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Development of On-line Sample Clean-up System for Organ-in-a-Column

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Abstract

Organoids are laboratory-grown 3D cell cultures that resemble organs and demonstrate the potential to model human development and disease as well as function as a tool in drug development. To overcome shortcomings associated with coupling organoid technologies to analytical methods like liquid chromatography-mass spectrometry (LC-MS), organ-in-a-column (OiC) have been developed, utilizing standardized equipment.

A problem with OiC is the lack of sample preparation and therefore this study aimed to expand the use of OiC by developing a robust on-line sample preparation method for OiC experiments for LC-MS analysis of drugs and their metabolites.

In this study an on-line sample clean-up system that can be coupled together with OiC has been developed, featuring a valve system for sampling, containing two loops, an automatic filtration/filter (back)flushing (AFFL) system used for cleaning and enriching injected cell medium standards of drugs and their metabolites, and LC analysis with electrospray ionization mass spectrometry (ESI-MS) detection.

Utilizing AFFL was shown to (1) increase the sensitivity of the method compared to not using AFFL and (2) reduce the signal intensity of background ions from the cell medium, both indicating sample clean-up and enrichment of injected samples. Moreover, including a wash step in the method is needed to reduce the observed amount of carry-over. Determination of the limit of detection (LOD, in the range of 0.013 ng/mL – 29 ng/mL) and limit of quantification (LOQ, in the range of 0.039 ng/mL – 87 ng/mL) were made.

Overall, the system and method developed, served as an on-line sample clean-up system improving sensitivity, and has successfully been used together with OiC.

Preface

The work presented in this thesis was performed at the section for Bioanalytical Chemistry at the Department of Chemistry, University of Oslo. This Master thesis has been supervised by Associate Professor Hanne Røberg-Larsen and PhD candidate Stian Kogler. Their guidance and support has been so helpful throughout this work. Thank you for always being available when I needed assistance and guidance.

I would also thank everyone else at the Bioanalytical group for creating an including environment and for all the conversations and lunches. I would like to thank engineer Inge Mikalsen for his technical support, helping me set up all instruments so that everything could work as supposed to.

A special thank you to my office partner Eva for a lot of joy and laughter, and for cheering me on through tough times writing this thesis.

Last, but not least, thank you to all my friends and family for your support and encouragement. A special thanks to my brother, sister and parents, for always being there for me no matter what.

Abbreviations

ACN	Acetonitrile
AFFL	Automatic filtration/filter backflushing
ESI	Electrospray ionization
FA	Formic acid
HPLC	High performance liquid chromatography
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceutical for Human Use
ID	Inner diameter
LC	Liquid chromatography
MeOH	Methanol
MP	Mobile phase
MRM	Multiple reaction monitoring
MS	Mass spectrometry
OD	Outer diameter
OiC	Organ-in-a-column
OoC	Organ-on-a-chip
SPE	Solid phase extraction
SS	Stainless steel
UV	Ultraviolet
V1	10 port valve number 1 (containing two 5 µL sampling loops)

V2	10 port valve number 2 (containing the AFFL part)

Contents

1 Introduction

1.1 Drug development

The process of drug development, from the discovery to the approval, can be described in four steps. This includes drug discovery, preclinical and clinical research/trials, and regulatory approval (**Figure 1)**. In the drug discovery step, new potential drugs undergo early testing like screening for inhibitors or activators for a specific target and show how selective they are for the specific target. Only compounds that show enough promise enters preclinical research, i.e. the stage at which *in vitro* and *in vivo* (animal studies) testing is carried out to test for toxicity and efficacy. These studies are mandatory before human trials can be conducted. The clinical trials consist of three phases, I, II, and III, and are used for examining potential side effects and evaluating the effectiveness of a drug. In phase I, 20-100 healthy volunteers are tested to evaluate the dosage and safety. In phase II, 100-300 people are tested for efficacy and side effects, before efficacy and monitoring of adverse reactions are carried out on 300-3000 participants in phase III. The last step is to get regulatory approval for the drug before it can reach the market [1].

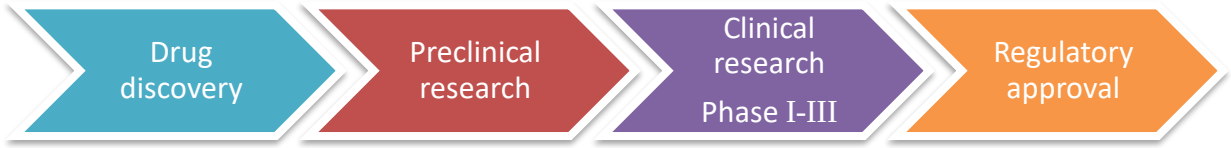

Figure 1: The four main steps in drug development, from the discovery of a compound to regulatory approval.

Drug development is a costly and lengthy process, where only a very few compounds make it through the entire process. The lack of our ability to anticipate efficacy and toxicity prior to clinical trials is one of the main reasons for the very low success rate. For example, humans and animals do not have the exact same physiology, and it is known that their cells respond differently to chemicals [2]. Therefore, results from using animal models often fail to represent the actual results in humans. Additionally, *in vitro* models often fail to recapitulate the function of *in vivo* tissues [3]. Hence, there is a need for better, cheaper models to better represent human physiology and increase animal welfare.

1.2 Organoids as a tool for solving the challenges in drug development?

Organoids are laboratory-grown 3D cell cultures that resemble an organ such as the liver, brain, and kidney [4-6]. It is possible to utilize both human stem cells and also patient-derived stem cells to grow organoids. Because of this, organoids serve as a model for organs and have a huge potential to model human development and disease as well as function as a tool in drug development/testing, and in the future even serve the purpose of organ replacement [4].

