phases. Since the resistance associated with the organic phase is dominant, it limits the current through the system. In order to fine-tune the system, the conductivity of the phases can be altered by changing the composition/polarity. The donor phase consisted therefore of 33% methanol: the addition of methanol decreased its conductivity and thus increased its electric field and the analyte velocity in the donor phase during 3-phase EE.

Initially, during 3-phase EE, ions migrate from the donor phase towards the organic filter interface at a velocity imposed by their electrophoretic mobility (μ) and the electric field strength (E) in the donor phase[19]–[21]. Once arrived in the organic filter phase, ions are subjected to a much higher electric field strength due to the low conductivity. As a consequence, ions in the organic phase migrate at a higher velocity and concentrate just after the organic filter-acceptor interface, as the electric field strength in the aqueous acceptor phase is much lower. A practical limitation of the applied electric field strength is the stability of the hanging droplet. In our experiments at voltages higher than 250 V the droplet is pulled off due to electrostatic force. Therefore we used 140 V to obtain reproducible results, to be sure of not losing the droplet even at extraction times up to 10 minutes.

(2) The distribution of the analytes based on the partition coefficient K_1 at the donor-organic filter interface plays an important role in 3-phase EE as well. Due to the low electric field strength in the donor phase the ions will arrive at the interface at moderate velocity, therefore K_1 determines the transport rate of the ions from the donor into the organic filter phase. In

addition, in our setup the total migration length (thus migration time) in the donor is only a few millimeters therefore further minimizes the influence of electrophoretic mobility on the transport into the organic filter phase. The partition coefficient K_2 at the organic filter-acceptor interface does only play a role when the analytes are rather apolar since ions are crossing this interface at high velocity due the high electric field strength in the organic filter phase. As a consequence for the rather polar analytes K_1 and the rather apolar analytes K_2 causes the selectivity of the system, which is elaborated in the next paragraphs.
Electrochemical reactions of analytes could occur at the surfaces of both electrodes. In all the experiments no significant gas formation was observed with the extraction voltage used, except during the longest extraction times of 10 minutes. This was tackled by aspiring the formed gas bubble into the pipette tip, but was not required at the conditions finally chosen.

Calculation of Enrichment.
The enrichment factor EF of 3-phase EE was calculated according to equation 1 for each analyte:

$$EF = \frac{[acceptor]_{normalized\ intensity}}{[donor]_{beforeEE,normalized\ intensity}} \quad (1)$$

where the normalized intensity was obtained by dividing the absolute intensity of the extracted analyte by the absolute intensity of the internal standard spiked to the acceptor phase prior to 3-phase EE. The normalized response of the donor phase was obtained by spiking that phase with internal standard and infuse it without the 3-phase EE step. To justify these calculations, the absolute intensities of the internal standard were checked; they did not significantly change during experiments because of possible electrochemical degradation or diffusion into the organic filter phase. For a good comparison, the donor was analyzed under the same conditions as the acceptor.

Effect of extraction time and voltage on enrichment
Seven carnitines with increasing size (and decreasing electrophoretic mobility[22]) and decreasing polarity were selected as model analytes (Supporting Table 1). Using these model analytes the dominant influence of the polarity of the analytes and thus the partition coefficient K_1 and K_2, in 3-phase EE could be demonstrated. In these experiments, the enrichment factor of the model analytes was studied as a function of 3-phase EE time and voltage. Enrichment in this setup was achieved by extracting the analytes from 50 µL donor to a 2 µL acceptor droplet. As a consequence the theoretical maximum enrichment factor was 25. The carnitines were always charged, since they are quaternary ammonium compounds. The acceptor phase was spiked with 1 µM butyrylcarnitine D_3 which was used as an internal standard for normalization. Experiments were performed with extraction times of 0.5, 1, 2, 3, 5 and 10 minutes at a voltage of 70 (Figure 3A) and 140 V (Figure 3B).
Applying an electrical potential difference of 140 V, several analytes were enriched close to the maximum enrichment factor, already within 3 minutes. In order to explore the depletion of the donor phase, three subsequent extractions were performed on the same donor and organic phase, refreshing only the acceptor phase after each extraction. These results show that decanoyl and octanoylcarnitine were hardly present after the first extraction indicating that for these analytes the donor was almost depleted. Another control experiment was conducted for transport based on 3 minutes of passive diffusion only, without applying the electrical potential difference. In this case none of the model analytes were detected. This proves that the

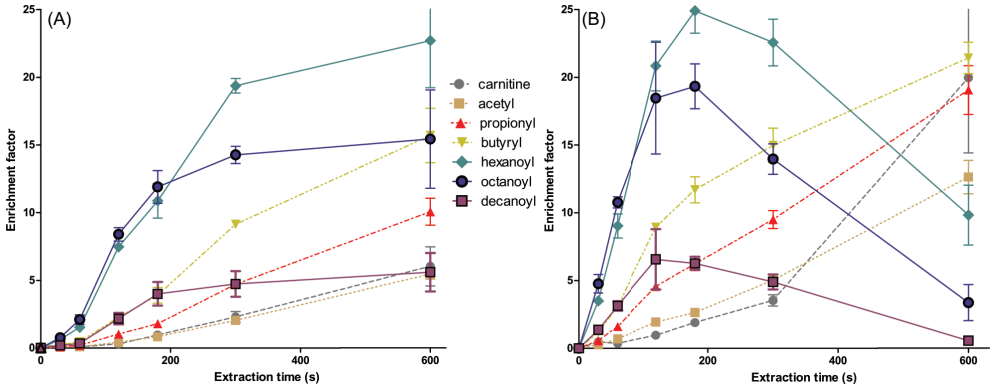


Figure 3: Average enrichment factor (n=3) versus extraction time of the 7 model analytes applying (A) 70 V and (B) 140 V.

transport speed from donor to acceptor phase was improved dramatically upon application of the voltage.

During the first 3 minutes the extraction rate (Δenrichment factor/Δextraction time) was higher at 140 V than when applying 70 V (Figure 3) which can be explained by the difference in the driving electrical field strength. In the first 3 minutes also the influence of both K_1 and K_2 is revealed: the most polar acylcarnitines (carnitine to hexanoylcarnitine) are extracted with an increased extraction rate as the polarity of the analyte increases, imposed by K_1. These results confirm that the partition coefficient K_1 across the aqueous-organic filter interface is the limiting factor for this settings, since hexanoyl-and octanoylcarntine were extracted faster than the smaller carnitines, while their electrophoretic mobilities are lower. For the most apolar carnitine (decanoylcarnitine) the extraction rate decreased with an increase in apolarity, which is caused by the influence of K_2, which becomes important for more apolar compounds. After 3 minutes the three most apolar carnitines started to decrease over time. This decrease was caused by back extraction from the acceptor phase into the organic filter phase, imposed by K_2. This is explained by the fact that the electrical field density is not homogeneously distributed around the acceptor droplet being significantly less dense at the top surfaces of the acceptor droplet (see finite element simulation, Supporting Figure 1). Analytes could reach the top of the acceptor droplet by diffusion and/or possible convection. To test this hypothesis, two follow-up experiments were performed. First we spiked 10 µM of the carnitines to the acceptor phase and using a blank donor when no electrical field was applied, more than 95% of the three most apolar carnitines were diffused into the organic filter phase after 10 minutes of extraction, solely caused by partition coefficient K_2. Secondly this experiment was repeated, but an electrical field was applied. Now the three most apolar carnitines were back extracted into the organic filter phase to about 50% of their total amount, which is comparable with the actual decrease we observed during 3-phase EE after 10 minutes of extraction. Other possible causes for the decrease such as electrochemical degradation and adhesion to the pipette tip were excluded (data not shown).

These characterization results indicate that for each application with its associated target analytes an optimal organic filter phase should be chosen (see next paragraph). Probably, possible back-extraction can be reduced by increasing the surface of the bottom electrode of the well and optimizing the geometry of the setup.

Influence of the organic filter phase composition on enrichment

To further show the influence of the partition coefficient K_1 for 3-phase EE, experiments with different organic filter phase compositions were conducted and the enrichment factor was calculated. By mixing organic solvents and by adding an ion pair modifier, the polarity of the organic filter phase, therefore K_1 and K_2, can be changed to tune selectivity of 3-phase EE. By adding an ion pair modifier (e.g. DEHP), the organic filter phase becomes more accessible for ions and therefore making K_1 more favorable towards polar analytes. Organic filter phases with increasing polarity were tested: EtoAC, EtoAC:MEtoAC (3:2), EtoAC+1% DEHP and EtoAC+5% DEHP. The acceptor phase was spiked with 1 µM butyrylcarnitine d_3 as an internal standard for normalization purposes. Figure 4 shows that by increasing the polarity of the organic filter phase, the optimum analyte apolarity shifts to lower values while seemingly narrowing the polarity window, and hence increasing selectivity. It can be observed that by using pure EtoAC the more apolar carnitines were most enriched, while transport of the smallest polar analytes was (almost) disabled at the same time due to the partition coefficient K_1. In contrast, by adding 5% of the modifier DEHP, the extraction was more selective towards the polar carnitines and the largest (apolar) decanoylcarnitine was hardly enriched. These findings lead to the important conclusion that selectivity in 3-phase EE can be tuned. As a consequence, the selection of the composition of the organic filter phase should be tuned according the analytes of interest. These observations are in agreement with applications of EME which reported that the chemical composition of the membrane influenced selectivity[3].

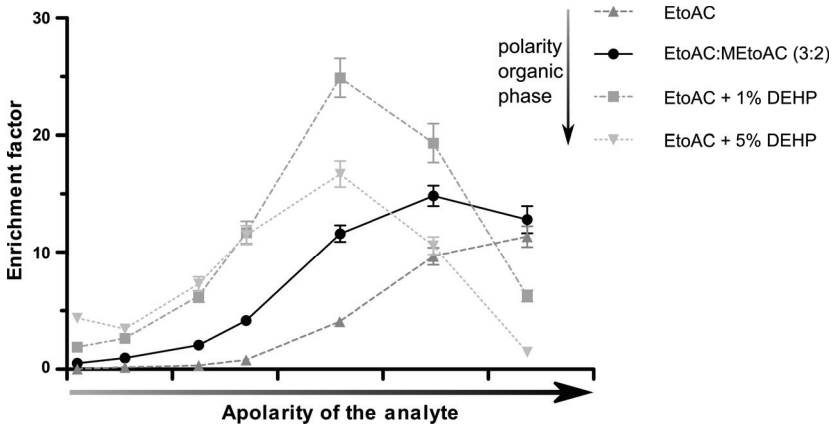


Figure 4: Average enrichment factor versus apolarity (expressed as log P) of the analyte and the influence of the organic filter phase ((n = 3), all RSD<15%).

Sample purification

In the following experiments an academic test solution (500 µg/mL) of a large model protein BSA (MW = 66.5 kDa) and and a smaller model protein Cyt C (MW = 12 kDa) was tested. BSA was chosen because albumine is the most predominant (55%) blood plasma protein[23] and Cyt C was added to assess the behavior of small proteins in the system. The protein standard solutions were subjected to 3-phase EE-DI-MS and was compared to DI-MS of the donor phase before 3-phase EE to which 5% FA added. Supporting Figure 2A shows that the MS spectrum of the BSA standard consists of a broad BSA envelope centered around 1500 *m/z*, obtained for the analysis of the donor phase which is not observed with 3-phase EE-DI-MS (Supporting Figure 2B). Supporting Figure 2C shows the MS spectrum of the Cyt C

standard consists of two smaller envelopes centered around 1537 *m/z* and 1752 *m/z*, opposed to the MS spectrum of the 3-phase EE extract (Supporting Figure 2D). These results show that the large BSA as well as the much smaller Cyt C are not transported into the acceptor phase during 3-phase EE. These results confirm the same selectivity principle (partition coefficient K_1) discussed in the previous paragraph: since the proteins do not dissolve (precipitation) in the used organic filter phase, they did not pass the donor-organic filter interface during 3-phase EE. Additionally, in the next paragraph the same mass range was scanned for the presence of proteins in spiked plasma. In these experiments protein precipitation was observed between the donor and organic filter phase.

Spiked plasma

To finish the evaluation of 3-phase EE, the acylcarnitines were spiked into samples of a pool of human blood plasma. By spiking the carnitines the plasma was diluted 10-fold in 33% methanol and 5% FA (pH=2.0), in order to make a valid comparison with the academic test mixture experiments. After dilution no protein precipitation was observed at this level of MeOH and FA. Subsequently the samples were subjected to 3-phase EE. The model analytes were effectively extracted from human plasma (Table 1). These results show that 3-phase EE can be used for the enrichment of complex samples. For all model analytes slightly lower enrichment values were obtained from human plasma samples compared with those of the academic test solution (Figure 4). This can possibly be explained by the plasma protein binding of the model analytes. Marzo *et al.* reported that carnitine and its short-chain esters did not interact with plasma proteins while octanoylcarnitine interacted to a poor extent (12-30%), and the larger carnitines were completely bound to plasma proteins[24]. Acidification of the donor phase with 5% FA appeared to be necessary, as without little enrichment was observed: upon acidification proteins are protonated, resulting in less binding of the model analytes. Since the plasma samples had high viscosity, high protein content and are more complex than the previously examined standard standard solutions, longer extraction times were needed to obtain higher enrichment (Figure 4). After 9 minutes of extraction, the smallest carnitines were enriched approximately 3x more (Table 1), whereas the largest carnitine did not benefit from the longer extraction time, due to the back extraction process into the organic phase as discussed earlier (Figure 3B).

Table 1: Enrichment values of the carnitines (500 nM equimolar) spiked in human plasma, (n = 3)[a],

analyte	EF_{3min}	EF_{9min}
carnitine	1.0*	2.8*
acetylcarnitine	0.7*	2.0*
propionylcarnitine	1.8	4.4
butyrylcarnitine	2.5	5.4
hexanoylcarnitine	3.9	7.0
octanoylcarnitine	5.1	6.1
decanoylcarnitine	4.7	3.1

[a]All RSD <15%; * = corrected for the endogenous level (Table 2).

As a reference the plasma donor phase spiked with 500 nM of carnitines was analyzed directly. This resulted in an unstable nanoelectrospray signal possibly caused by the abundant plasma proteins which was also visible as a broad envelope in the 1000-2000 *m/z* range, and moreover only a few model analytes could be detected close to their limit of detection. These findings demonstrated the potential of 3-phase EE as a rapid and effective sample cleanup and enrichment technique for bioanalysis.
Calibration curves were obtained by spiking the acylcarnitines (0, 10, 50, 100, 500 and 1000 nM) and 1 μM butyrylcarnitine d_3 as an internal standard to the plasma and for carnitine (0, 10, 50, 100, 500, 1000 and 5000 nM). The performance results are summarized in Table 2.

Table 2: Analytical performance of 3-phase EE-DI-MS of carnitines spiked to human plasma[a]

analyte	Linearity (R^2) 10-1000 nM	LOD donor solution (nM)	LOD blood* (nM)	Concentration blood* (μM)
carnitine	0.999	-	-	26
acetylcarnitine	0.997	-	-	0.8
propionylcarnitine	0.994	9	90	x
butyrylcarnitine	0.989	29	290	x
hexanoylcarnitine	0.987	33	33	x
octanoylcarnitine	0.995	28	280	x
decanoylcarnitine	0.992	29	290	x

[a] x< LOD; - = not possible to determine; * the donor solution was obtained by diluting 1:10, the LOD for blood and the concentration in blood was obtained by multiplying the concentrations obtained in the donor with 10. LOD was determined by $(Y_{blank} + 3SD_{blank} - intercept_{response\ curve})/slope_{response\ curve.}$

Linear behavior of 3-phase EE was observed by measuring the response of the analyte vs. their concentration over 2 orders of magnitude. The R^2-values of the linear regression line ranged from 0.987 to 0.999 for the compounds tested. Limits of detection[25] were in the nM range for most of the tested compounds and were improved 10-fold compared to the DI-MS analysis of the donor phase. The limit of detection of carnitine and acetylcarnitine could not be accurately determined, since no measurements could be done at levels close to the detection limit due to the relatively high endogenous levels[26]. In the future the overall limit of detection can easily be improved by using a larger donor volume and/or using a smaller acceptor volume, possibly at the cost of a longer extraction time. Also, future research will be directed to optimizing the organic filter phase composition to achieve a more efficient extraction. The repeatability of the 3-phase EE measurements in plasma showed RSD values below 15%, and in the future this can most likely be improved by automation.
The endogenous levels of free carnitine and acetylcarnitine were in agreement with literature[27], [28]. Interestingly, and also as expected, many other plasma components were observed in the MS spectra and by putative identification (based on exact mass), among others, several amino acids were annotated such as leucine and phenylalanine. This suggests that the used organic filter phase was also suitable for the 3-phase EE analysis of many amino acids. Future research has to be done in order to characterize 3-phase EE for nonpermanently charged compounds like amino acids and drugs.

Toward online 3-phase EE-nanoESI-DI-MS: proof of principle.
In the final experiments the first steps of the integration of 3-phase EE into a fully automated system using an automated nanoESI robot (Triversa NanoMate) were made.
A 96 polypropylene well plate was modified by removing the bottom of some of the wells and replacing it by a stainless steel plate which functioned as the anode (Supporting Figure 3). The mandrel of the NanoMate, which grabs the pipette tip and which is also electrically connected to the grabbed pipette tip, functioned as the cathode. Both electrodes were connected to a voltage source.
150 µL of the donor academic test mixture of 500 nM carnitines was pipetted in one of the wells containing a bottom electrode, followed by 250 µL of the organic filter phase on top. A sequence was programmed in the NanoMate Chipsoft software which (1) aspired 1 µL of the acceptor phase from a certain well, (2) moved the pipette tip to the extraction well in which it was positioned in the organic filter phase, (3) dispensed a 1 µL droplet and waited 3 minutes to perform the extraction, (4) subconsequently aspired the acceptor droplet back into the pipette tip and (5) performed nanoESI. The required extraction voltage was only 55 V in order to get the same extraction currents as for the off-line experiments and was manually turned on and off using the external voltage source, but in the future this can also be automated. The first proof of principle result is shown in Supporting Table 2 which revealed an about 5-fold increase of the enrichment factor, compared to the off-line results in Figure 4. This increase in the enrichment factor can be explained by the 2-fold smaller acceptor volume and the 3-fold larger donor volume. These results demonstrate the potential of 3-phase EE as an easy automated, high-throughput sample preparation method. Further research and development has to be done on improving the on-line setup into a fully automated, high-throughput screening platform.

CONCLUSIONS AND OUTLOOK

In this paper, we demonstrated the fast and selective electromigration of analytes from an aqueous sample through an immiscible organic filter phase into an aqueous acceptor phase. We demonstrated that this technique, which we described as 3-phase electroextraction (3-phase EE), can be efficiently used for sample purification in bioanalysis, since the organic phase prevents proteins from being transported into the acceptor phase. Selectivity can be tuned by proper selection of the composition of the organic filter phase, being an attractive feature of 3-phase EE. Enrichment of the analytes was demonstrated by extracting them from a 50 µL sample into a small 2 µL acceptor droplet. Using academic test solutions, several of the model compounds were enriched close to the expected enrichment maximum, already within 3 minutes. Moreover, 3-phase EE was found to be suitable for extracting and enriching spiked carnitines from human plasma samples. 3-phase EE coupled to direct infusion-MS of plasma showed a good linear response over two orders of magnitude and improved detection limits and importantly, resulted in a stable protein-free nanoelectrospray-MS signal. Since the maximum enrichment factor is proportional to the volume of the donor phase and inversely proportional to the volume of the acceptor phase, 3-phase EE could easily be used to achieve a 100-fold or more enrichment. In addition, future research will be done on optimizing the composition of the organic filter phase, on 3-phase EE geometries in order to maximize fast enrichment and on extending the type of analytes.
Finally, proof of principle of the integration of 3-phase EE in a commercially available on-line robotic nanoESI-DI-MS system was achieved. Next to coupling to DI-MS, 3-phase EE can also be integrated in other downstream (separation) applications such as LC and CE. Thanks

to its combination of sample purification, selective enrichment of analytes from biofluids to an aqueous phase and the simplicity of the setup, 3-phase EE holds the promise to become a core sample preparation module in fully automated, high-throughput bioanalysis.

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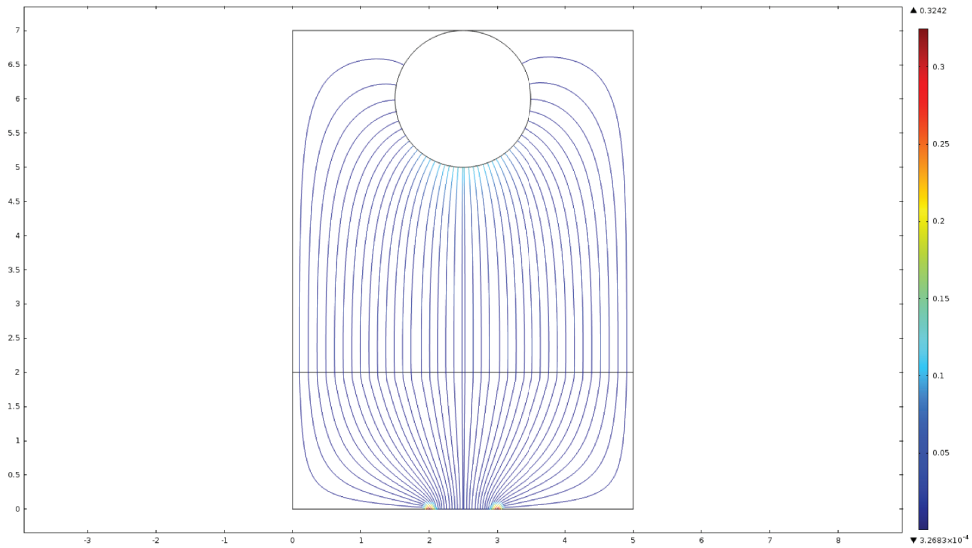
SUPPORTING INFORMATION

Supporting Table 1: Carnitines with increasing mass and increasing apolarity.

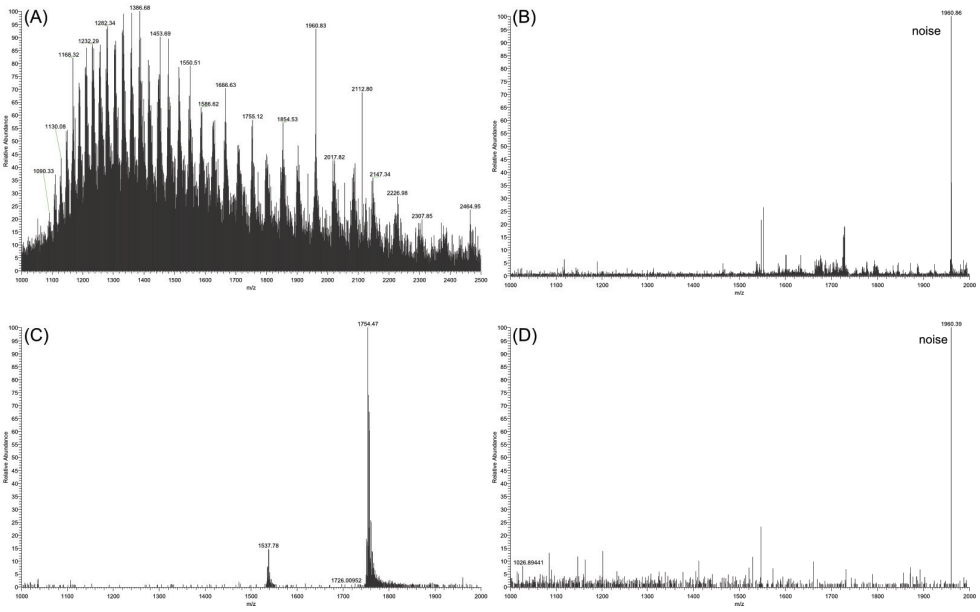
analyte	MW
carnitine	161.2
acetylcarnitine	203.2
propionylcarnitine	217.2
butyrylcarnitine	231.3
hexanoylcarnitine	259.3
octanoylcarnitine	287.4
decanoylcarnitine	315.1

Supporting Table 2: Enrichment factor of online 3-phase EE on an academic test mixture of 500 nM acylcarnitines (n = 1).

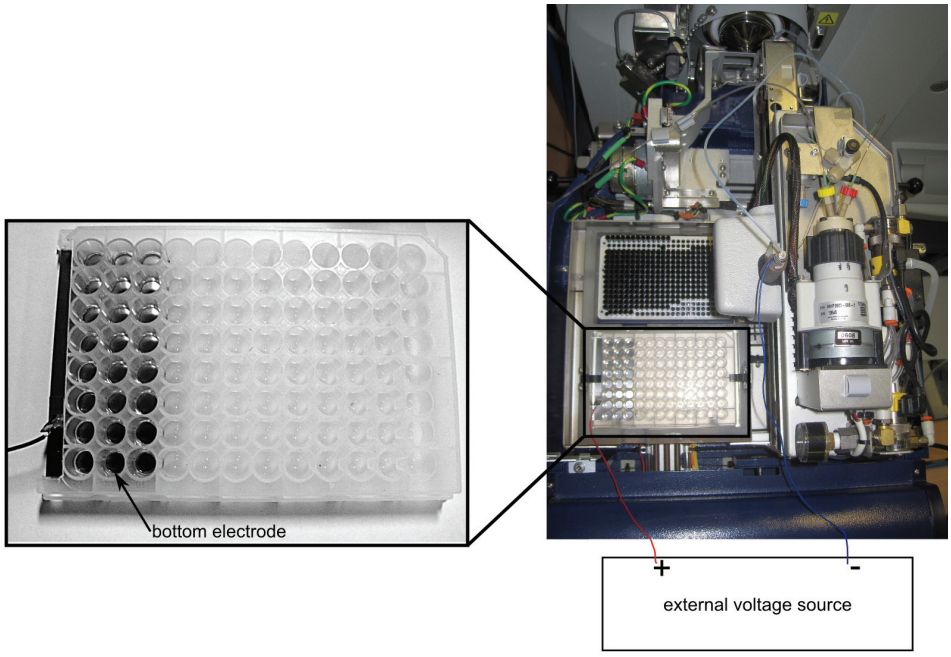
analyte	EF
carnitine	3.4
acetylcarnitine	5.1
propionylcarnitine	11.8
butyrylcarnitine	25.9
hexanoylcarnitine	70.5
octanoylcarnitine	77.3
decanoylcarnitine	81.9



Supporting Figure 1: Finite element simulation of the off-line 3-phase EE setup showing the electric field density distribution. As depicted, the electric field density is significantly lower at the top surface of the acceptor droplet, resulting in possible back-extraction processes imposed by K_2.



Supporting Figure 2: Averaged mass spectra of (A) BSA standard (500 µg/mL) (B) 3-phase EE of BSA standard (500 µg/mL) (C) Cyt C standard (500 µg/mL) (D) 3-phase EE of Cyt C standard (500 µg/mL)



Supporting Figure 3: Photo of a modified 96 polypropylene well plate with an electrode at the bottom of three well-rows integrated in the automated nanoESI robot.

Chapter 5

Gas Pressure Assisted Micro-Liquid-Liquid Extraction coupled Online to Direct Infusion Mass Spectrometry: a New Automated Screening Platform for Bioanalysis.

ABSTRACT

In the field of bioanalysis there is an increasing demand for miniaturized, automated and robust sample pretreatment procedures which can be easily connected to direct-infusion mass spectrometry (DI-MS) in order to allow the high-throughput screening of drugs and/or their metabolites in complex body fluids like plasma. Liquid-Liquid extraction (LLE) is a common sample pretreatment technique often used for complex aqueous samples in bioanalysis. Despite significant developments that have been made in automated and miniaturized LLE procedures, fully-automated LLE techniques allowing high-throughput bio-analytical studies on small-volume samples using direct infusion mass spectrometry, have not been matured yet. Here, we introduce a new fully-automated micro-LLE technique based on gas-pressure assisted mixing followed by passive phase separation, coupled online to nanoelectrospray-DI-MS. Our method was characterized by varying the gas flow and its duration through the solvent mixture. For evaluation of the analytical performance four drugs were spiked to human plasma, resulting in highly acceptable precision (RSD down to 9%) and linearity (R^2 ranging from 0.990 to 0.998). We demonstrate that our new method does not only allow the reliable extraction of analytes from small sample volumes of a few microliters in an automated and high-throughput manner, but also performs comparable or better than conventional offline LLE in which the handling of small volumes remains challenging. Finally, we demonstrate the applicability of our method for drug screening on dried blood spots showing excellent linearity (R^2 of 0.998) and precision (RSD of 9%). In conclusion, we present the proof of principe of a high-throughput screening platform for bioanalysis based on a new automated micro-LLE method, coupled online to a commercially available nano-ESI-DI-MS.

Based on

R.J. Raterink, Y. Witkam, R.J. Vreeken, R. Ramautar and T. Hankemeier, *"Gas pressure assisted micro-liquid-liquid extraction coupled online to direct infusion mass spectrometry: a new automated screening platform for bioanalysis."*

Submitted to Analytical Chemistry

INTRODUCTION

In the last decades there has been a strong demand for high-throughput MS-based technologies for bioanalytical and pharmaceutical screening studies such as in drug discovery and development[1]. Direct infusion-mass spectrometry (DI-MS) is an analytical technique which is in principle very suitable as a rapid and global high-throughput screening platform since it does not include often time consuming separation methods prior to MS detection[2]. However, as a consequence, DI-MS is susceptible to potential ion suppression effects due to interfering, co-eluting matrix components in the samples, especially when using electrospray ionization (ESI)[3]. By employing nano-electrospray (nanoESI) ionization instead of conventional ESI, the ionization efficiency can be somewhat improved as ion suppression is considerably reduced under low flow-rate conditions [4]. However, nano-ESI nozzles (with diameters of typically a few μm) are prone to clogging as a result of protein precipitation, salt crystallization and dust/impurities which asks for an efficient sample pretreatment prior to analysis. Moreover, when an effective sample pretreatment procedure is carried out, ion suppression may be further reduced, since the extract contains fewer molecules when co-introduced into the MS. Plasma is a complex sample due to the presence of proteins, salts and a wide range of (endogenous) metabolites with a chemistry which may be similar to the analytes of interest and/or causing ion suppression. Therefore, an efficient sample pretreatment procedure for the clean-up and selective extraction of the compounds of interest is often required for the reliable analysis of drugs in complex samples like plasma by DI-MS-based approaches.

With sample pretreatment being one of the major challenges in a typical (DI)-MS-based screening pipeline, significant progress has been made in the improvement of sample pretreatment procedures over the past few years[5]. New developments for sample pretreatment methods are often directed towards simplification, automation, miniaturization, integration in well-plate formats, more environment friendly and specificity enhancements of the clean-up and enrichment process[6][7]. Liquid-liquid extraction (LLE), next to solid-phase extraction (SPE), is a common sample pretreatment technique[8] which can be used to extract relatively apolar molecules from a complex aqueous biological sample phase into an immiscible organic phase. After the LLE procedure both fractions, containing polar and apolar compounds, can then be independently analyzed. Although LLE techniques are commonly used, their use in online systems is much less than e.g. SPE techniques. Moreover, most of the reported LLE systems are rather difficult to automate especially for small volume-samples of a few microliters due to the fact that conventional LLE requires a mixing and often a centrifugation step which are challenging to automate (e.g. in a multi-well format)[9]. Off-line sample pretreatment can be considered a limiting step for high-throughput bioanalysis of especially small sample volumes, indicating a clear need for on-line sample pretreatment procedures to shorten the total sample handling and analysis time and to reduce the possibilities for errors. In addition, when samples in a well plate are processed in a parallel manner prior to analysis, the handling time per sample is slow and variable and evaporation of volatile solvents and analyte degradation could be an issue.

An emerging trend in the field of biomedical and pharmaceutical analysis is to analyze drugs in limited sample amounts, such as dried blood spots (DBS) and plasma of mice (especially in the case of longitudinal studies the amount of plasma which can be collected from the same mouse, usually via the tail, is only a few microliters)[1]. Therefore, miniaturization and automation of the sample pretreatment procedure is needed in order to allow the analysis of drugs in these types of small samples in a very effective and high-throughput manner by MS. So

far, various miniaturized LLE strategies have been developed such as liquid-phase microextraction (LPME) approaches [7]. In LPME, extraction generally takes place from an aqueous sample phase into a small amount of a water-immiscible organic acceptor phase, and reducing the acceptor-to-donor ratio with typical enrichment factors between one and two orders of magnitude[10]. LPME has been developed in a variety of configurations such as single drop microextraction (SDME)[11][12][13], headspace LPME[14], hollow fiber (HF)-LPME[15][16], continuous flow LPME[17], solvent bar microextraction[18][19], dispersive liquid-liquid microextraction (DLLME)[20][21] and parallel artificial liquid membrane extraction (PALME) [22][23], among others. However, most of these LPME methods need a form of (off-line) agitation in order to get efficient extractions which will make it less straightforward to implement in a fully-automated high-throughput analytical platform. Interestingly, the on-line coupling of (micro)LLE procedures to nanoESI-DI-MS has, to our knowledge, not been explored yet.

Here, we present the proof principle of a new and simple approach to miniaturized and automated LLE, so-called gas pressure assisted micro liquid-liquid-extraction (GPA-μLLE) coupled online to nanoESI-DI-MS. Rapid extraction is realized by dispensing an aqueous sample phase into DCM after which both phases are mixed thoroughly by applying nitrogen gas pressure and flowing gas through the liquid phases. After passive settling of the phases under the mentioned conditions, 2 μL of DCM is aspired and consecutively analyzed with nanoESI-DI-MS. As a consequence, our method is easy to automate as it bypasses the need for off-line vortexing and centrifugation as is the current standard. Furthermore, as GPA-μLLE is performed in 384-well plate, it is compatible with handling small sample volumes. In addition, by the integration of GPA-μLLE in a commercially available nanoESI robot, GPA-μLLE is coupled on-line to DI-MS making it very suitable for a high-throughput screening platform. Moreover, in our method, every sample is sequentially processed which results in a short and constant handling time per sample which is also very suitable for volatile solvents such as DCM. The GPA-μLLE procedure is characterized by studying the effect of the gas pressure and its duration on the recovery of test analytes. Subsequently, the potential of micro-LLE coupled online to nanoESI-DI-MS for the extraction and screening of drugs from human plasma is demonstrated. A comparison between GPA-μLLE and conventional (off-line/manual) LLE is made, as well as with 'dilute and shoot' methods. Finally, the utility of GPA-μLLE for the screening of lidocaine in a DBS sample is shown.

EXPERIMENTAL SECTION

Chemicals and materials

Water (H_2O) and methanol was bought from Actu-All Chemicals (Randmeer, The Netherlands). Dichloromethane (DCM), crystal violet, fluorescein, lidocaine, propranolol, verapamil, loperamide were obtained from Sigma-Aldrich (Steinheim, Germany). Diltiazem and chlorpromazine were from Sigma-Aldrich (Steinheim, Germany) and used as internal standards. Diltiazem was used for normalizing lidocaine and chlorpromazine was used for normalizing propranolol, verapamil and loperamide. All solvents were HPLC grade. Human plasma (heparin) and whole blood (heparin) were obtained from adult healthy volunteers.