Organoids grown *in vitro* are not ideal due to differences in the environment compared to that *in vivo*. To cope with this, organ-on-a-chip (OoC) has been developed [7]. OoC consists of a chip with several small channels and chambers that allows liquids and gases to flow through them, creating an environment more similar to that *in vivo*. Organoids can be grown or placed inside the chambers of the chip fulfilling the OoC. The chip design allows for dissolved nutrients in the liquid to flow through the channels and interact with the cell culture in a similar way as blood flowing through blood vessels in the body [7, 8].

The combination of organ-specific cells and an environment similar to that *in vivo* makes OoC ideal for recreating human organ physiology and possess incredible possibilities to improve toxicity and efficacy testing in drug development, as well as better modeling human organ disease [9]. Another advantage of using OoC is that several different chips may be connected together, forming multi-organ-chips also referred to as body-on-chip. This allows interactions between different types of organoid cell cultures and can mimic more complex responses during toxicology and drug screens [3].

However, there are several challenges related to the use of organoids. The full functional repertoire and complexity of organs are not reproduced by different established organoid systems and may be due to the lack of vascularization and consistent cellular organization in the organoids. Moreover, organoids have limited lifespans, and this results in organoids failing to mature beyond a fetal phenotype because of the vast disagreement in the timing of human and other *in vivo* organogenesis [10]. Analysis of organoid systems is also difficult. For instance, assessing the functionality of hepatic liver organoids can be carried out by performing analyses of metabolites from endogenous and exogenous compounds. The presence of these analytes in individual organoids is typically limited to small quantities being available for study and thus makes their analysis challenging [10].

Eventually, if these, and other challenges are dealt with, organoids may replace animal models in research and drug development since they resemble human physiology better [4-6]. In addition, they may help to advance precision medicine efforts for specific individuals, allowing personalized medicine approaches [9]. Lastly, OoC and body-on-chip systems have the potential to greatly decrease the cost of drug development by providing a "fail early, fail cheaply" approach [3].

1.2.1 Difficulties with coupling organ-on-a-chip to mass spectrometry and possible solution

Liquid chromatography-mass spectrometry (LC-MS, discussed in sections 1.4 and 1.3 respectively) is usually the analytical tool of choice for measuring drugs, their metabolites, and other small molecules due to its great sensitivity and selectivity. Analysis of metabolites from/in organoids with mass spectrometry is typically accomplished with indirect (off-line) methods [11-14], which are associated with increased time consumption, and hence, increased cost.

As well as off-line systems, there are also on-line systems where there is no need for manual steps after injection of sample into the system, thus serving as a direct method for analysis of samples. In contrast to off-line methods, on-line systems reduce time consumption and, at the same time, offer advantages like increased precision, easily automatable, and less chance of outside interferences [15]. Therefore, on-line analysis of OoC would be advantageous. However, it is difficult to integrate OoC with LC-MS because there is a lack of standardized chips and couplings; the design varies for different types of organoids and the chips are custom-made in labs. Moreover, the organoids do not tolerate the high pressures needed in LC.

To overcome the difficulties with coupling organ-on-a-chip to mass spectrometry, Kogler et al. have developed organ-in-a-column (OiC), utilizing standardized equipment such as tubing, ports, fittings, screws, etc. In their setup, liver organoids and glass beads were packed inside a 10 cm long PFTE/PFA tubing which was assembled with nuts, ferrules, screens, and unions. This OiC was then coupled to an LC-MS system (**Figure 2**) which allowed for selective and automated on-line monitoring of drug metabolism of organoids while at the same time not exposing them to the high pressures in the LC system [16]. In this early stage, only one

organoid type (liver organoids) was utilized. However, future setups might contain different types of organoids to mimic the full body.

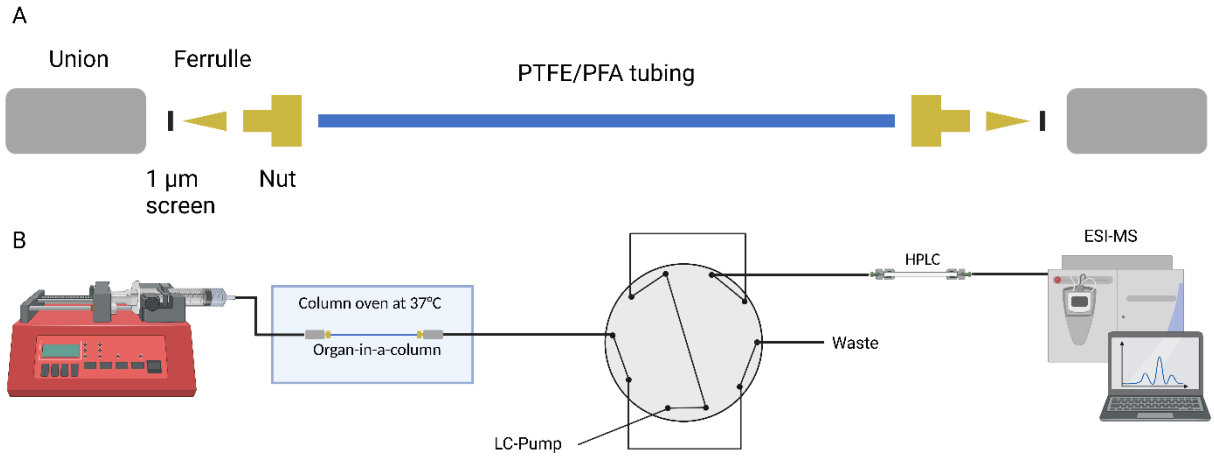

Figure 2: (A) Representation of the column housing for organ-in-a-column. (B) Illustration of the organ-in-a-column coupled on-line to liquid chromatography-mass spectrometry (LC-MS). Adapted from [16]. Made with BioRender.com