GPA-µLLE

All the GPA-µLLE-DI-MS steps were programmed and performed in a Nanomate robot (Advion Triversa NanoMate, Ithaca, USA) in a fully automated manner, except of the prefilling of the well-plate. The steps used for GPA-µLLE are illustrated in Figure 1. The GPA-µLLE was performed according to the following procedure: in a 384-well plate, two adjacent wells were prefilled with 14 µL aqueous sample (blue), consisting of 14-fold diluted plasma dissolved in 71.5% methanol, and 10 µL DCM (orange) as an immiscible organic phase (Figure 1A). The aqueous sample phase consisted of 71.5% methanol which was based on a Bligh and Dyer[24] extraction in order to get an efficient extraction and protein precipitation. Moreover, after mixing, some of the methanol and H_2O was dissolved in the DCM, making it suitable for nanoESI (pure DCM could not be electrosprayed). The aqueous phase was dispensed into the DCM from the bottom of the well (Figure 1B). Thorough mixing of both phases was accomplished by applying a gas pressure of nitrogen (in our system typically 0.5 psi, or stated otherwise) on a pipette tip (inner diameter of 0.5 mm) inserted at the bottom of the well for a certain time duration (typically 20 sec, or stated otherwise), see Figure 1C. During a short period of time (90 seconds), both phases were allowed to settle and separate (Figure 1D) and, subsequently, 2 µL of the DCM phase was aspired into a new pipette tip (Figure 1E) and transferred for analysis (Figure 1F).

Comparison with 'dilute and shoot' methods

The 'dilute and shoot' experiment was carried out after spinning down the precipitated proteins of the aqueous phase. After centrifugation, the supernatant mixture was directly infused into the MS using nanoelectrospray ionization using the same MS settings as for the direct infusion of the DCM extract after GPA-µLLE.

Dried blood spots

20 µL of whole blood spiked with lidocaine was pipetted on standard blood spot cards obtained from RIVM (the Netherlands). The dried blood spots (DBS) were punched out (3 mm diameter) from the middle of the spot and inserted into the bottom of a 384-well plate, which was placed in the NanoMate. 20 µL of aqueous solvent (71.5% methanol) including 100 nM diltiazem as an internal standard was added to the DBS and 30 minutes of passive extraction was chosen prior to GPA-µLLE-DI-MS.

Mass spectrometry

The GPA-µLLE on-line coupled to DI-MS (positive mode) were carried out using an automated chip-based nanoESI (Advion Triversa NanoMate, Ithaca, USA) source coupled to a LTQ-Orbitrap XL (Thermo Fisher Scientific). In the NanoMate ChipSoft software (version 4.3.3.1108) the Advanced User Interface (AUI) was enabled in order to program and preform all the steps needed for µ-LLE. Finally, a 2 µL extract was aspired and infused with a back pressure of 0.4 psi and an electrospray voltage of 1.47 kV in the positive mode. The well-plate was set to a temperature of 4°C in order to reduce the evaporation rate of DCM and to ensure a fixed temperature. At this temperature no visible loss of DCM was observed within the experimental time of around 2 minutes. To further control evaporation, a well-plate cover foil could be used. The inlet capillary temperature was 120°C, the capillary voltage and the tube lens voltage was 30 and 100 V, respectively. The automatic gain control and injection waveforms were enabled. Mass spectra were recorded at a resolution of 100,000 using one microscan and 10-20 scans were averaged in order to further analyze the data. The response of the drug compounds analyzed was normalized with the appropriate internal standard by

calculating the ratio of intensity of the drug and intensity of the internal standard.

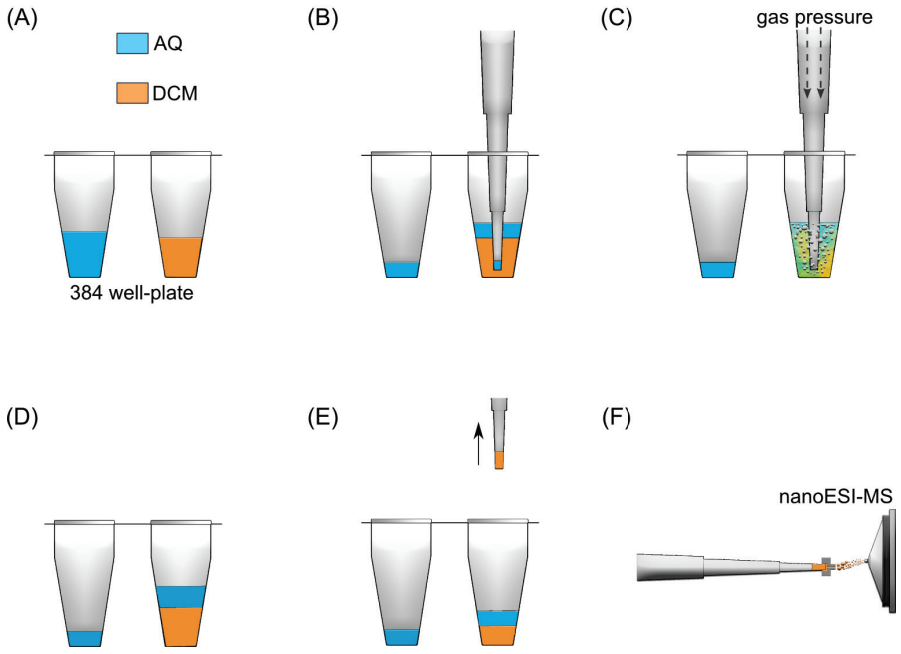


Figure 1: Schematic illustration of the individual steps of the GPA-μLLE method. (A) A 384 well plate is prefilled with an aqueous methanol-water (blue) sample and dichloromethane (DCM) (orange). (B) 14 μL of the AQ sample is pipetted into the bottom of the DCM layer. (C) Vigorous mixing is achieved by applying nitrogen gas pressure onto the pipette tip while the pipet is immersed in the liquid. (D) After mixing, both phases are allowed to settle and separate. (E) 2 μL of the DCM phase is pipetted and (F)directly infused into MS using nanoelectrospray ionization.

RESULTS AND DISCUSSION

In this section the proof of principle of GPA-μLLE is demonstrated followed by the characterization of two important parameters, i.e. the gas flow through the liquid mixture controlled by the applied gas pressure and its duration. Next, the performance of GPA-μLLE was evaluated and compared to conventional LLE and 'dilute and shoot' methods. Finally the utility of GPA-μLLE applied to DBS for drug screening was demonstrated.

Proof-of-principle of GPA-μLLE

The general working principle of GPA-μLLE is illustrated in Figure 1. For a visual proof of principle of the general operation, an experiment was conducted using a (green) mix of 59 μM crystal violet and 200 μM fluorescein as an aqueous sample. The results are shown in Figure 2. The aqueous sample was aspired from its well and dispensed at the bottom of the prefilled DCM well after which a bi-phasic system was generated (Figure 2B). After this step already some, but limited, extraction of crystal violet into the DCM could be observed, whereas, after 10 seconds of nitrogen gas-pressurized mixing (Figure 2C), the majority of the crystal violet was extracted. After 90 seconds the two phases were settled into a bi-layer system (Figure 2E).

During the mixing step the surface contact between both phases is enhanced and therefore the extraction efficiency is improved. From Figure 2D (taken 2 seconds after mixing) it can be seen that during mixing an emulsion of the organic phase and the aqueous phase was formed. Furthermore, the precipitated proteins are distributed in the aqueous phase, but not in the organic DCM phase, as can be observed in Figure 2F.

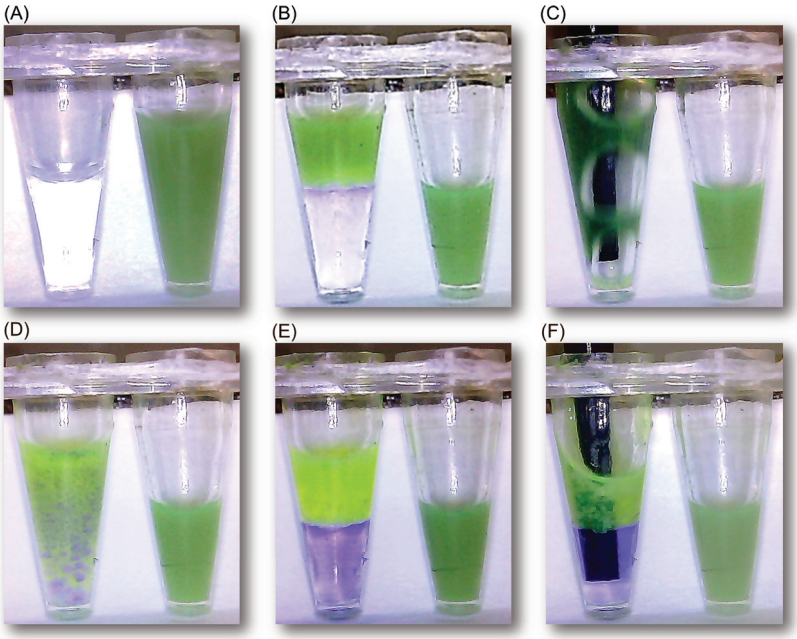


Figure 2 : Video stills showing the proof of principle of GPA-µLLE of a plasma sample: (A) 10 µL of DCM and 20uL of the aqueous sample are prefilled in adjacent 384-wells. (B) Bi-layer system after pipetting 14µL of the aqueous phase (consisting of 71.5% methanol containing a green mix of crystal violet and fluorescein and 14-fold diluted plasma) into the DCM. (C) Nitrogen Gas pressure assisted mixing of the phases. (D) After 2 second after mixing the created emulsion is clearly observed in which the crystal violet has been extracted from the aqueous phase into the organic phase, while fluorescein remains in the aqueous phase. (E) Bi-layer system after passive phase separation (typically around 90 seconds after the end of the mixing). (F) 2 µL is pipetted from the bottom of the DCM layer in order to be analysed by nanoESI-DI-MS. The precipitated proteins are visible in the aqueous phase.

Effect of gas pressure and its duration on extraction

To study the mixing due to the gas flow, the effect of gas pressure and its duration on the extraction efficiency during extraction was studied with four selected drugs as model analytes with varying size, pKa and log P (Supporting Table 1 and Figure 3). An equimolar mix of these drugs was spiked to human plasma (14-fold dilution in 71.5% methanol) at a concentration of 500 nM. Preliminary experiments using lower dilution factors resulted in less robust results (data not shown), probably due to the high protein content of human plasma.

To determine the extraction recovery, the steps after GPA-µLLE were modified: 2 µL of the DCM phase was mixed with 2 µL of DCM containing 1 µM of the internal standards and 2 µL of this mix was infused for DI-MS analysis. By adding the internal standards only after the extraction, we ensured that the internal standards could not be extracted into the aqueous phase during GPA-µLLE. As a consequence, we determined the relative recovery of the 4 drugs as the ratio of the intensity of drug and intensity of internal standard. Since methyl tert-butyl ether (MTBE) is a less toxic alternative to DCM[26], preliminary experiments with MTBE

instead of DCM were performed; however the infusion of MTBE extracts resulted in less stable nanoelectrospray currents (data not shown). Therefore, in all subsequent experiments DCM was used as an organic phase.
The gas flow through the tip was varied by choosing gas pressures of 0, 0.1, 0.3 and 0.5 psi for 2, 10 and 20 seconds (Figure 3). A practical limitation of the applied gas pressure with the used volumes is the sputtering of liquid out of the well, which can cause contamination of other wells. In our system at pressures higher than 0.5 psi, small amounts of liquid were sputtering

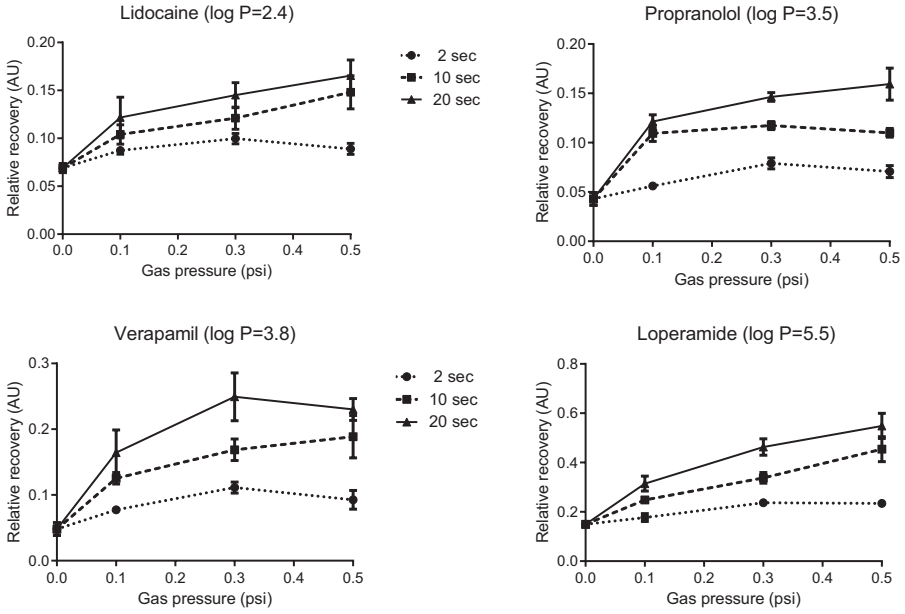


Figure 3 : Average of the relative recovery (ratio of intensity of drug and its internal standard) versus the gas pressure and its duration of the 4 drugs. Internal standards are added only after the extraction, see text; n=3, error bars are expressed as the S.E.M.

out of the well. Therefore 0.5 psi was the maximum pressure used in order to obtain reproducible results. As expected, by applying no pressure (0.0 psi) the relative recovery of the drugs was minimal (as already shown in Figure 2B). The fact that at 0 psi already some extraction was observed was caused by dispensing the sample at the bottom of the DCM layer (Figure 1B). From 0.3 psi upwards the recovery of the drugs did not seem to increase significantly, indicating that a higher gas flow at a higher pressure did not increase surface contact between both phases significantly any further. The duration of the applied gas pressure had a positive effect on the extraction: a duration of 2 seconds resulted in the lowest recovery, whereas a duration of 20 seconds provided the highest recovery. These results demonstrate that extraction of the drugs was improved significantly upon application of the gas pressure. Moreover, these results indicate that applying the gas pressure longer than 20 seconds would probably result in even higher recoveries. However, as there is a trade-off between speed and recovery we have chosen to minimize the time duration while still obtaining acceptable results.

Performance evaluation of GPA-µLLE

In the following experiments the analytical performance of GPA-µLLE was tested and compared with conventional off-line LLE, and direct infusion of the aqueous sample ('dilute and shoot') after spinning down the precipitated proteins. For the comparison of these methods the same nanoESI and MS settings were used. Calibration curves were obtained by spiking the four model drugs to 14-fold diluted plasma at the 0, 25, 50, 100, 250, 500 and 1000 nM level with diltiazem and chlorpromazine as an internal standard (500 nM). Again, after spiking the drugs, the aqueous sample phase consisted of 71.5% methanol. Subsequently, the aqueous samples were subjected to analysis by GPA-µLLE -DI-MS, conventional off-line LLE/DI-MS and 'dilute and shoot' DI-MS after protein spin-down of the aqueous sample. In order to compare GPA-µLLE with conventional LLE, the same aqueous: DCM solvent ratio as in GPA-µLLE was used, only the volumes were up-scaled in order to be able to perform the vortexing and centrifugation in 1.5 mL eppendorf tubes. After 30 seconds of vortexing followed by 10 minutes of centrifugation at 15 krcf, the DCM extract was carefully pipetted out of the tube and subsequently analyzed by DI-MS using the same settings as for GPA-µLLE. It should be mentioned that we also have tested a 'dilute and shoot' direct infusion of the aqueous phase without precipitation of proteins; however, as expected, we could not obtain a stable spray probably due to clogging of the nanoESI emitter caused by abundant plasma proteins.

For the 'dilute and shoot' method the same aqueous sample phase (after protein precipitation) was analyzed whereas for both GPA-µLLE and conventional off-line LLE the DCM phase was analyzed as described above. From Table 1 it can be seen that for the drugs tested, our new GPA-µLLE method performed comparable or better than the conventional off-line LLE in terms of repeatability, linearity and limit of detection. Linear behavior was observed by measuring the normalized response of the analyte versus their concentration up to 2-orders of magnitude. The R^2-values of the linear regression line of GPA-µLLE was good and ranged from 0.990 to 0.998 for the compounds tested. The precision of the GPA-µLLE measurements in plasma at 50 nM showed RSD values below 15%, except for lidocaine. This could be caused by a lower recovery due to lidocaine lowest log P and pKa, as extraction was carried out at neutral pH, (Supporting Table 1) and/or a lower ionization efficiency. The fact that the precision of our GPA-µLLE was improved over conventional LLE can be explained by the omission of the manual pipetting steps. The estimated LOD and LLOQ of GPA-µLLE seemed comparable with conventional, off-line LLE. The estimated LOD and LLOQ of loperamide were significantly higher than those of the other drugs. This is explained by the fact that a protonated ion with the same mass as loperamide was detected in the blank plasma, opposed to the other 3 drugs. In Figure 4, typical averaged mass spectra, zoomed in at the verapamil

Table 1: Analytical characteristics of GPA-µLLE of 14-fold diluted plasma compared with conventional off-line LLE; n=5.

analyte	precision(%) @ 50 nM[a]		linearity (25 - 1000 nM)		LOD (nM)[b]		LLOQ (nM)[c]	
	conv. LLE	GPA-µLLE	conv. LLE	GPA-µLLE	conv. LLE	GPA-µLLE	conv. LLE	GPA-µLLE
lidocaine	43	21	0.992	0.990	5	9	17	30
propranolol	11	9	0.990	0.996	3	4	10	13
verapamil	26	9	0.993	0.995	2	3	7	10
loperamide	20	12	0.989	0.998	25	30	80	99

[a] concentration in 14-fold diluted plasma. [b] LOD was estimated by $3(SD_{25nM} + SD_{blank}) / slope_{response\ curve}$.
[c] LLOQ was estimated by 3.3LOD.

m/z range from blank and spiked human plasma, after GPA-μLLE-nanoESI-DI-MS analysis are shown. It can be observed that for verapamil no protonated ion or carry over was detected in the blank plasma. Moreover, at the lowest dilution of 25 nM a protonated ion was clearly detected. It should be mentioned that the GPA-μLLE method did not require a centrifugation after mixing in order to facilitate phase settling as is required for conventional LLE methods. This was the advantage of the miniaturization of the whole LLE procedure, since only 2 μL of DCM had to be phase-separated for successful nanoESI-DI-MS analysis, which was well-achieved within 90 seconds under our conditions.

Since we analyzed the aqueous phase with 'dilute and shoot' instead of the DCM in both LLE methods, we did not include these results in Table 1. However, with precision up to 62%, the 'dilute and shoot' method after protein precipitation performed significantly worse than both LLE methods, as expected. This demonstrates that effective fractionation/pretreatment is important for the analysis of complex samples using DI-MS. These findings demonstrate the potential of GPA-μLLE as a rapid, fully-automated screening platform for small molecules in bioanalysis with excellent performance comparable with conventional off-line LLE. Moreover, as GPA-μLLE was integrated in a 384-well plate, it was compatible with handling small volumes in order of 2 μL with good precision. In principle, GPA-μLLE can be downscaled even more when smaller well-dimensions are used. Next to the analysis of the organic DCM phase after GPA-μLLE, the aqueous phase could in principle also be analyzed, depending on the application and the used solvents. However, due to high salt concentrations in a typical biological sample, ion suppression effects and potential clogging could occur when analyzing

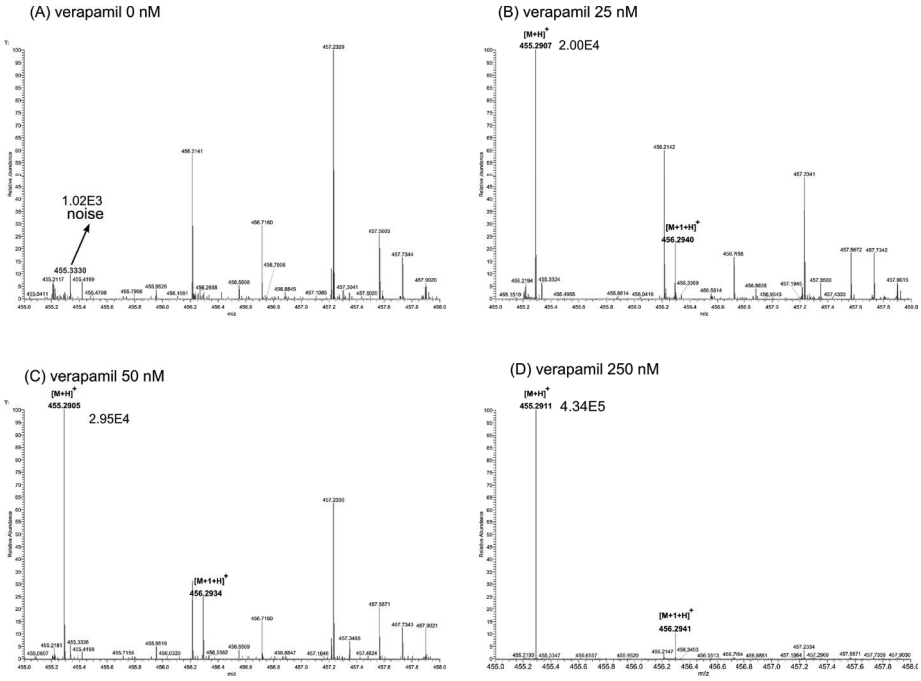


Figure 4: Typical averaged mass spectra zoomed in at verapamil (455-458 *m/z*) of blank and spiked human plasma analyzed with GPA-μLLE-nanoESI-DIMS. (A) In blank plasma, no protonated ion of verapamil was observed, while at (B) 25 nM concentration the protonated ion of verapamil as well as its isotopic ions can clearly be observed. As expected, at higher concentrations (C),(D) the intensities of the protonated and isotopic ions of verapamil is observed at higher levels.

the aqueous phase, especially when using DI-MS.
After mixing and settling of the phases, the precipitated plasma proteins were present in the aqueous phase (as observed in Figure 1F). During the last step of µ-LLE, in which 2 µL of the bottom DCM-phase is aspired for consecutive DI-MS analysis, the pipette tip punched through the protein layer. Although this step could lead to protein contamination into the pipette tip, no indications of proteins in the DCM extract were observed: the resulting nanoESI spray was stable (around 10 nA) and moreover, in the 1000-2000 *m/z* range, no protein envelopes in the mass spectra could be observed.

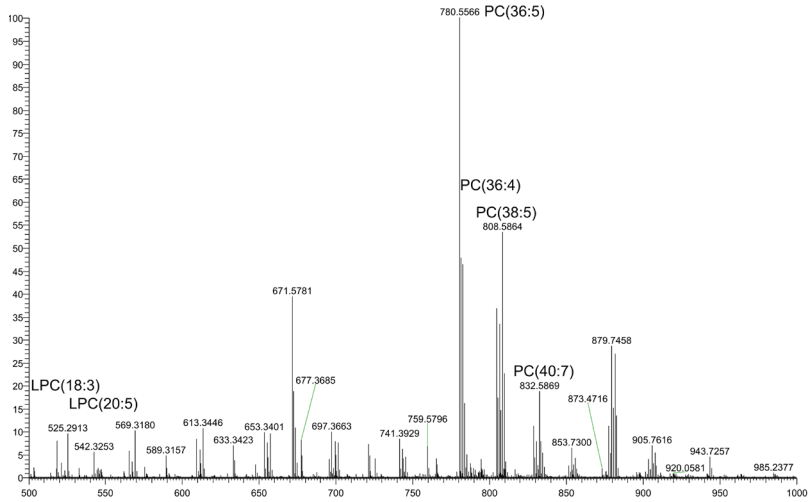


Figure 5: Typical averaged mass spectrum of the 500-1000 *m/z* mass range of (blank) human plasma analyzed with GPA-µLLE-nanoESI-DIMS. Several lipids were putatively annotated based on exact mass.

Interestingly and also expected, many other plasma components were observed in the MS spectra and by tentative identification (based on exact mass), e.g. several plasma lipids were identified in the higher mass range (500-1000 *m/z*) (Figure 5), suggesting that GPA-µLLE might also be suitable for lipid profiling. On the other hand, these observations suggest that ion suppression effects could occur during the DCM analysis on the tested drugs. In future, by fine-tuning the solvents used for extraction, an optimized method could be developed in order to minimize ion suppression effects in terms of sensitivity. Moreover, the use of stable isotopically labeled internal standards is expected to improve precision. In order to further verify the suitability of our method for lipid analysis, we calculated the precision of 4 typical plasma lipids: LPC (18:3), LPC (20:5), PC (38:5) and PC (40:7) from the blank plasma replicates with good RSD being 6, 9, 2 and 3%, respectively. Since no internal standard specifically for lipids was spiked, we normalized the 4 lipids with another endogenous abundant lipid, i.e. PC (36:5).
Since in GPA-µLLE the whole LLE procedure is automated, GPA-µLLE could be very suitable for rapid optimization of LLE procedures. Moreover, because in our method the samples are processed sequentially, the sample handling time is constant, making it also very suitable for extractive derivatization procedures. In this paper GPA-µLLE was integrated in an automated nanoESI-DI-MS robot and coupled online to MS. However, in the future GPA-µLLE could also be integrated into other platforms such as LC, CE and GC-MS, as long as the used auto-

sampler/ sample preparation robot has the ability to apply a (controlled) gas flow through the liquid mixture.

Application of GPA-µLLE to dried blood spot analysis

The developed GPA-µLLE was also applied to dried blood spots (DBS) samples because of the simplicity of the sample preparation procedure. A calibration experiment was carried out by spiking whole blood with lidocaine (0, 25, 50, 100, 250, 500, 1000 and 2500 nM) and spotting these on a card and drying it for 7 days. In these experiments, the blood was not diluted. Linear behavior was observed by measuring the normalized response of lidocaine versus its concentration over 3-orders of magnitude (Supporting Figure 1). The R^2-value of 0.998 of the linear regression line of GPA-µLLE was excellent. The precision at 1000 nM was 9%, which is a favorable value to determine lidocaine in in a complex sample. Actually, the therapeutic window of lidocaine in plasma is at an even higher concentration range, i.e. between 6 to 25 µM[27], proving the suitability of our method for clinical use. The aliquot of blood of the DBS punch was only 2.8 µL, resulting in a 7-fold dilution after adding the aqueous solvent.

These results demonstrate that GPA-µLLE is also very promising for the rapid screening of drugs in DBS which could be useful in the pharmacokinetic screening in volume limited samples such as mouse plasma. However, it should be mentioned that the punching of the spot was performed manually and in an off-line manner. In future, further miniaturization of the punched spot size could be aimed for, which would enable more samples from one DBS, possibly for other analytical measurements, or for backup samples.

CONCLUSIONS AND OUTLOOK

In this paper, we have presented a miniaturized and fully-automated LLE method for profiling of small molecules based on gas flow mixing via a pipette through the solvent mixture followed by passive phase separation, coupled to nanoESI direct infusion mass spectrometry. We have shown that this method, which we called gas pressure assisted micro liquid-liquid extraction (GPA-µLLE), can be effectively used for the online clean-up of complex samples which was needed for reliable drug screening using nanoESI-DI-MS. The coupling of GPA-µLLE to nanoESI-DI-MS was found to be suitable for the rapid extraction and analysis of spiked drugs to human plasma samples and importantly, resulted in a stable protein-free nanoESI-MS signal. Moreover, compared to conventional LLE, GPA-µLLE showed an improved or comparable precision, linearity and sensitivity. Furthermore, the extraction can be optimized by varying the gas flow and its duration. Finally, the utility of the GPA-µLLE-nanoESI-DI-MS was demonstrated for the analysis of lidocaine in DBS samples. In future, research could be directed towards further improvements of GPA-µLLE in terms of speed. By adding ultrasonic transducers below the well plate, extraction and phase settling times will probably be shortened. In addition, by using salting-out effect by addition of salt, the LLE procedure might also be faster and more efficient, only in case the organic phase is to be analyzed. Furthermore, GPA-µLLE is very suitable for performing rapid LLE optimization studies in which typical LLE parameters could be tested. Next to coupling to DI-MS, GPA-µLLE can also be integrated in other applications such as LC-MS, GC-MS. Moreover, in principle both aqueous as organic phase could be analyzed, depending on the application and solvents used. When large amount of samples needs to be processed, the well plate can be covered with a foil and/or the organic phase could be pipetted from a stock, in order to further control possible evaporation of solvents. Thanks to its combination of simplicity of the fully-automated setup and the ability to downscale LLE volumes, GPA-µLLE holds the promise to be integrated in many sample pretreatment modules for high-throughput screening in bioanalysis.

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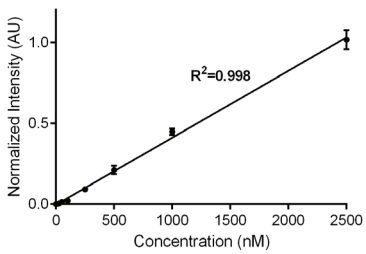
SUPPORTING INFORMATION

Supporting Table 1: Drugs with varying mass, log P and pKa values and the used internal standards. (* data from [25])

drug	Log P*	pKa*	MW
lidocaine	2.4	8.0	234.3
propranolol	3.5	9.4	259.3
verapamil	3.8	8.9	454.6
loperamide	5.5	9.4	477.0
chlorpromazine	5.4	9.3	318.1
diltiazem	2.8	8.2	414.2



Supporting Figure 1 : Calibration curve of lidocaine spiked to whole blood (undiluted) and applied as DBS, analyzed with GPA-μLLE-DI-MS. A linear regression line is fitted through the average (n=4) of the normalized response; error bars are expressed as the S.E.M.

Chapter 6

Testing tuberculosis drug efficacy in a zebrafish high-throughput translational medicine screen

ABSTRACT

The translational value of zebrafish high throughput screens can be improved when more knowledge is available on uptake characteristics of potential drugs. We investigated reference antibiotics and 15 preclinical compounds in a translational zebrafish-rodent screening system for tuberculosis. As a major advance we have developed a new tool for testing drug uptake in the zebrafish model. This is important because despite the many applications of assessing drug efficacy in zebrafish research, the current methods for measuring uptake using mass spectrometry do not take into account the possible adherence of drugs to the surface. Our approach combines nanoliter-sampling from the yolk using a micro-needle, followed by mass spectrometric analysis. As of to date no single physico-chemical property has been identified to accurately predict compound uptake; our method offers a great possibility to monitor how any novel compound behaves within the system. We have correlated the uptake data with high-throughput drug screening data from *M. marinum* infected zebrafish larvae. As a result, we present an improved zebrafish larvae drug screening platform which offers new insights into drug efficacy and identifies potential false negatives and drugs that are effective in zebrafish and rodents. Thereby we demonstrate that this improved zebrafish drug screening platform can complement conventional models of *in vivo M. tuberculosis* infected rodent assays. The detailed comparison of two vertebrate systems, fish and rodent, may give more predictive value for efficacy of drugs in humans.

Based on

R.J. Raterink*, A. Ordas*, H.J. Jansen, F. Cunningham, Malgorzata I. Wiweger, S.Jong-Raadsen, R.H. Bates, D. Barros, A.H. Meijer, R.J. Vreeken, L. Ballell-Pages, R.P. Dirks, T. Hankemeier and H.P. Spaink, *"Testing tuberculosis drug efficacy in a zebrafish high-throughput translational medicine screen"*

*shared first authorship
manuscript submitted to Antimicrobial Agents and Chemotherapy

INTRODUCTION

Studies in zebrafish larvae are increasingly used for modeling human diseases with the expectation that they will be able to contribute towards bridging the gap between *in vitro* cell based assays and *in vivo* mammalian disease models[1]–[3]. Zebrafish larvae have become a popular vertebrate model due to: i) their anatomical, molecular and genetic similarity to humans, ii) the availability of a large genetic toolbox, iii) their easy and low cost maintenance, iv) the optical transparency of embryos and larvae enabling massive phenotype-based screens, v) very few ethical issues associated with the use of larvae up to the feeding stage and vi) the occurrence of many pathological processes resembling various human diseases. Amongst numerous other human diseases, tuberculosis (TB) has been modeled in zebrafish as a potential complement to the currently established *in vitro* and *in vivo* models proven to recapitulate the pathology of the human disease progression in zebrafish larvae[4]–[7]. In these systems efficacy of a drug can be accurately measured by its effect on the number of bacteria and their presence and survival inside immune cells[8]–[12]. As a result, zebrafish larvae have already yielded significant new insights into the understanding of the pathogenesis of TB in humans and contributed to the development of novel strategies for disease treatment[7]. Such a gain in understanding is very important since therapy of TB is becoming increasingly difficult with the emergence of new strains of bacteria resistant towards currently used antibiotic regimes (MDR, XDR, TDR)[13]–[17]. Although new drugs are being discovered the progress in FDA-approval of anti-TB medicines is very limited[18]. Moreover, there is also a lack of effective vaccination against TB pending new approaches and strategies that are under evaluation for further improvement[13]. Therefore the need for more efficient novel drugs, targeting either the pathogen or the host, remains as high and urgent as ever[17], [19]. This task can only be accomplished by bringing together academic institutes and pharmaceutical companies with the aim of quickly implementing novel tools that have the potential of accelerating the drug discovery process. As an example of this strategy, GlaxoSmithKline (GSK) recently made publically available the structure and anti-TB properties of a set of 177 potent anti-tubercular compounds, making samples available to the industrial and academic research communities to stimulate early-stage TB drug discovery activities[20].

Given its previously mentioned attributes, zebrafish larvae can be potentially used at various stages of the drug discovery process, ranging from target identification and lead optimization to preclinical and clinical development[21]. The easy accessibility to large larvae numbers and their small size makes this model particularly suitable for high-throughput *in vivo* screening of drugs added to the medium[1], [2], [8], [22]–[24], significantly reducing material requirements for testing. However, caution must be taken when interpreting the data in the absence of knowledge on the way of absorption of drugs in the tissues of zebrafish larvae [25].

While the pharmaceutical industry is increasingly gaining confidence in the use of zebrafish larvae in a number of toxicity studies[21], [26]–[28], progress in the application of zebrafish as a possible alternative or complement to traditional *in vitro* and *in vivo* drug efficacy models has been limited[11], [29], [30]. Despite the previously mentioned benefits, a possible explanation behind this lack of progress is the limited, or altogether missing understanding on the basic pharmacological parameters in zebrafish driving compound efficacy, namely uptake, distribution and metabolism[31].

Mass spectrometry (MS) is an analytical technique which is very suitable for the quantification and qualification of small molecules such as drugs and its metabolites. In the current state-of-the-art zebrafish assays, larvae are treated by adding the compound into their water container, and uptake is assessed by LC–MS analysis of whole larval lysates[28], [31]–[33].