One of the problems with the organ-in-a-column, however, is the lack of sample preparation. The OiC allows salts, proteins, and other contamination from the cell medium to be transferred directly from the organoid column into the LC-MS. This can cause ion suppression in the electrospray ionization (ESI, see section 1.3.1), and possible clogging of the LC column. Additionally, it leads to MS downtime due to increased maintenance. Hence, there is a need for a sample clean-up system that can be coupled on-line with OiC.

1.3 Drugs as model analytes for liver organoid metabolism

The liver is the most important site for drug metabolism, both quantitative and qualitative [17]. Therefore, when studying the metabolism of different drugs from organoid cell cultures, liver (hepatic) organoids should be utilized.

Several drugs are used in metabolism studies to determine enzyme activity [18]. The three drugs phenacetine, tolbutamide, and fluoxetine, are all metabolized by preferably one specific enzyme and can be considered telltale drugs for metabolism studies. Phenacetine is metabolized to acetaminophen by primarily CYP1A2 [19], tolbutamide is metabolized to 4-

hydroxytolbutamide by primarily CYP2C9 [20], and fluoxetine is metabolized to norfluoxetine by primarily CYP2D6 [21]. The structure of the different compounds is shown in **figure 3**. These drugs have shown to be metabolized by liver organoids [22], and may therefore be used as model analytes for drug metabolism studies utilizing organ-in-a-column.

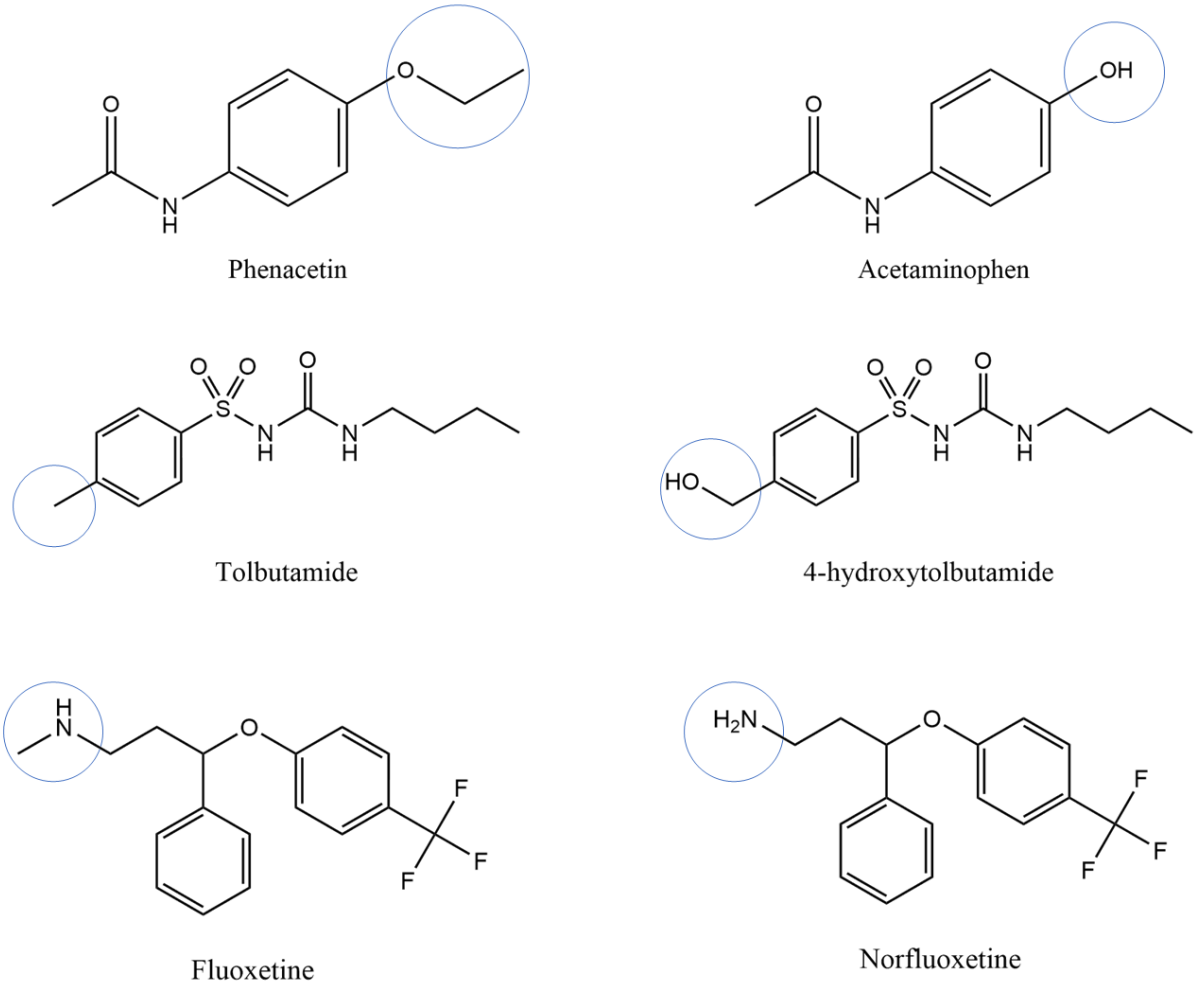

Figure 3: The structure of the drugs phenacetine, tolbutamide, and fluoxetine together with their metabolites acetaminophen, 4-hydroxytolbutamide, and norfluoxetine. The structural differences between the drug and its metabolite are highlighted.