Here we demonstrate that the whole larvae lysis method is not always suitable for determining the uptake of drugs. This is due to the capacity of some molecules to persistently adhere to the skin, even after thorough washing, resulting in artificially high background readouts and false positive results for drug uptake. To palliate this, we present an alternative drug uptake evaluation methodology in zebrafish larvae based on micro-needle sampling from the yolk followed by mass spectrometric analysis. Using this novel method two antituberculars in clinical use, rifampicin and moxifloxacin, and 15 preclinical lead compounds developed by GSK were examined. We correlate *in vivo* uptake levels with the efficacy of the compounds as measured in *M. marinum* and *M. tuberculosis in vitro* culture inhibition assays. Furthermore, the data obtained is also correlated with *in vivo* drug efficacy tests in a murine model of TB infection as well as with a high-throughput drug screening on *M. marinum* infected zebrafish (Figure 1). The results demonstrate the importance of standardized drug uptake studies in order to be able to understand and correlate the results obtained in zebrafish with other, commonly employed rodent efficacy models.

MATERIALS AND METHODS

Animal experiments ethic statement

All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals.

Zebrafish husbandry and compound treatment

Zebrafish of AB/TL wild-type strain were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Embryos were collected from family crosses and grown at 28 °C in egg water (60 µg/mL Instant Ocean sea salts, Sera Marin) in dark. At 3 day post-fertilization (dpf) 20-30 larvae were transferred to small, 35 x 10 mm Petri dishes each containing 3-4 mL of egg water supplemented with the following compounds: rifampicin (RIF; Sigma-Aldrich) and moxifloxacin (MOX; Santa Cruz) each at 150 µM, and GSK compounds (GlaxoSmithKline Pharmaceuticals) at 10 µM concentration. For controls dimethyl sulfoxide (DMSO) at 0,1% was used. Larvae were exposed to the compounds for 17 and 40 hours. Controls were exposed for few seconds (T=0h). Compounds were administered separately to the larvae or in combinations. Compounds were refreshed every day.

Assessing compound quantities in zebrafish larvae with lysis method

Larvae were transferred to 2 mL micro-centrifuge tubes one by one; excessive compound was removed, washed three times briefly with 1 mL 50 % methanol and then prepared for lysis. Lysis was performed based on snap freezing in liquid nitrogen followed by sonication, as previously described[39].

Micro-needle sampling from the yolk of zebrafish larvae

Before taking samples, larvae were transferred to 2 mL micro-centrifuge tubes, and then washed three times briefly with 1 mL 50 % methanol one by one. After washing, one larva was placed on 1% agarose plate and excessive methanol was removed. Under stereomicroscope at 20 x magnification larvae were punctured in the yolk with a micro-needle (manually pulled borosilicate glass capillary, Harvard Apparatus) placed in the capillary holder of CellTram Oil micro-injector (Eppendorf) mounted onto a micro-manipulator workstation. Vacuum was generated manually via a rotating knob and the content of the yolk was extracted. When

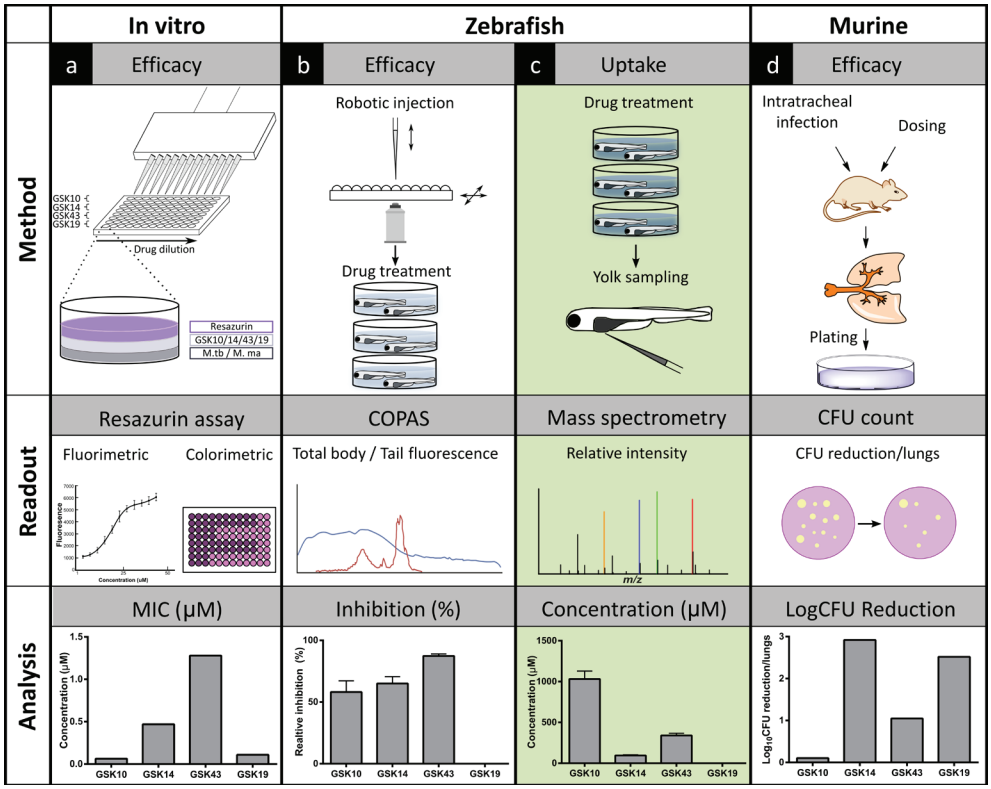


Figure 1: Scheme of drug efficacy methods: antibacterial activity and uptake assays in the zebrafish model integrated in conventional disease screening pipeline. Different models are used in our workflow to determine the efficacy of antitubercular compounds (from left to right): (a) Initially, the *in vitro* efficacy of the tested compounds are determined by their minimum inhibitory concentration (MIC) against *Mycobacterium marinum* and *Mycobacterium tuberculosis* cultures using fluorimetric and colorimetric read-outs from resazurin assay. Subsequently, biological validation is performed in *in vivo* models (b-d). (b) First, compound efficacy is screened in *M. marinum* infected zebrafish larvae. Embryos are robotically injected and following compound treatment the percentage of inhibition is determined using fluorescence read-out of COPAS system. (c) To unravel whether certain compounds fail to be active in zebrafish larvae due to lack of antibacterial activity or poor uptake, our micro-needle sampling method combined with mass spectrometry is used to assess uptake levels from samples of the yolk. (d) As gold standard in antitubercular drug development, compound efficacy is established by determining the rate of the colony forming units (CFU) reduction in the lungs of *M. tuberculosis* infected rodents. After setting (arbitrary) cut-offs in all models, compounds could be categorized into positive or negative groups. By the comparison of these groups along the pipeline, our improved zebrafish platform may give more predictive value for human efficacy of drugs.

the most of the content was removed from the yolk the suction was balanced and stopped. To recover the sample from the micro-needle into a micro-centrifuge tube a pressure was generated by turning the knob in the opposite direction.

Determination of the sample volume

Since individual larva can differ in their yolk size and volume; and the manually pulled micro-needles used for sampling were not identical in diameter, the sampled volume was not always the same per larva. In order to quantify the drugs in the sample, the volume has to be known. As a consequence, images of every sample were taken including the micro-needle containing

the sample. The volume of the samples was determined by modeling the needle as a cone and measuring the diameter and the length on the image, using the scale bar as a reference. Since the angle for the capillary holder was always adjusted in a 45° angle we also adjusted the model for this projection. This way the results were normalized with the according sample volume. Sample volumes were typically around 50 nL, varying between 20 - 200 nL.

Sample preparation
After recovering the sample from the micro-needle into the micro-centrifuge tube, 75% methanol including internal standards (IS) were added to the sample (rifabutin and levofloxacin as a IS for rifampicin and moxifloxacin respectively, and ampicillin as an IS for the GSK compounds). For the micro-needle samples an IS concentration of 20 nM was used. For the whole larvae samples the spiked IS resulted in a concentration of 250 nM. After vortexing for 2 minutes the precipitated proteins were spun down by centrifuging at 16.1 krcf for 5 minutes at 0°C. The supernatant was used for LC-MS analysis.

Mass Spectrometry
A Surveyor Plus UPLC system was used and 15 µL sample was injected on a Acquity C18 T3 column (2.1x100 mm, 1.8 µm). Mobile phase A was 99% H_2O and 1% acetonitrile with 0.3% formic acid. Mobile phase B was 90% acetonitrile and 10% H_2O with 0.3% formic acid and the flow rate was 500 µL/min. A LTQ Orbitrap XL (Thermo Fisher Scientific) was used for detection of the ions in positive electrospray mode. The electrospray voltage was 4.5 kV. The capillary temperature was 375°C and the capillary and tube lens voltage was 29 V and 120 V, respectively. The sheath gas flow rate and aux gas flow rate was 35 and 10 units, respectively. Resolution was set to 7500 in order to reduce the scan time and thus increase the number of data points. For the quantification of the samples an academic calibration curve was constructed within every batch.

***M. marinum* infections in zebrafish *in vivo* model**
Infection experiments were performed on zebrafish embryos at early developmental stages (up to 1,024-cell stage) injected with 40 CFU *Mycobacterium marinum M* strain into the yolk using automated robotic injection system as described in Carvalho *et al.*[8]. As a control an equal volume of carrier solution was injected. After injection, embryos were incubated at 280°C in egg water in Petri dish. Starting at 3 days post-fertilization (dpf) 200 larvae were treated with the following compounds till 5 dpf by adding the compounds into egg water: 200 µM rifampicin (Sigma-Aldrich), 10 µM GSK compounds and dimethyl sulfoxide (DMSO) (0,1%) as negative control. Water with compounds was refreshed once daily. Bacterial infection was quantified using Complex Object Parametric Analyzer and Sorter (COPAS) XL (Union Biometrica) as described previously[8]. Fluorescence in the posterior half of the larvae was determined using a custom perl script that analyses the COPAS extinction profile to determine the posterior half and then sums the fluorescent values of all data points for that half.

***M. tuberculosis* H37Rv inhibition assay**
The measurement of the minimum inhibitory concentration (MIC) was performed as described in Ballell *et al.*[20]. For each compounds measurements were performed in 96-well flat-bottom polystyrene micro-titer plates. Ten twofold drug dilutions in neat DMSO starting at 50 mM were performed. These drug solutions (5 µL) were added to 95 µL Middlebrook

7H9 medium (lines A–H, rows 1–10 of the plate layout). Isoniazid was used as a positive control; eight two-fold dilutions of isoniazid starting at 160 µg/mL were prepared, and this control curve (5 µL) was added to 95 µl Middlebrook 7H9 medium (row 11, lines A–H). Neat DMSO (5 µL) was added to row 12 (growth and blank controls). The inoculums was standardized to ~1x10^7 CFU/mL and diluted 1:100 in Middlebrook 7H9 broth (Middlebrook ADC enrichment, a dehydrated culture medium which supports growth of mycobacterial species, available from Becton–Dickinson, cat.# 211887), to produce the final inoculum of H37Rv strain (ATCC25618). This inoculum (100 µL) was added to the entire plate except G-12 and H-12 wells (blank controls). All plates were placed in a sealed box to prevent drying out of the peripheral wells and were incubated at 370°C without shaking for six days. A resazurin solution was prepared by dissolving one tablet of resazurin (VWR International Ltd., Resazurin Tablets for Milk Testing, cat.# 330884Y') in 30 mL sterile phosphate-buffered saline (PBS). Of this solution, 25 mL were added to each well. Fluorescence was measured (Spectramax M5, Molecular Devices; λ_{ex}=530 nm, λ_{em}=590 nm) after 48 h to determine the MIC value.

***M. marinum* inhibition assays**

The measurement of the minimum inhibitory concentration (MIC) was performed as described above in for the *M. tuberculosis* H37Rv inhibition assay with minor changes. Each compound measurement was performed in 96-well flat-bottom polystyrene microtiter plates. Ten two-fold drug dilutions in neat DMSO starting at 50 µM were performed. These drug solutions (2 µL) were added to 98 µL Middlebrook 7H9 medium (lines A–H, rows 1–10 of the plate layout). Isoniazid was used as a positive control; eight two-fold dilutions of isoniazid starting at 100 µM were prepared, and this control curve (2 µL) was added to 98 µL Middlebrook 7H9 medium (row 11, lines A–H). Neat DMSO (2 µL) was added to row 12 (growth and blank controls). The inoculum was standardized to ~2x10^5 CFU/mL and diluted 1:1 in Middlebrook 7H9 broth (Middlebrook ADC enrichment, a dehydrated culture medium which supports growth of mycobacterial species, available from Becton–Dickinson, cat.# 211887), to produce the final inoculum. This inoculum (100 µL) was added to the entire plate except C-12 and D-12 wells (blank controls). All plates were placed in a sealed box to prevent drying out of the peripheral wells and were incubated at 280°C without shaking for three days. A resazurin solution was prepared by dissolving one tablet of resazurin (VWR International Ltd., Resazurin Tablets for Milk Testing, cat.# 330884Y') in 30 mL sterile phosphate-buffered saline (PBS). Of this solution, 25 µL were added to each well. Fluorescence was measured (Tecan infinite 200Pro; λ_{ex}=535 nm, λ_{em}=590 nm) after 48 h to determine the MIC value.

***M. tuberculosis* infections in mouse *in vivo* model**

Infections of mice with *Mycobacterium tuberculosis* was initiated by nonsurgical intratracheal instillation of *M. tuberculosis* H37Rv as previous described by Rullas *et al.*[29]. In brief, 8- to 10-week-old female mice were anesthetized with 3% isoflurane and intubated with a metal probe (catalog number 27134; Unimed SA, Lausanne, Switzerland). The inoculum (10^5 CFU/mouse suspended in 50 µL of phosphate-buffered saline) was put into the probe and delivered through forced inhalation with a syringe. Treatment was started 24 hours after infection, to allow for phagocytosis of instilled bacteria, and lasted up to 7 days post infection. Finally, an additional 24 h was allowed for clearance of compounds before organ harvesting. To measure infection burden in lungs, all lobes were aseptically removed and homogenized. The homogenates were supplemented with 5% glycerol and stored frozen (-80 °C) until plating. After 14 days of culture, colonies were counted using an automatic colony counter (aCOLyte-Su-

percount; Synoptics Ltd., Cambridge, United Kingdom) and confirmed by visual inspection to correct potential misreadings. Bacterial growth of about 2 logs over the initial inoculum was determined to be a level that would provide enough dynamic range to detect statistically significant growth inhibition.

Statistical Analysis

COPAS data were analysed (Prism 4.0, GraphPad Software) using nonparametric, two-tailed Wilcoxon signed-rank test, and the uptake data were analysed using unpaired, two-tailed t-test. P values shown are: *P<0.05, **P<0.01, and ***P<0.001.

RESULTS

Assessing rifampicin and moxifloxacin quantities in zebrafish by lysis of whole larvae

We have recently reported an automated high-throughput drug screening protocol using *Mycobacterium marinum* infected zebrafish larvae[8]. To better understand the efficacy of antituberculosis compounds used in this test system, we performed uptake experiments using mass spectrometry. This improved zebrafish drug screening platform could then be integrated into the conventional disease screening pipeline and give more predictive value for human efficacy of antitubercular compounds (Figure 1).

To assess the quantity of drugs present in zebrafish, larvae were exposed to rifampicin, a first-line anti-TB drug used as a positive control in our drug screening experiments[8], [22], as well as to moxifloxacin, a gyrase inhibitor currently under exploration for novel antitubercular regime development to shorten the duration of TB treatment[34].

First we measured the quantities of the aforementioned compounds using the previously described whole larvae lysis method followed by LC-MS analysis[28]. After different exposure times (17 and 40 hours) larvae were washed with 50% methanol and lysed individually to determine drug concentration using LC-MS (Figure 2a). A steady increase was detectable in the level of moxifloxacin during the treatment period from 3.1 pmol/larva at 17 hours of exposure and increasing further to 13.6 pmol/larva at 40 hours of exposure. On the other hand, rifampicin seemed to have reached a steady state level already at 17 hours of exposure with 6.2 pmol/larva and 6.6 pmol/larva at the 40 hour exposure time. As a control we quickly exposed the larvae to the drugs for only a few seconds, then washed them immediately with 50% methanol and lysed them for analysis (T=0 h). Surprisingly, the presence of both drugs was detected even after these few seconds of exposure, at which time rifampicin was measured at 4.6 pmol/larva and moxifloxacin at 0.8 pmol/larva. Washing after drug exposure was also performed using other solvents (embryo medium, 5% and 50% ethanol), which resulted in the same quantitities as were detected after 50% methanol wash (data not shown). Detection of rifampicin and moxifloxacin in the T=0 h control lysates was presumably due to the tendency of these drugs to adhere to the skin of the larvae. These results suggest that the whole larvae lysis method is not suitable for monitoring drug uptake.

Determining uptake of rifampicin and moxifloxacin using micro-needle sampling

Since the larvae lysis method cannot be used for correctly monitoring the uptake of certain drugs, we aimed to overcome this issue by taking samples (in the nanoliter range) from the yolk of zebrafish larvae using a micro-needle attached to an Eppendorf CellTram Oil. As detailed in the material and methods section we also have developed a method to accurately determine the sample volume needed for quantitative mass spectrometric analysis. This method was used to analyze samples in the same time-course experiment as applied in the lysis method (17 and 40 hours of exposure). We observed a gradual increase in the uptake

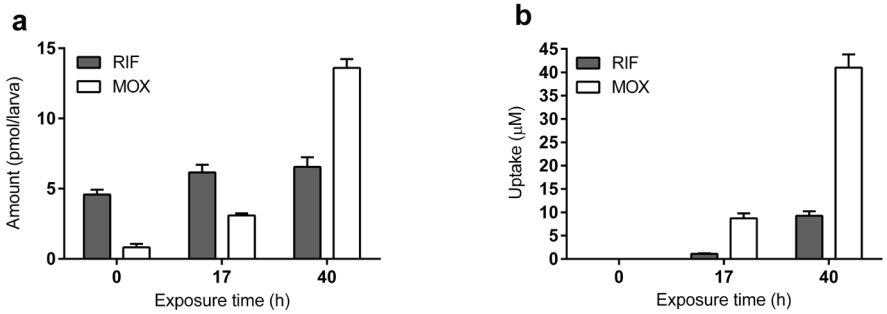


Figure 2: Rifampicin and moxifloxacin quantities measured by (a) whole zebrafish larvae lysis method and (b) micro-needle sampling method from the yolk. Three day-old zebrafish larvae were exposed to the combination of rifampicin (RIF; grey bars) and moxifloxacin (MOX; white bars) dosed at a 150 µM concentration for 0, 17 and 40 hours (n = 10). Note that as a control at 0 time point (T=0 h) the zebrafish larvae were exposed to the compounds (at the same dose) for only few seconds. This short exposure was followed by three consecutive washing steps with 50% methanol. In the read-out, (a) after lysis of the whole larvae the presence of compounds is detected even at T=0 h, however (b) via our micro-needle method the compounds are undetectable in the yolk at T=0h. All data are expressed as the mean ± S.E.M.

level of both rifampicin (17 hours of exposure at 1.1 µM, 40 hours of exposure at 9.2 µM) and moxifloxacin (17 hours of exposure at 8.7 µM, 40 hours of exposure at 41 µM) (Figure 2b). Neither rifampicin nor moxifloxacin was detected at the control time point (T=0 h), indicating that our new method eliminates the issue of drug adherence. The results show that our method can measure drug uptake with high accuracy and sensitivity from samples in the nanoliter range and is also suitable for compounds that persistently stick to the surface of larvae such as rifampicin.in the nanoliter range and is also suitable for compounds that persistently stick to the surface of larvae such as rifampicin.

Uptake of preclinical anti-tuberculosis compounds and correlation with their efficacy on bacterial burden in infected zebrafish larvae

In order to correlate the uptake of a drug with its *in vivo* effect on microbial infection using drugs that were not previously tested in our zebrafish model, we performed an anti-TB drug screening on *M. marinum* infected zebrafish larvae using 15 preclinical compounds provided by GSK. These compounds were pre-screened *in vitro* for their antibacterial activity on *M. tuberculosis* and *M. marinum* cultures showing a large gradient of activities (Supporting Table 1). For the *in vivo* test, zebrafish embryos were robotically injected with fluorescently labeled bacteria at early stages of development and hatched larvae were treated with compounds from 3 days post-infection (dpi) following the protocol as published by Carvalho *et al.*[8]. The antibacterial efficacy of the compounds was assessed by monitoring the fluorescence intensity correlating with bacterial burden present either in the total body or only in the tail region of 5 day-old zebrafish larvae using COPAS analysis. The efficacy was expressed as percentage of inhibition relative to DMSO treated control groups (Figure 3). The tail region is representative of a part of the body where mycobacteria are mainly enclosed by immune cells leading to granulomas and therefore presents a good measure for disseminated disease[8], [35]. The tail measurements compared to the total body are an indicator for potential problems with drug distribution. Compared to the control we identified compounds reducing bacterial load significantly and others that had moderate or no significant effect on infection (Figure 3). The results show that at least 3 compounds were significantly active on reducing bacterial burden both in the total body and the tail region. In order to better understand the reason for a com-

pound failing to lower bacterial load in the zebrafish, we determined GSK compound levels in samples taken from yolk of larvae at 5 dpf after 40 hours treatment (Figure 3a). The comparison revealed a correlation between the uptake and the antibacterial efficacy of compounds both in the total body and the tail region (Figure 3a, b, c).

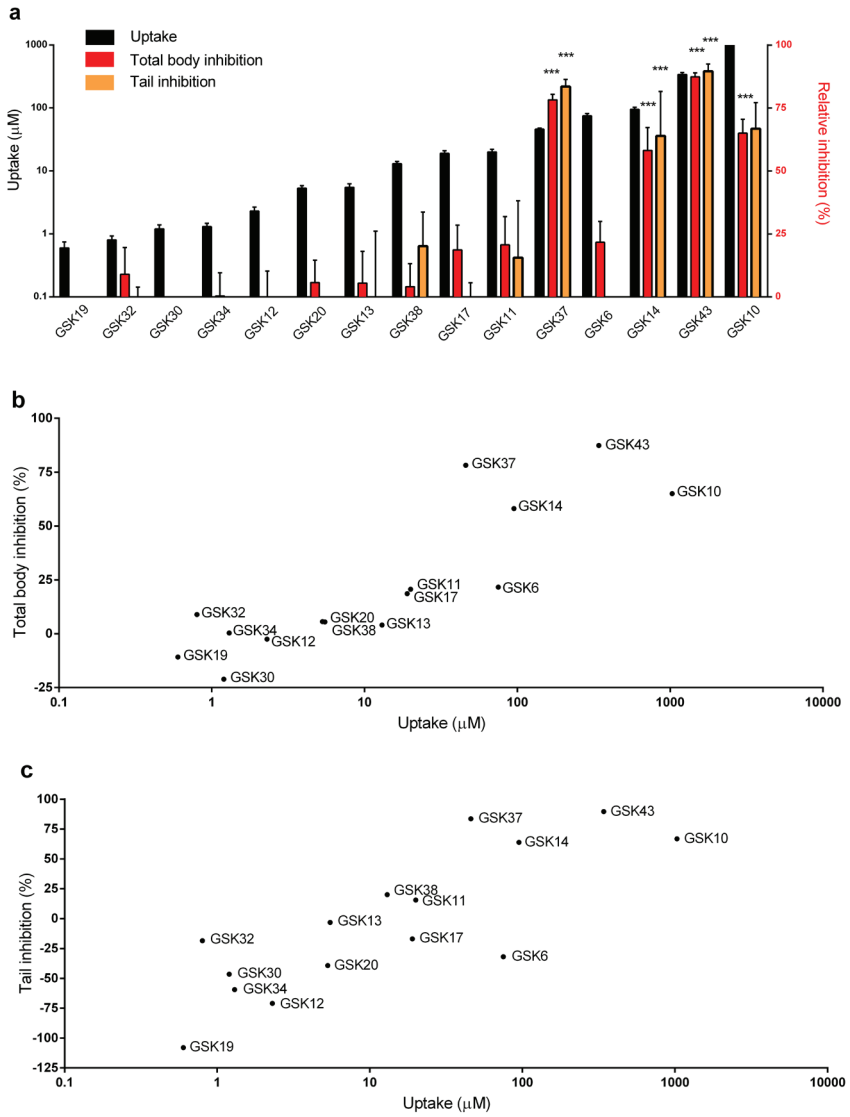


Figure 3: Correlation between the uptake level of preclinical antitubercular compounds and their efficacy in *M. marinum* zebrafish infection model. (a) Uptake levels of 15 preclinical antitubercular GSK compounds were measured from samples taken from the yolk of 5 day old zebrafish larvae after 40 hours exposure at 10 µM concentration (black bars) (n=10). Efficacy of the compounds was assessed by monitoring fluorescent bacterial burden in the total body (red bars) or tail region (orange bars) of 5 dpi larvae using the COPAS system after 40 hours treatment (n=200). Efficacy is expressed as percentage of inhibition relative to DMSO treated control groups. The bar graphs depict the correlation between the uptake and the relative inhibition, both in the total body and the tail region. Significance in inhibition is indicated with asterisks (***P<0.001). (b, c) Correlation between the efficacy (b) in the total body or (c) in the tail and the uptake of the GSK compounds.

Correlation between drug uptake levels and physico-chemical properties

It is important to determine if the uptake level of a compound can be correlated with its physico-chemical properties; if such a relationship was identified it would allow effective prediction of the uptake of compounds in this model. We compared a number of common metrics used in predictive strategies to the uptake levels measured in our assays. In our case no relationship was found for the majority of the metrics examined (molecular weight, molecular volume, number of rotatable bonds, hydrogen bond acceptors/donors, number of sp3 carbons, number of aromatic rings, number of Lipinski H-bond acceptors/donors, and polar surface area; see Supporting Figure 2). One metric did show a weak relationship with the measured uptake, chromlogD – which represents the lipophilicity of a compound (Figure 4). This, experimentally determined property, is the chromatographic hydrophobicity index (CHI)[36], a retention time of the compound at pH 7.4 on a fast gradient reverse phase HPLC column, modified by a conversion factor which has been shown to be more accurate than classical OW partitioning[37]. Only a weak correlation was identified between high chromlogD (above 3.5) and uptake. This is in line with other findings and confirms that it is hard to accurately predict uptake of compounds solely based on their physico-chemical properties such as lipophilicity[21], [31]. Our micro-needle sampling method gives valuable additional information on transportability of particular drugs through epithelial layers and could lead to better theoretical models for use in combination with physico-chemical predictions.

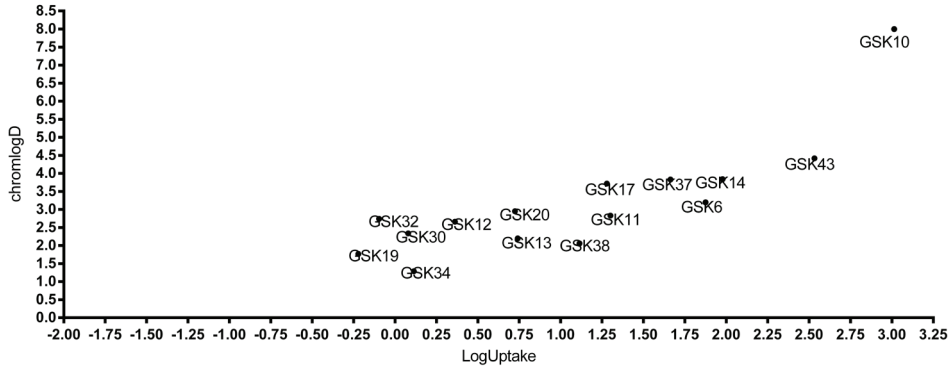


Figure 4: Correlation between uptake levels and compound hydrophobicity. ChromlogD is the representation of the hydrophobicity and therefore the solubility/ lipophilicity of a compound. To examine the relationship between ChromlogD of the compounds and their uptake ChromlogD is plotted against uptake levels measured in 5 day old zebrafish larvae after 40 hours exposure. A weak correlation is observed between high chromlogD (above 3.5) and uptake.

Comparison of uptake levels with *in vitro* and *in vivo* antibacterial efficacy models used in our pipeline of anti-TB drug discovery

We aimed to elucidate if the uptake and antibacterial efficacy results obtained from the zebrafish model translate to other models commonly used in the drug development pipeline. For this reason, we first compared zebrafish data to *in vitro M. marinum* antibacterial efficacy models.

To determine if there is correlation between uptake and the antibacterial activities, first we normalized the data for the uptake with the minimum inhibitory concentration (MIC) of compounds against *M. marinum* (Figure 5). This Log(uptake/MIC) ratio identifies which compounds are present in the larvae at concentrations in excess of their MIC values. Using this data a plot of the Log(uptake/MIC) against the observed percentage of inhibition in the zebrafish assay was produced (Figure 5). As expected, none of the 3 compounds of which

the Log(uptake/MIC) was below zero significantly reduced the bacterial burden in the entire body in zebrafish (GSK12, GSK13, GSK19). However, from the 12 compounds, which were present in concentrations well above their MIC values, only 4 were active (indicated with green) (GSK 10, GSK14, GSK37, GSK43).

Furthermore, we extended our comparison with *in vitro M. tuberculosis* antibacterial efficacy data as well as with *in vivo M. tuberculosis* infected mouse efficacy data, the latter being a gold standard in anti-tuberculosis drug development (Figure1). In the acute mouse model the inactive compounds are defined as compounds that produced less than a 2 log reduction in CFU count[29] (Supporting Table 2). Conversely, active compounds are those that produced a greater than 2 log reduction in the CFU count in the acute mouse model. Of the 4 active compounds identified via the zebrafish screening only 2 (GSK14, GSK37) are considered as active in mouse experiments, whereas the other 2 compounds (GSK10, GSK43) were inactive in the mouse model. Of the 11 compounds detected as inactive in zebrafish assay 6 proved to be inactive in mouse as well (GSK6, GSK12, GSK13, GSK30, GSK34, GSK38) showing that both *in vivo* models yield very different results from the *in vitro* MIC data.

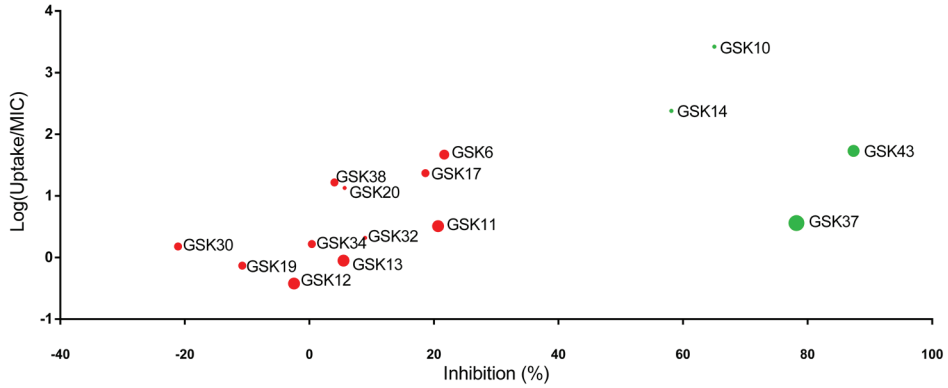


Figure 5: Correlation between uptake and in vitro minimum inhibitory concentration and *in vivo* zebrafish antibacterial activities of compounds. The measured uptake data in zebrafish larvae is normalized with *in vitro* minimum inhibitory concentration (MIC) of compounds against *M. marinum* (Log(uptake/MIC)). This ratio is plotted against the observed percentage of inhibition in the zebrafish *M. marinum* infection assay. Size of the circles indicates their MIC values. Green colour represents compounds significantly reducing bacterial burden in zebrafish infection model, considered as active compounds, while red colour indicates inactive compounds.

DISCUSSION

Zebrafish larval screening systems have increasingly been used for testing the effects of drugs. Especially, in the context of drug screening against tuberculosis, current methodologies based on bacterial load reduction are accurate and can be automated. In the case of antibiotic screens this has led to valuable information on the efficacy of existing and new antibiotics using *M. marinum* infection as a test system[8], [11], [12], [38]. However, the reasons behind the failure of some compounds to lower bacterial load in *M. marinum* infected zebrafish larvae were unclear and we wanted to test whether this was caused by poor compound uptake. To date, the mass spectrometric methods currently in use fail to correctly monitor uptake levels in zebrafish. In order to address this problem, we decided to explore an alternative methodology that could measure uptake of compounds from the yolk of zebrafish larvae by micro-needle sampling followed by mass spectrometric analysis. The results show that micro-needle sampling of volumes in the nanoliter range from the yolk allows accurate and sensitive analysis of uptake of drugs by individual larvae.

Our method is based on the assumption that taking samples from the yolk is relevant for predicting limitations of drug efficacy in the rest of the body of zebrafish larvae. This assumption is supported by measured quantities in the yolk and whole larvae lysis experiments for moxifloxacin (Supporting Figure 1). We have compared antimicrobial efficacy in the entire body with the tail region in order to check whether spreading of the tested drugs through the body could present a problem (Figure 3). These results do not show any indication that the effect of the 15 tested compounds is limited by distribution through the body and therefore indicate that the yolk is a representative location for measuring drug uptake.

Our results show that there is a high variety of uptake of the various tested compounds. As expected, only compounds that reach uptake levels that are greater than their MIC are active in our antimicrobial test system. In some cases uptake shows to be a limiting factor for reaching the MIC as determined in vitro (Figure 5). The fact that this outcome could not be predicted solely based on physico-chemical properties like hydrophobicity or mass shows the importance of our test system. At least in one case (compound GSK19) it is clear that the difference with the effect in the rodent test system can be explained by problems with uptake by the zebrafish larva (Figure 1). By explaining false negative test results from large screens, our microsampling method opens new possibilities for drug screening using zebrafish larvae in a preclinical test set up. In the future our screening pipeline can also be the basis for in depth studies of the mechanisms behind this and other false negative test results in zebrafish. It is also interesting to study why some drugs that did show high efficacy in the zebrafish larvae model were inactive in the mouse model. This could be examined for instance by testing other drug administration methods, e.g. drugs can be injected directly inside the body or added to the food instead of the medium. This can show whether the negative results in the rodent test model are caused by degradation of drugs added to the food in the acidic environment of the stomach.

In addition to highlighting the importance of identifying potential false negative and positive test results, our results show that some drugs that come out as most positive in the combined uptake and efficacy zebrafish test system are also active in the *in vivo* rodent model. This is of significance since zebrafish larvae do represent a very different test system as compared to rodents for instance with respect to (1) method of application (in the medium versus application in the food in the rodent system), (2) metabolic rate of these test system (where zebrafish present a metabolic rate that is much slower than rodents), and (3) different levels of genetic polymorphisms (the zebrafish strains are wild types with very high number of polymorphisms whereas the mouse strains are highly inbred and devoid of polymorphisms). We therefore believe that the zebrafish model provides added value to the rodent system since, in comparisons with the human population, test models with a larger number of polymorphisms and a lower metabolic rate are of relevance. In a broader perspective the observation that compounds that are active in two highly different test systems (in this case a fish versus a rodent) could provide additional confidence that the compound will also provide high efficacy in human subjects with varying background genotypes. Some of the compounds tested in this study (such as GSK14, which was shown to have good uptake and high efficacy in the zebrafish system) are members of series of compounds which are currently progressing to extensive medicinal chemistry programs that hopefully lead to clinical applications in the near future.