Detection of these three drugs and their metabolites can be carried out using both ultraviolet (UV) detection and mass spectrometry (MS) detection due to the presence of chromophores (conjugated pi system) and ionizable functional groups, respectively (section 1.4).

1.4 Detection techniques in liquid chromatography

1.4.1 Ultra violet detection

UV detection is widely used in LC analysis due to several advantages compared to other detection techniques. It is easy to use, requires low maintenance costs, and is overall inexpensive.

For compounds to be detected by a UV detector, they must absorb radiation/light in the UV spectra, i.e. the compound must contain a chromophore. This is the part of the compound that absorbs the light and usually consists of conjugated double bonds (conjugated pi systems), like aromatic species. Light with a narrow range of wavelengths, referred to as monochromatic light, is passed through the sample containing compound(s) with chromophores (**figure 4**). The amount of light absorbed by the compound(s) is measured by the detector.

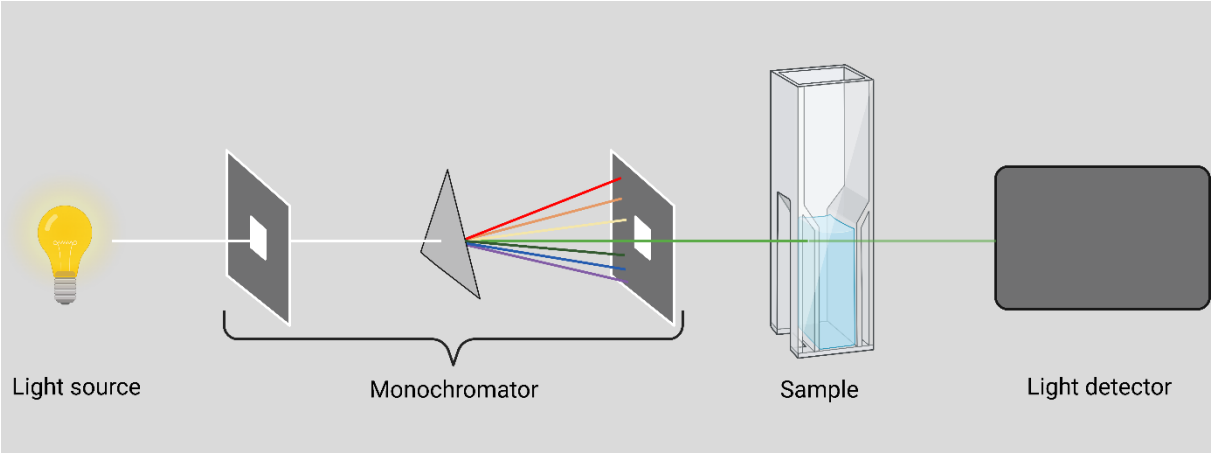

Figure 4: Illustration of the minimum requirements for a spectrophotometer, consisting of a light source, wavelength selector (monochromator), sample with a specific path length, and a light detector. Created with BioRender.com.

The absorbance is important because it is directly proportional to the concentration of light-absorbent species in the sample, as stated by Beer's law (**equation 1**),

$$A = \epsilon bc \qquad (1)$$

where A is the absorption, b is the path length, and c is the concentration. ϵ is the molar absorptivity and tells how much light is absorbed by a particular substance at a given

wavelength [23]. Beers law works well with the use of monochromatic radiation for dilute samples (≤ 0.01M). However, most compounds absorb radiation in the lower part of the UV spectra, and variations in pH and solvent may also influence UV absorption properties [23].

Although UV is most used traditionally, biological samples may require better selectivity, and sensitivity because of complex matrices with the presence of other UV absorptive compounds interfering. MS detection provides better selectivity, resolution, and sensitivity and is widely used for analyses of biological samples [24].

1.4.2 Mass spectrometry

Mass spectrometry (MS) is an analytical technique used to detect different compounds in a sample and is widely used as a detector in bioanalysis. MS is a powerful technique to get both quantitative and qualitative information about different compounds in a sample. The basic principle is that the MS separates gas phase ions according to their mass-to-charge ratio (m/z), i.e. compounds need to be transferred to gas phase ions in order to be separated and detected by the mass spectrometer. This is done by an ion source, one of the main components in an MS instrument, before the ions are separated in the mass analyzer and a signal is generated and amplified in the detector (**Figure 5**).

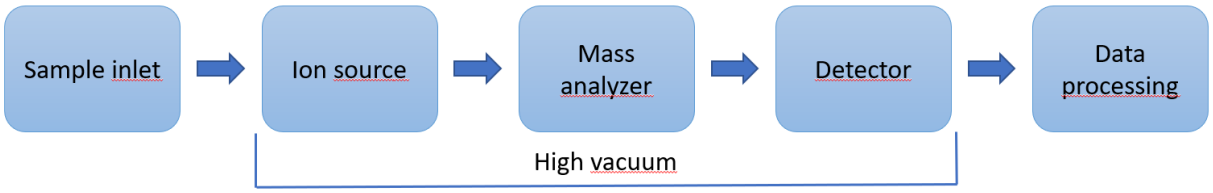

Figure 5: Illustration of the main components of an MS instrument. The inlet introduces the sample to the ion source where compounds are transferred to gas-phase ions. Next, the mass analyzer separates the ions due to their mass-to-charge ratio (m/z) with the possibility of further fragmenting of the ions in a collision cell and then analyzing the fragments. Ions then reach the detector which produces an electrical signal related to the ion stream. The signal is then interpreted by the data processing system.