We therefore anticipate that the novel uptake measurement technique presented here makes important inroads to our understanding of zebrafish infection model data and opens up new opportunities for zebrafish larval systems to be more widely employed in the field of pharmacology. It should be of notice that our uptake method is also highly applicable for toxicity

testing in zebrafish larvae testing and in this case it will also be of great added value to rodent toxicity test systems. We hope that our results will stimulate future detailed follow-up pharmacokinetic studies in this model system. In conclusion, our results are a good starting point to undertake more detailed PK/PD studies together with studies of reference compounds that eventually can lead to full acceptance of the zebrafish as a model in pharmacological screening pipelines in industry.

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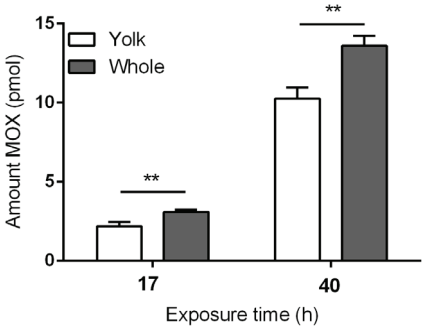
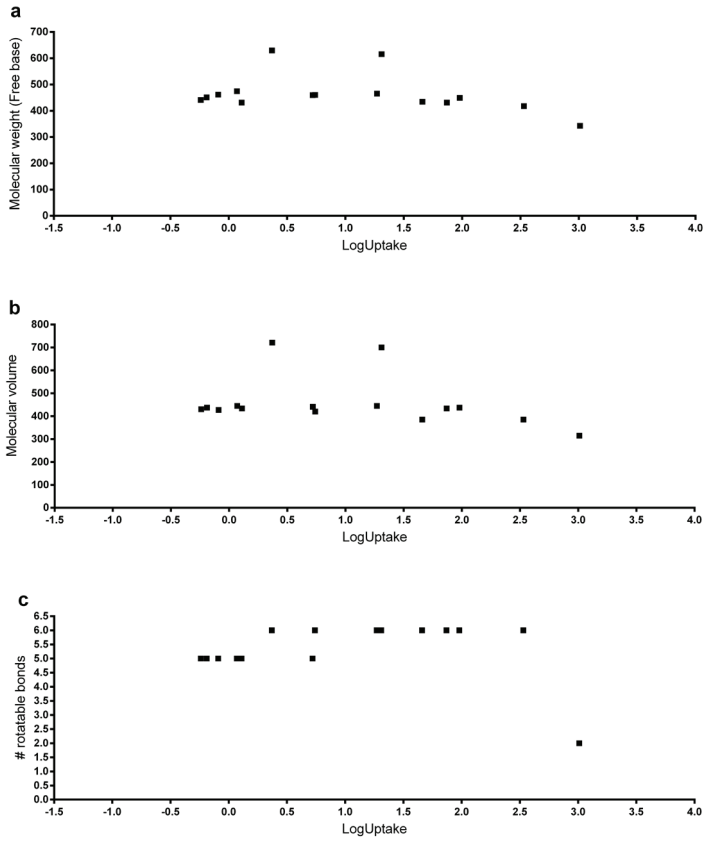
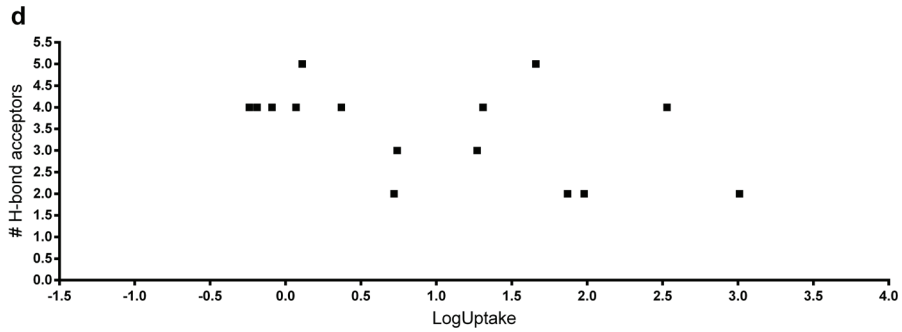
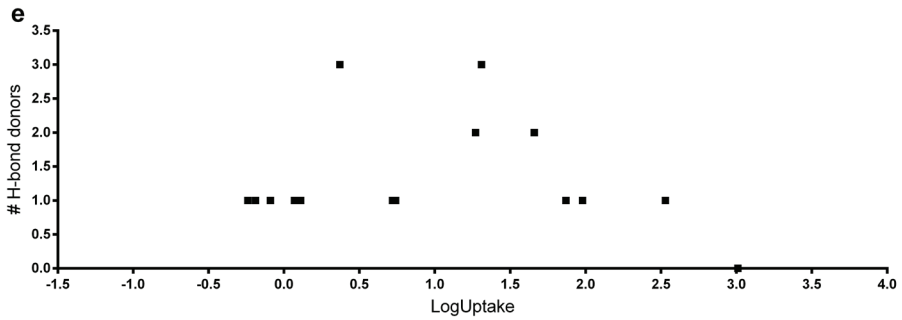
SUPPORTING INFORMATION



Supporting Figure 1: Comparison of the amount of moxifloxacin measured in the samples from the yolk and lysates of whole zebrafish larvae. Level of moxifloxacin was measured after 17 (4 day old larvae) and 40 hours (5 day old larvae) of drug exposure using samples from the yolk (white bars) and from the lysates of whole larvae (grey bars) (n=10). Data are expressed as the mean ± S.E.M. Significance is indicated with asterisks (**P<0.01).




d
H-bond acceptors
LogUptake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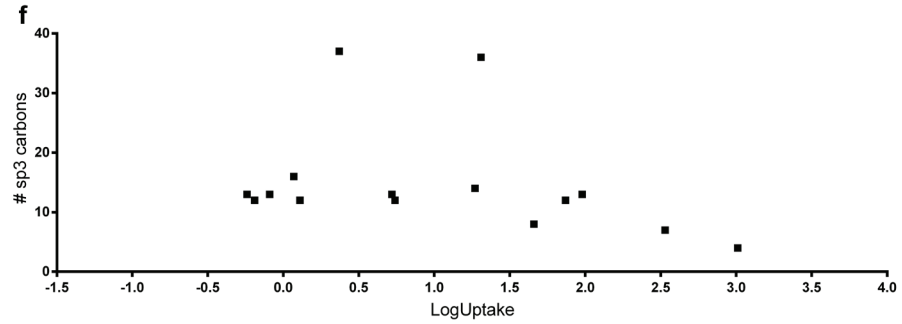
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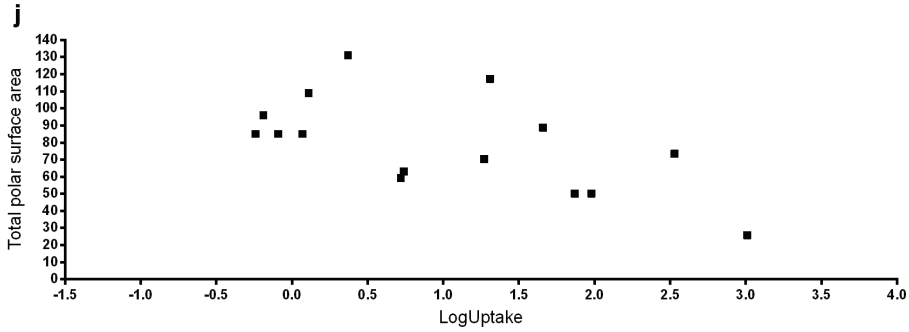
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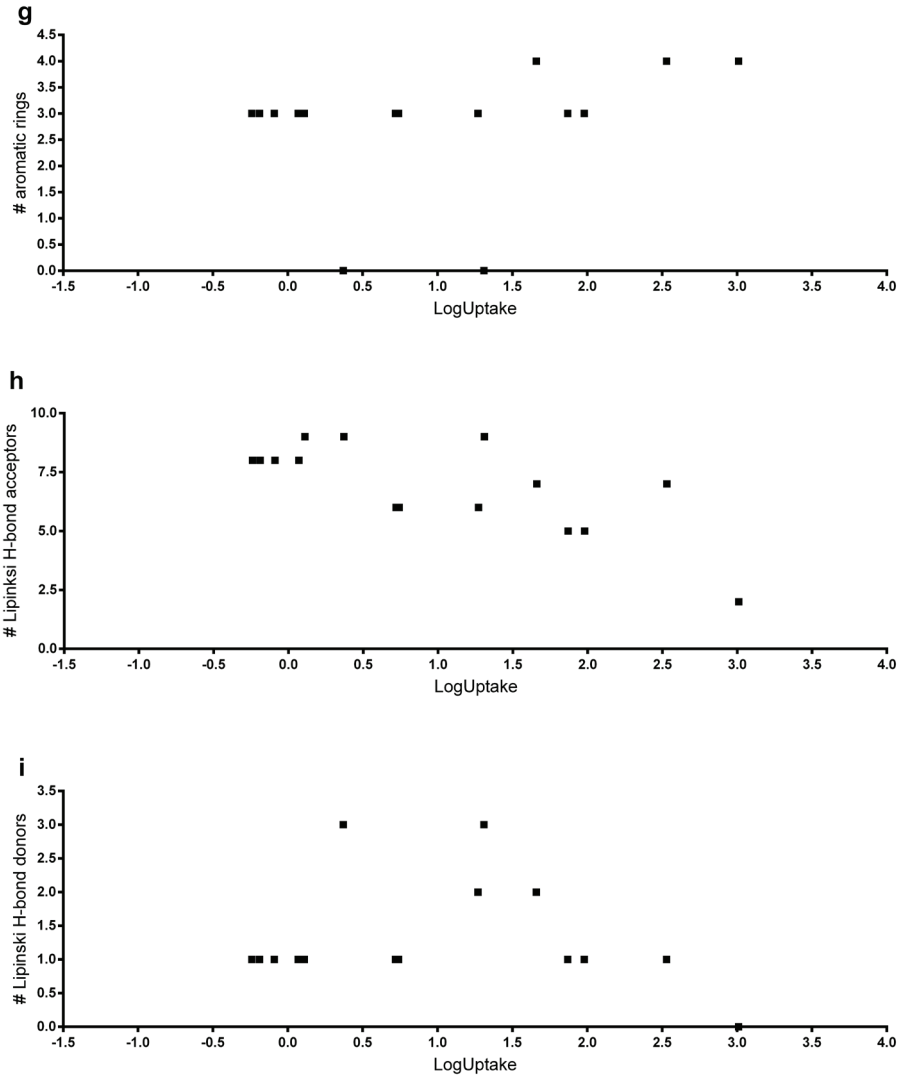
Total polar surface area
LogUptake



Supporting Figure 2: Uptake versus physico-chemical properties. Common metrics used in predictive strategies was compared to the uptake levels of the 15 compounds used in our assays. In the examination uptake level was compared to (a) molecular weight, (b) molecular volume, (c) number of rotatable bonds, (d) hydrogen bond acceptors and (e) donors, (f) number of sp3 carbons, (g) number of aromatic rings, (h) number of Lipinski H-bond acceptors and (i) donors, and (j) polar surface area.

Supporting Table 1: Minimum inhibitory concentration of compounds

Compound	MIC *M.tuberculosis* (μM)	MIC *M.marinum* (μM)
GSK6	0,08	1,56
GSK10	0,06	0,39
GSK11	1,11	6,25
GSK12	1,59	6,25
GSK13	0,9	6,25
GSK14	0,47	0,39
GSK17	0,03	0,78
GSK19	0,12	0,78
GSK20	0,04	0,39
GSK30	0,06	0,78
GSK32	0,04	0,39
GSK34	0,08	0,78
GSK37	0,22	12,5
GSK38	0,04	0,78
GSK43	1,28	6,25

Supporting Table 2: Log reduction in CFU count in the acute mouse model

Compound	CFU reduction
GSK6	1,65
GSK10	0,1
GSK11	2,85
GSK12	1,76
GSK13	0,03
GSK14	2,92
GSK17	2,98
GSK19	2,52
GSK20	4,65
GSK30	1,95
GSK32	3,7
GSK34	0,99
GSK37	2,3
GSK38	1,14
GSK43	1,06

Chapter 7

Conclusions and Perspectives, Samenvatting

CONCLUSIONS AND PERSPECTIVES

With the persistent efforts to improve healthcare while the costs are ever-increasing, there is a growing demand for healthcare innovations including faster and cheaper analytical methods to support clinical decisions. The in-depth phenotyping of a biological system, such as comprehensive small-molecule profiling, will be key for breakthroughs in biomedical and pharmacological research towards the development of personalized medicine. However, there is often an inverse relation between sensitivity (coverage of the number and type of small-molecules) and the speed and costs of the assay. Although classical small-molecule assays such as microscopy- and enzyme-linked immunosorbent assays predominantly rely on single analyte readouts at a possible high-throughput manner, there is a need for more in-depth small-molecule assays while maintaining reasonable throughput. Yet the comprehensive, high-throughput profiling of a wide variety of small molecules is a major challenge, especially in the field of metabolomics.

In this thesis the development of new methods towards high-throughput, mass spectrometry-based profiling of small molecules in complex biological samples is presented. One option for high-throughput mass spectrometric profiling is direct infusion in which no separation techniques are employed prior to MS detection. With the pre-analytical part being a major bottleneck in a typical direct-infusion MS-based analytical workflow, the focus was set on the development of new sample pretreatment and sampling procedures, including miniaturized and automated concepts with the potential for high-throughput application.

In **Chapter 2** the current state-of-the-art in sample pretreatment techniques for mass spectrometry-based metabolomics has been reviewed. Although to date no universal sample pretreatment technique is available for the comprehensive analysis of the metabolome, the combination of protein and lipid removal is often reported to be an effective strategy to improve metabolite coverage and reproducibility. It has been described that the recent innovations in automated offline well-plate extraction (including protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have allowed fast sample cleanup and partly removed the bottlenecks associated with sample pretreatment. It has been suggested that in the future sample pretreatment procedures should not only be automated and fast, but also quantitative and standardized so that metabolomics data of different studies are comparable within and between labs. This is especially important as some metabolites can be partly bound to proteins or other factors in blood, and are partly free in solution.

In **Chapter 3** the rapid phenotyping of early zebrafish embryogenesis using nanoESI high resolution (HR) direct infusion mass spectrometry (DI-MS) has been described. It has been demonstrated that with limited but efficient sample pretreatment in-depth profiles could be obtained which could distinguish five developmental stages of early embryogenesis with a time resolution of 1 hour. It has been shown that with thorough data cleanup, 102 molecular features proved relevant which reflected the onset of (earlier reported) gene expression and the increase in energy requirement. It was concluded that effective and preferably fast sample pretreatment procedures are desirable to obtain more reproducible in-depth information about metabolites at lower concentrations.

By using limited sample preparation and proper data processing, in-depth metabolic profiles of complex samples can be obtained using nanoESI-DI-MS. Still, in order to exploit the full potential of DI-MS, effective sample pretreatment/fractionation methods should be developed (as proposed in **Chapter 4** and **5**) which preferably includes the extraction of small polar molecules and its separation from interfering (endogenous) salts, as proposed in **Chapter 2.**

In **Chapter 4**, three-phase electroextraction (3-phase EE) into a two microliter droplet, which was hanging from a conductive pipette tip, has been described. By the electromigration of analytes from a larger volume of aqueous sample, through an immiscible organic filter phase, into a small-volume of aqueous acceptor phase, a fast analyte enrichment of metabolites has been achieved. The organic filter phase composition imposed the selectivity of 3-phase EE, a feature which is useful in tuning the extraction into the analyte window of interest, and thus preventing the migration of proteins into the acceptor phase. Therefore, 3-phase EE has great versatility as analyte enrichment and purification (deproteinization) can be integrated in one single method. Proof of principle towards the online integration in an automated nanoESI-DI-MS platform has been realized which shows the potential of 3-phase EE in HTS.
It is expected that the range of analytes compatible with 3-phase EE may be extended by the optimization of the organic filter phase composition which may make 3-phase more suitable for comprehensive profiling purposes. In addition, though not demonstrated in this thesis, 3-phase EE might have desalting properties caused by the organic filter phase. Furthermore, by the optimization of 3-phase EE geometries, enrichment could be further improved. In future, by using microfluidic-chip technologies, 3-phase EE could be further downscaled and the potential of massive parallelization in the context of high-throughput screening (HTS) could be explored.
In **Chapter 5** a miniaturized automated LLE has been developed in a 384 well-plate based on gas pressure-assisted mixing followed by passive phase separation. Our method enabled the whole extraction procedure being executed and integrated in an automated nanoESI-DI-MS robot. Through varying the gas pressure and its duration, the effectiveness of the mixing procedure in terms of recovery has been optimized. It has been demonstrated that for drugs in human plasma, this new platform proved excellent analytical characteristics in terms of precision and linearity compared to e.g. a conventional LLE procedure. These experiments show the potential of this new fully-automated platform for the analysis of drugs in complex (volume-limited) samples such as plasma and dried blood spots.
As demonstrated in this thesis, the dichloromethane phase can be successfully analyzed after the LLE procedure, which makes this method very suitable for the analysis of relatively apolar, small molecules such as drugs but possibly also lipids. In principle, both phases could be infused as the analysis of the (cleaned-up) aqueous phase might also be useful for measuring the more polar molecules. However, caution must be exercised when the aqueous phase contains high salt concentrations and dilution might be an extra step which should be taken into account.
In **Chapter 6** a miniaturized sampling method using a microneedle has been described which enabled monitoring drug uptake in the (small-volume) yolk of zebrafish larvae using MS analysis. It has been demonstrated that the current methods based on embryo lysis did not take into account the possible adherence of drugs to the skin, resulting in false-positive uptake readouts. It has been shown that our new method offers a great possibility to monitor how any novel compound behaves within the zebrafish system, as to date no single physicochemical property has been identified to accurately predict compound uptake. With our new method, the uptake data of two commonly used anti-tuberculars, together with 15 preclinical compounds, have been monitored and correlated to high-throughput screening data from M. marinum infected zebrafish larvae as well as to other conventional *in vivo* and *in vitro* models. It has been proposed that our improved zebrafish drug screening platform could obtain new insights into interpretation and prediction of drug efficacy.
It is expected that this method opens a doors for the use of zebrafish embryos for in-depth

pharmacological assessment of activity (such as PK modelling) and therefore enables a wider acceptance for the zebrafish in the field of drug discovery and development. Moreover, next to the targeted drug analysis, a comprehensive profiling of small molecules could be performed in order to gain extra phenotypic information such as in the field of metabolomics and systems pharmacology.