Electrospray ionization

A challenge when coupling LC to an MS is that the sample needs to get from the liquid phase to the gaseous phase to be able to detect the analytes. One way to do this is to use electrospray ionization (ESI) as the ionization source. Also, polarity and size determine which ion source is best suited as the interface between the LC and MS, and ESI is well suited when using LC

for separating compounds with polar groups, like biomolecules, many drugs, and their metabolites [25].

In ESI, the actual ionization process of compounds usually occurs in the mobile phase, while the conversion into the gas phase is carried out in the ESI interface. The compounds flow with the mobile phase and enter a capillary, where there is applied a high voltage (**Figure 6**). Together with a nebulizing gas at the outlet of the capillary, it allows for spraying the liquid and facilitating the formation of small droplets. In the opposite direction, a drying gas is introduced, providing better evaporation of the charged droplets. The charge density in the droplets becomes so large that the repulsion forces are greater than the surface tension (Rayleigh limit), causing the droplets to explode into smaller droplets, in a repetitive process. Eventually, this results in gas phase ions entering the MS [25].

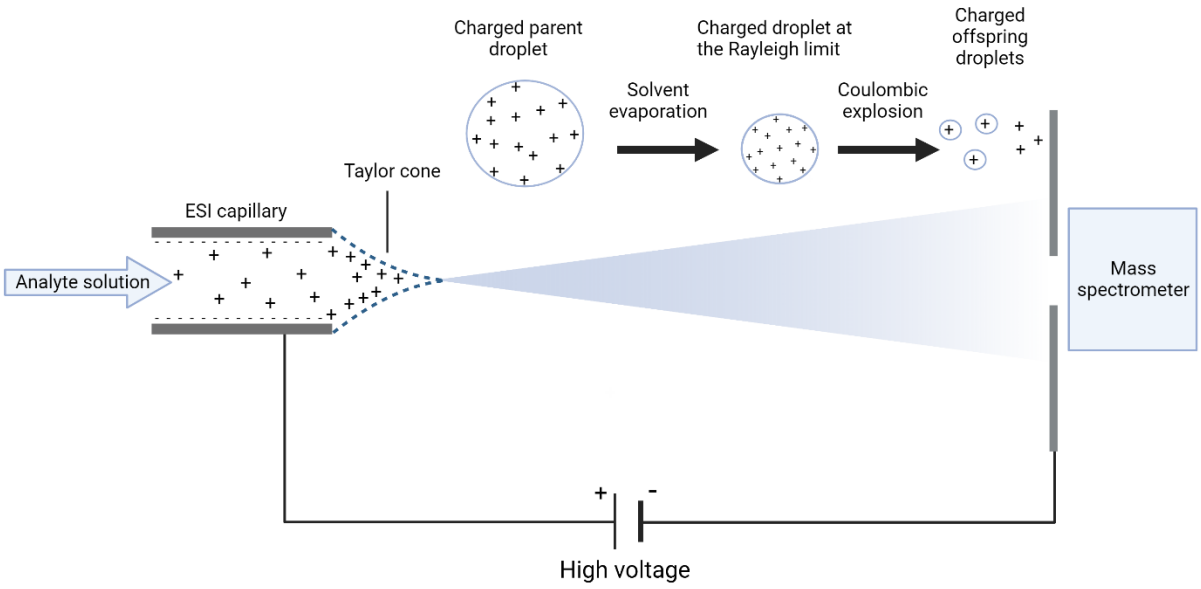

Figure 6: Illustration of the ESI ion source in positive mode. Adapted from *[25]*. Made with BioRender.com.

In ESI, the most important concern is ion suppression, which can cause the signal to be suppressed or enhanced [26]. Ion suppression is caused by nonvolatile and less volatile solutes that change droplet evaporation or droplet formation. This further affects the number of gas phase ions that reach the detector. These compounds can be salts, endogenous compounds, and drugs/metabolites [27].

Tandem MS and triple quadrupole mass analyzer

Post ionization, analytes enter the MS through lenses and skimmers providing focusing of the ion beam. There are several different MS instruments available, which offer different advantages by combining different types of mass analyzers, creating hybrid MS instruments [28-30]. Such instruments allow the use of tandem MS (MS/MS) as one advantage. In tandem MS, ions are first separated by their m/z ratio followed by fragmentation (often by collision-induced dissociation in a collision cell). Then the fragments generated can again be separated according to their m/z.

One common MS instrument for acquiring MS/MS data is the triple quadrupole, containing three quadrupole mass analyzers in series. The quadrupole consists of four parallel rods creating an oscillating electrical field upon applying both a direct current (DC) and a radio frequency (RF) to a pair of the rods, and the opposite DC and RF on the other pair. This electrical field can be tuned to stabilize or destabilize ions with different m/z, resulting in a trajectory causing either detection or collision with one of the rods, and hence no detection (**Figure 7**). By changing the DC and RF values in a controlled manner while maintaining a constant ratio, ions with different m/z are detected one at a time, and a full mass spectrum (full scan) can be obtained [25].

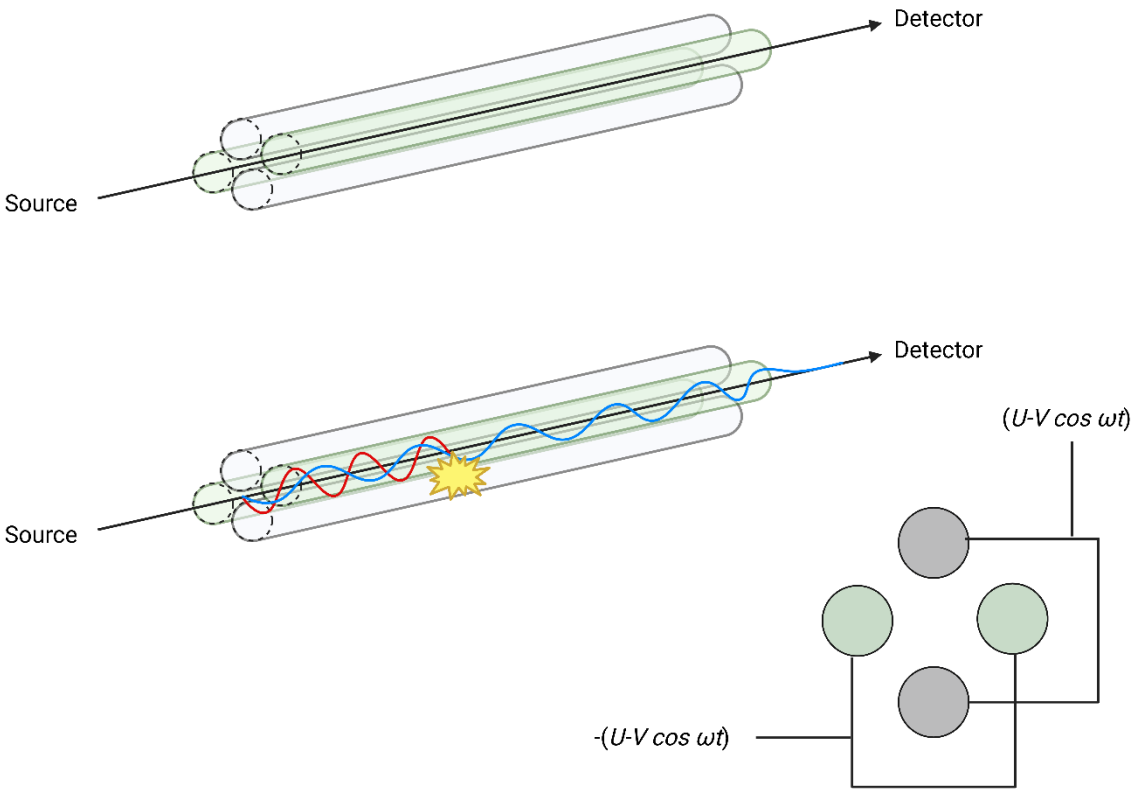

Figure 7: Illustration of a quadrupole mass analyzer consisting of four parallel rods with opposite DC and RF applied to the two pairs of rods. Ions move in the z-direction, from the ion source towards the detector. Due to the oscillating electrical field applied, ions start to oscillate in the xy-directions. Stable ions reach the detector whilst unstable ions do not. By controlling the DC and RF, it is possible to decide which ions will be detected. Adapted from [25].

Due to sample complexity causing matrix effects and other interferences, a pre-separation step, like LC, is favorable. Compounds enter the MS at different time periods which means that the retention time can be evaluated as an addition to the m/z to increase the selectivity. Hence, the addition of LC (section 1.5) will increase the sensitivity and selectivity of a method [26].

1.5 Liquid chromatography

Liquid chromatography (LC) is an analytical technique serving the purpose of separating compounds in a sample from each other by utilizing different separation principles [25]. Compounds are transferred through a column by a mobile phase (MP), and separated due to interactions with a stationary phase (SP). The most common separation principle used in LC is reversed phase (RP) LC. RP-LC is suitable for making hydrophobic interactions with analytes, and hence separate compounds based on their differences in hydrophobicity. RP is

normally the first choice for separating both neutral and ionic compounds using columns packed with fully porous silica-based particles bonded with alkyl chains, like C18 (**Figure 8**) and C8. Bonded C18 alkyl chains are the most usual stationary phase in RP-LC [31]. The mobile phase is an aqueous phase containing a mixture of water and an organic solvent, like methanol or acetonitrile, and an acid or buffer for pH control. The use of RP-LC will allow separation of several biomolecules, drugs, and their metabolites. However, for very complex samples there may be a need for sample preparation and clean-up prior to LC analyses.