High-throughput small molecule profiling requires new approaches in sample preparation to allow fast DI-MS analysis. In summary, in this thesis first steps of new methods have been presented which are promising in small-molecule analysis using DI-MS. In **Chapter 4** and **5** new concepts regarding sample pretreatment have been developed. In general, the potential of both 3-phase EE and the described micro-LLE method might be improved when a derivatization method can be integrated prior to extraction. By altering the physico-chemical properties of the relatively polar target molecules it might enable effective desalting and improved ionization efficiencies.

Most of the methods presented in this thesis were integrated with direct infusion MS, as it is potentially the most suitable MS-based platform for comprehensive, high-throughput profiling of small molecules. However, as described in **Chapter 6**, the small-volume yolk samples were analysed with LC-MS. Future research could be directed towards its coupling to DI-MS for which 3-phase EE might be a promising candidate since analytes can be purified and enriched in a small volume as described in **Chapter 4**.

When the presented high-throughput concepts are further optimized, it is expected that these methods can be used in biomedical and pharmacological screening which eventually may result in the development of personalized medicine.

SAMENVATTING

In de gezondheidszorg wordt continu gestreefd naar verbetering en speelt kostenbesparing een steeds belangrijkere rol. Om de zorgkosten te beperken, is er een sterke behoefte aan zorginnovaties. Hierin zijn snellere en goedkopere analytische meetmethoden cruciaal. Het ultieme doel van deze nieuwe meetmethoden is het mogelijk maken van therapie op maat: 'personalized medicine'. Voor dit doel zijn er doorbraken nodig in biomedisch en farmacologisch onderzoek. Het snelle fenotyperen van een biologisch systeem door middel van het globale profileren van kleine moleculen is hierin belangrijk.

Er is vaak een omgekeerd evenredig verband tussen de gevoeligheid (het aantal te detecteren moleculen en molecuulklasses) en de snelheid en kosten van de meetmethode. Klassieke meetmethoden voor kleine moleculen, zoals bijvoorbeeld microscopie of immunoassays, analyseren voornamelijk een enkel molecuul met hoge meetsnelheid. Er is echter behoefte aan meer diepgaande meetmethoden waarbij vele kleine moleculen tegelijkertijd gemeten kunnen worden, met een acceptabele meetsnelheid. Tot dusver is het globale en tegelijkertijd snelle profileren van een breed scala aan kleine moleculen een uitdaging, vooral in het metabolomics veld.

In dit proefschrift wordt de ontwikkeling van nieuwe methoden voor het snelle profileren van kleine moleculen in complexe biologische monsters, door middel van massaspectrometrie (MS) beschreven. Hiervoor is direkte infusie (DI)-MS een aangewezen, snelle techniek, omdat er voorafgaand aan MS detectie geen scheidingsmethode wordt gebruikt. Voor een succesvolle DI-MS meetmethode is het pre-analytische gedeelte een zeer belangrijke, maar tot dusver onderbelichte factor. De focus van dit proefschrift lag daarom op het ontwikkelen van nieuwe monstervoorbewerkingstechnieken waarbij, met betrekking tot de meetsnelheid, miniaturisatie en automatisering de rode draad waren.

Hoofdstuk 2 geeft een overzicht van de huidige monstervoorbewerkingstechnieken voor metabolomics uitgevoerd met een MS. Hoewel er op dit moment geen enkele universele monstervoorbewerkingstechniek beschikbaar is voor de globale analyse van het gehele metaboloom, lijkt de combinatie van onteiwitten en het verwijderen van lipiden een goede strategie om een groter aantal metabolieten te meten en hun reproduceerbaarheid te verbeteren. Door recente ontwikkelingen op het gebied van geautomatiseerde off-line well-plate extractietechnieken (onteiwitting, vloeistof-vloeistof extractie en vaste-stof extractie) is een snelle monstervoorbewerking al goed mogelijk. In de toekomst moeten monstervoorbewerkingstechnieken niet alleen geautomatiseerd en snel zijn, maar ook kwantitatief en gestandaardiseerd, zodat metabolomics data van verschillende studies uit verschillende labs vergeleken kunnen worden. Dit is vooral belangrijk voor metabolieten die deels gebonden kunnen zijn aan eiwitten of andere factoren in bloed.

In **Hoofdstuk 3** wordt de snelle fenotypering van zebravis embryogenese beschreven door middel van nanoelectrospray en hoge resolutie (HR) DI-MS. Met behulp van beperkte, maar efficiënte monstervoorbewerking werd gedemonstreerd dat gedetailleerde metabolietprofielen konden worden verkregen. Hierin konden vijf vroege ontwikkelingstadia met een tijdsresolutie van slechts 1 uur worden onderscheiden. Met behulp van gedegen dataverwerking werden 102 relevante moleculaire variabelen aangetoond. Deze variabelen weerspiegelen de

start van bepaalde genexpressie zoals bekend uit de literatuur en de toenemende energiebehoefte. In de toekomst zijn effectievere en bij voorkeur snelle monstervoorbewerkingstechnieken wenselijk om nog meer (reproduceerbare) informatie te verkrijgen over metabolieten met een lage concentratie.
Uit de studie in **Hoofdstuk 3** valt te concluderen dat met beperkte monstervoorbewerking en nanoelectrospray-DI-MS in combinatie met goede dataverwerking, gedetailleerde metabolietprofielen van complexe monsters verkregen kunnen worden. Om de volledige potentie van DI-MS te benutten zouden er andere effectievere monstervoorbewerkingstechnieken moeten worden ontwikkeld (zoals bijvoorbeeld voorgesteld in **Hoofdstuk 4** en **5**) die bij voorkeur kleine polaire moleculen kunnen extraheren en scheiden van interfererende (endogene) zouten, zoals gesuggereerd in **Hoofdstuk 2**.

In **Hoofdstuk 4** wordt drie-fase elektroextractie (3-fase EE) naar een twee microliter druppel hangende aan een geleidende pipetpunt beschreven. Door middel van de elektromigratie van analieten in een waterige monsterfase met groter volume, door een niet-mengbare organische filter fase, naar een waterige acceptorfase met een klein volume, werd een snelle verrijking van carnitines gerealiseerd. De samenstelling van de organische filterfase bepaalde de selectiviteit van 3-fase EE. Deze eigenschap is niet alleen nuttig voor het afstemmen van de extractie van het aantal verschillende metabolieten naar keuze, maar ook om migratie van eiwitten naar de acceptordruppel te voorkomen. Hierdoor is 3-fase EE veelzijdig, omdat selectieve verrijking en tegelijkertijd purificatie van analieten in een enkele methode geïntegreerd worden. Tenslotte werd de potentie van 3-fase EE voor high-throughput screening gedemonstreerd, door een *proof of principle* van de online integratie van 3-fase EE in een commercieel en geautomatiseerde nanoelectrospray-DI-MS platform.
Het aantal verschillende metabolieten dat met 3-fase EE gemeten kan worden, kan in vervolgonderzoek worden uitgebreid door de samenstelling van de organische filterfase te optimaliseren. Op deze manier zou 3-fase EE ook geschikt kunnen worden voor globale profilering. Bovendien valt het te voorzien dat 3-fase EE ontzoutings eigenschappen kan hebben vanwege de organische filter fase, hoewel dit niet is aangetoond in dit proefschrift. Verder zou de verrijkingsfactor verbeterd kunnen worden door de dimensies van 3-fase EE te optimaliseren. Ook zouden er in de toekomst microfluïdische chiptechnologieën ingezet kunnen worden om 3-fase EE verder te miniaturiseren. Hierbij kan de potentie van massief-parallelisatie (in het kader van high-throughput screening) verder verkend worden.

In **Hoofdstuk 5** wordt een geminiaturiseerde vloeistof-vloeistof extractie beschreven, door middel van gasdruk-geassisteerd mengen, gevolgd door passieve faseschei ding. Deze methode zorgde ervoor dat de gehele extractieprocedure uitgevoerd kon worden in een 384-well plaat, geïntegreerd in een commerciële en geautomatiseerde nanoESI-DI-MS robot. Door de gasdruk en tijdsduur te variëren kon de effectiviteit van het mengproces geoptimaliseerd worden. Voor de extractie van geneesmiddelen uit menselijk plasma toonde dit platform, in vergelijking met een conventionele, manuele vloeistof-vloeistof extractie procedure, goede analytische eigenschappen met betrekking tot precisie en lineariteit. De resultaten demonstreerden de potentie van dit nieuwe volledig geautomatiseerde platform voor de analyse van geneesmiddelen in complexe (klein-volume) monsters zoals plasma en gedroogde bloedspots. De dichloormethaan fase is na vloeistof-vloeistof extractie succesvol geanalyseerd met nanoelectrospray-DI-MS. Hierdoor is onze methode zeer geschikt voor de analyse van relatief apolaire verbindingen zoals geneesmiddelen, maar bijvoorbeeld ook lipiden. In principe kunnen

beide fases geïnfuseerd worden na extractie. Dit kan interessant zijn voor de de analyse van polaire verbindingen in de (opgeschoonde) waterige fase. Wel moet dan rekening gehouden worden met een mogelijk hoge concentratie van (endogene) zouten in de waterige fase en zou een additionele verdunningsstap nodig kunnen zijn.

Tot slot wordt in **Hoofdstuk 6** een geminiaturiseerde samplingmethode beschreven waarbij met behulp van een micronaald de opname van geneesmiddelen in de (klein-volume) dooier van zebravislarven gemonitord wordt met massaspectrometrie. Eerst werd aangetoond dat de huidige methoden door middel van gehele embryo lyse geen rekening houden met mogelijk adsorptie van geneesmiddelen aan de huid. Dit kan resulteren in vals-positieve opnameuitkomsten. Onze micronaaldmethode biedt wèl de mogelijk om de opname van in principe elke nieuwe verbinding in de zebravis correct te monitoren. Dit is erg belangrijk omdat geen enkele physisch-chemische eigenschap gebruikt kan worden om de opname van de verbinding in de zebravis accuraat genoeg te voorspellen. Met onze methode werd de opname van 15 pre-klinische verbindingen en twee veel gebruikte anti-tuberculosis geneesmiddelen gemonitord en gecorreleerd aan high-throughput screening data van *M. Marinum* geïnfecteerde zebravislarven en aan andere conventionele *in vivo* en i*n vitro* modellen. Met ons verbeterde zebravis geneesmiddel-screening platform kunnnen nieuwe inzichten verkregen worden in de interpretatie en voorspelling van de effectiviteit van een geneesmiddel.
Het ligt in de lijn der verwachting dat deze methode een deur opent voor het gebruik van zebravislarven in gedetailleerde farmacologische bepaling van aktiviteit (zoals PK modellering) en dat het een bredere acceptatie van de zebravis in het ontdekken en ontwikkelen van nieuwe geneesmiddelen teweegbrengt. Ook zou er naast de gerichte analyse van geneesmiddelen uit het microsample een globale profilering van kleine moleculen uitgevoerd kunnen worden, waarbij men extra informatie over het fenotype krijgt met betrekking tot metabolomics en systeemfarmacologie.

De profilering van kleine moleculen vereist nieuwe monstervoorbewerkingstechnieken om snelle DI-MS analyses mogelijk te maken. Samenvattend werden in dit proefschrift de eerste stappen gezet voor nieuwe methoden die veelbelovend zijn in de analyses van kleine moleculen met DI-MS. In **Hoofdstuk 4** en **5** werden nieuwe concepten met betrekking tot monstervoorbewerking gepresenteerd. In het algemeen zou de potentie van zowel 3-fase EE, als de micro vloeistof-vloeistof-extractie methode beter benut kunnen worden wanneer alvorens extractie een derivatiseringsmethode geïntegreerd wordt. Door de physisch-chemische eigenschappen van relatief polaire verbindingen gunstig te veranderen zijn effectieve ontzouting en verbeterde ionizatie efficiëncies mogelijk.
De meeste methoden in dit proefschrift werden geïntegreerd met DI-MS, omdat deze MS-techniek in potentie erg geschikt is voor het globale en snelle profileren van een groot aantal kleine moleculen. In **Hoofdstuk 6** werden de (klein-volume) dooiermonsters echter geanalyseerd met vloeistof-chromatografie-MS. In vervolgonderzoek zou de koppeling met DI-MS onderzocht kunnen worden, waarbij 3-fase EE wellicht een goede monstervoorbewerkingstechniek is, omdat analieten hierbij verrijkt en gepurificeerd kunnen worden, zoals beschreven in **Hoofdstuk 4**.
Wanneer de gepresenteerde high-throughput concepten verder uitgewerkt en geoptimaliseerd worden, zouden deze methoden gebruikt kunnen worden in biomedische en farmacologische screeningstoepassingen. Dit kan uiteindelijk bijdragen aan de ontwikkeling van 'personalized medicine'.

Appendix

Dankwoord, Curriculum Vitae, List of Publications

DANKWOORD

Promoveren doe je niet in je eentje. Nu mijn onderzoek tot een proefschrift heeft geleid, wil ik graag diegenen bedanken die hebben bijgedragen aan mijn promotietijd waarin ik op meerdere vlakken veel heb geleerd.

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Vanuit de moleculaire celbiologie groep wil ik Herman bedanken voor de zeer prettige samenwerking en zijn aanstekelijke enthousiasme. Je had gelijk: ik heb geen spijt gekregen. Anita, met jou heb ik prettig samengewerkt in het drug uptake project. Dit begon als zijprojectje, maar groeide steeds meer uit. Ik verwacht zeker dat hier in de toekomst meer projecten uit voort zullen vloeien.

Ik heb het genoegen gehad met diverse studenten te hebben gewerkt die geïnteresseerd waren in mijn onderzoek. Koen en Yoeri, fijn dat jullie je hebben ingezet voor mijn onderzoek.

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Voor hun immer-gastvrije ontvangst in Zeeland en het meedenken in allerlei zaken in de afgelopen jaren ben ik Marijke en Henk dankbaar.

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CURRICULUM VITAE

Robert-Jan Raterink was born 1982, April 7th, in Dronten, The Netherlands. After receiving his pre-university Atheneum diploma at the Lambert Franckens College in Elburg in 2000, he studied electrical engineering at University of Twente. By completing his study in October 2008, Robert-Jan received the degree Master of Science. He wrote his master's thesis in the BIOS lab-on-a-chip group of prof. dr. ir. Albert van den Berg about the detection of human spermatozoa using electrical conductivity measurements. During this period he developed interest in the combination of engineering, life sciences and chemistry.
In October 2009, Robert-Jan started his PhD research in prof. dr. Thomas Hankemeier's Analytical BioSciences group, as a part of the Netherlands Metabolomics Centre, at Leiden University. Robert-Jan's research on 3-phase electroextraction was featured as a cover article in leading journal Analytical Chemistry and was awarded with the Roland W. Frei award at the international symposium on LC/MS in Montreux, Switzerland.
After his PhD project, Robert-Jan joined the Netherlands Forensic Institute in August 2014.

LIST OF PUBLICATIONS AND PATENTS

R.J. Raterink*, F. M. Kloet*, J. Li, N. A. Wattel, M. J. M. Schaaf, H. P. Spaink, R. Berger, R. J. Vreeken, and T. Hankemeier, "*Rapid Metabolic Screening of Early Zebrafish Embryogenesis based on Direct Infusion-nanoESI-FTMS*", Metabolomics, vol. 9, no. 4, pp. 864–873, 2013.

R.J. Raterink, P. W. Lindenburg, R. J. Vreeken, and T. Hankemeier, "*Three-phase Electroextraction: a New (Online) Sample Purification and Enrichment Method for Bioanalysis*", Analytical Chemistry, vol. 85, no. 16, pp. 7762–8, 2013.[1]
Cover article.

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Accepted for publication in Trends in Analytical Chemistry

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Submitted to Antimicrobial Agents and Chemotherapy

* Authors contributed equally to this work

[1] Patent : 3-phase ELECTROEXTRACTION patent, No. 2008662

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L.I. Segerink, R.J. Raterink, A.J. Sprenkels, I. Vermes and A. van den Berg, "*Spermatozoa detection and counting on chip*", Nederlands Tijdschrift voor Klinische chemie en Labgeneeskunde, vol. 34, 254-255, 2009.