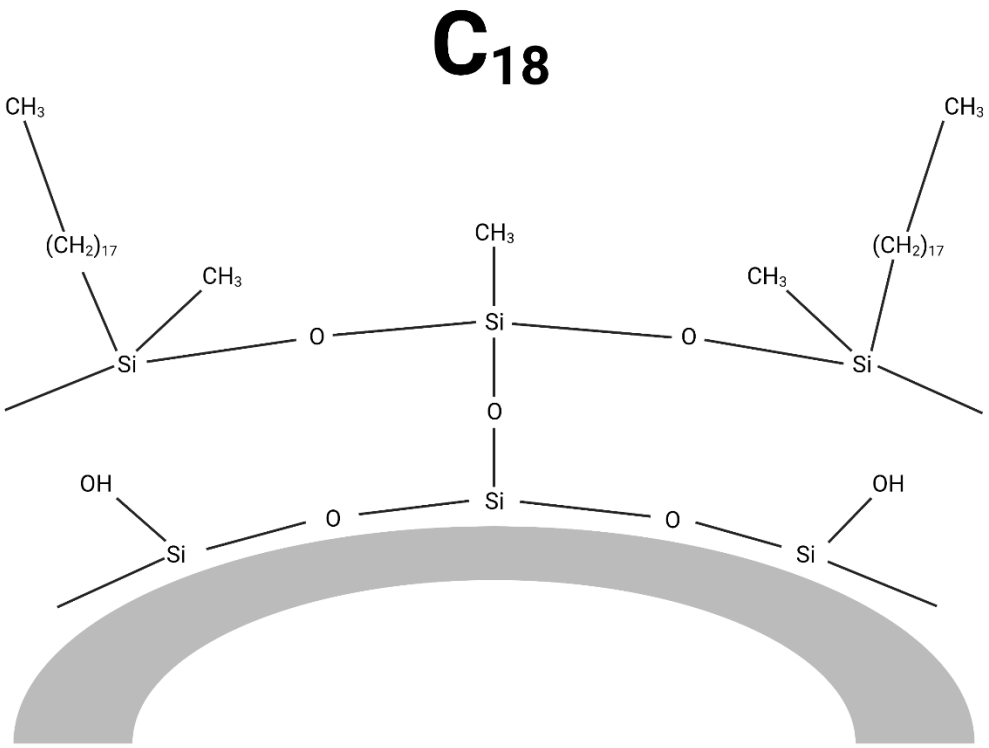

Figure 8: Illustration of bonded C18 (octadecyl) and C8 (octyl) alkyl groups to polymeric silica-based materials. Adapted from [25].

1.5.1 Band broadening and resolution

When solute is moving through a chromatographic column, it tends to spread out in a Gaussian shape due to longitudinal diffusion, eddy dispersion, and resistance to mass transfer in the MP and the SP [23]. This leads to band broadening and results in poorer separation of the peaks associated with the different compounds. Good separation of the compounds in liquid chromatography is essential for qualitative and quantitative analyses, since it increases both selectivity and sensitivity for the method. When compounds are well separated, meaning

they elute at different time periods and no overlapping of analyte bands occurs, the retention time can be used as a parameter for the detection of these compounds.

The resolution between two nearly eluting peaks can be calculated according to **equation 2:**

$$Resolution = \frac{0.589\ \Delta t_R}{w_{0.5\ avg}} \quad (2)$$

where $\Delta t_R$is the difference in retention times for the two peaks, and $w_{0.5\ avg}$ is the average peak width at half height for the peaks. A resolution of > 1.5 is considered baseline separation and is highly desirable for quantitative analysis [23]. With a lower resolution of 0.75, the peaks overlap and the selectivity drops (**Figure 9**).

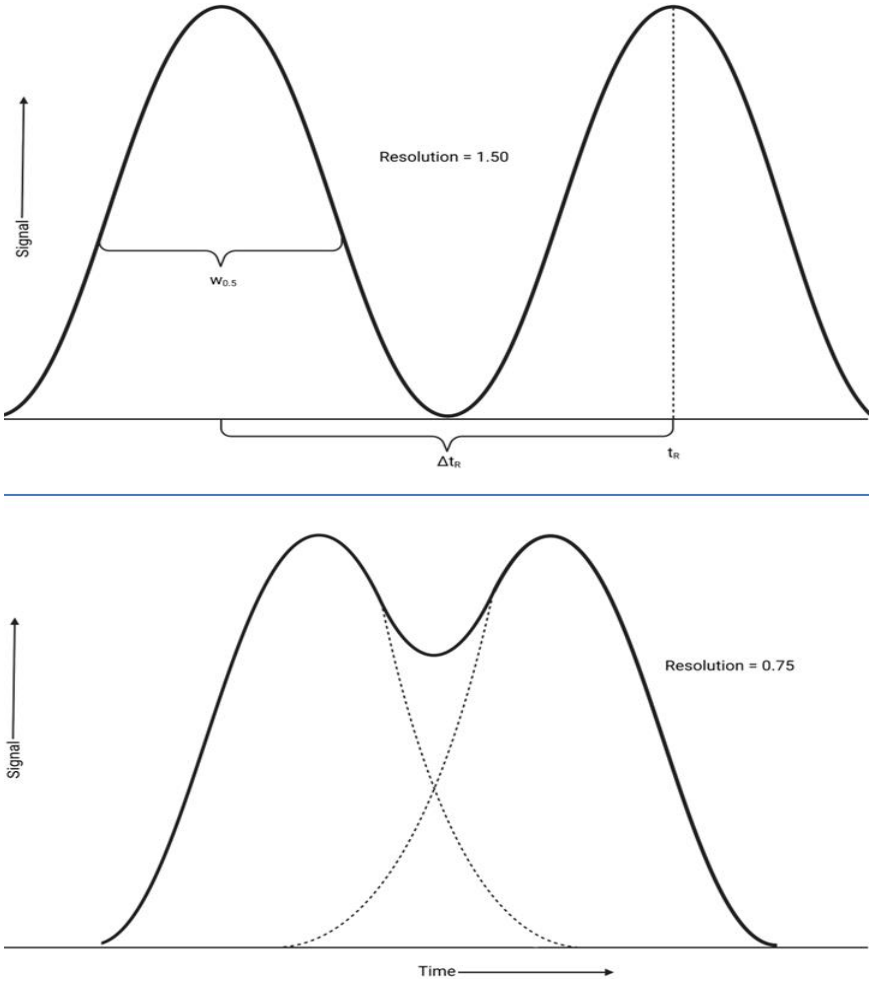

Figure 9: Illustration of the resolution of 1.50 and 0.75 for two closely eluting peaks with equal area and amplitude. The interpretations of the parameters, $w_{0.5}$ and Δt_R, used for calculating the resolution are also illustrated. Adapted from [23].

1.6 On-line sample preparation

Sample preparation serves the purpose to provide isolation and concentration of analytes in addition to simplification of the matrix. Several techniques, such as liquid-liquid extraction, solid-phase extraction, and protein precipitation, are widely used in bioanalysis. The use of these techniques is characterized by being time-consuming, labor-intensive, and a bottleneck for high throughput. On-line sample preparation methods effectively handle these limitations [32, 33].

1.6.1 Solid phase extraction

Solid phase extraction (SPE) is a sample preparation technique that allows sample clean-up and enrichment, while at the same time achieving extraction recoveries close to 100% [34]. Analytes (and other compounds) are retained on a stationary phase bonded to a sorbent material, due to interactions between the analytes and stationary phase, while the rest is removed. These interactions can e.g. be hydrophobic, polar, or ionic interactions, depending on the separation principle utilized, allowing retention of analytes with preferred characteristics.

A normal (off-line) SPE procedure consists of four steps (**Figure 10**). The first step is *conditioning* of the column to make it ready for application. Second, the sample is *loaded* by flushing the sample through the column so that analytes are retained, but matrix components are flushed through. The third step is *washing* the column with different solvents to remove additional matrix components and hence contribute to sample clean-up. Lastly, *elution* of the analytes (and other retained compounds) is performed and the eluate is collected.

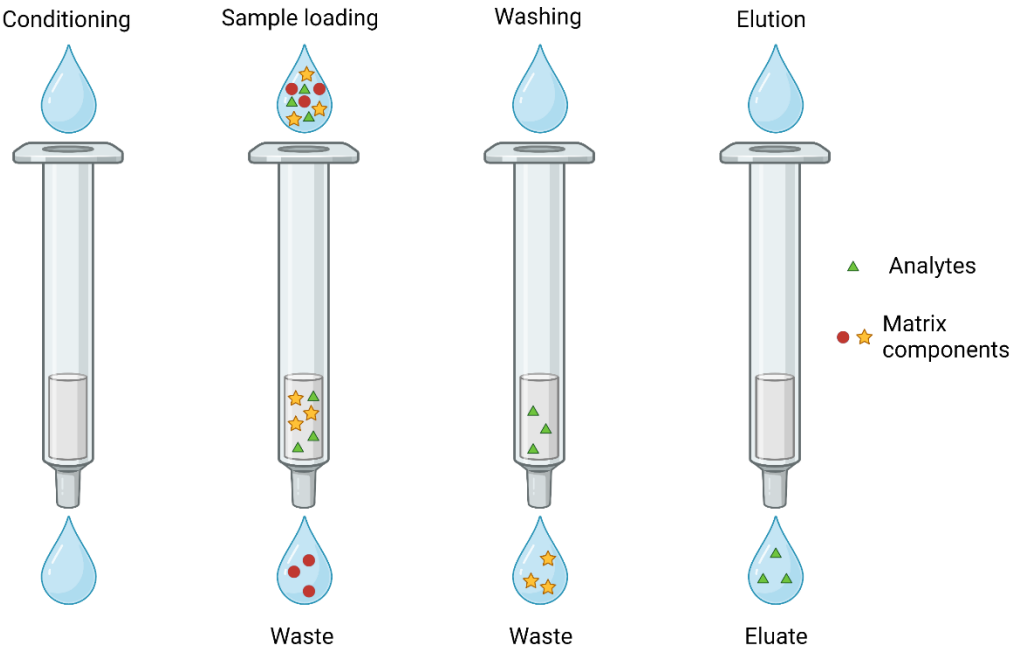

Figure 10: Principle of solid phase extraction. After conditioning the column, the sample is loaded onto the column where analytes and other matrix components are retained on the sorbent material. Then, matrix components are washed away followed by elution of the analytes. Made with BioRender.com.

This off-line procedure requires several steps and is therefore time-consuming. An off-line approach can also lead to possible sample loss, contamination, and/or reduced precision, due to human errors [35]. To overcome these features, an on-line version of SPE may be beneficial, e.g. by utilizing a column-switching system.

1.6.2 On-line solid phase extraction liquid chromatography

By using a column switching system, an SPE column can be coupled on-line together with LC-MS instrumentation, allowing sample preparation without the need for manual steps and with the following advantages as mentioned earlier.

Today, on-line SPE systems are used for routine applications in proteomics and make it easier to inject larger volumes (compared to few nL) at narrow columns. It is not widely used in metabolomics and sample clean-up due to bad robustness, i.e. the SPE column easily gets clogged and leads to backpressure buildup. To avoid this, a modification of column switching, named automatic filtration and filter flush (AFFL) was introduced in 2011 by Svendsen et.al. [35]. The system has been further modified by Roen et al. [36] and applied to sample clean-up of various biosamples and environmental samples [32, 36-42]. The AFFL setup includes an

in-line filter prior to the SPE column to remove particle debris from the sample matrix before it reaches the SPE. This will prevent clogging and therefore avoid backpressure buildup. Upon column switching, the filter is backflushed so that particles are removed before the next injection, while the sample analytes are introduced to the LC-MS for separation and determination (**Figure 11**). Because the filter is backflushed, there is no need for daily replacement due to clogging of the filter as it would otherwise [35]. Hence, the addition of an AFFL system to an on-line SPE method makes a promising tool for robust on-line sample clean-up from OiC.

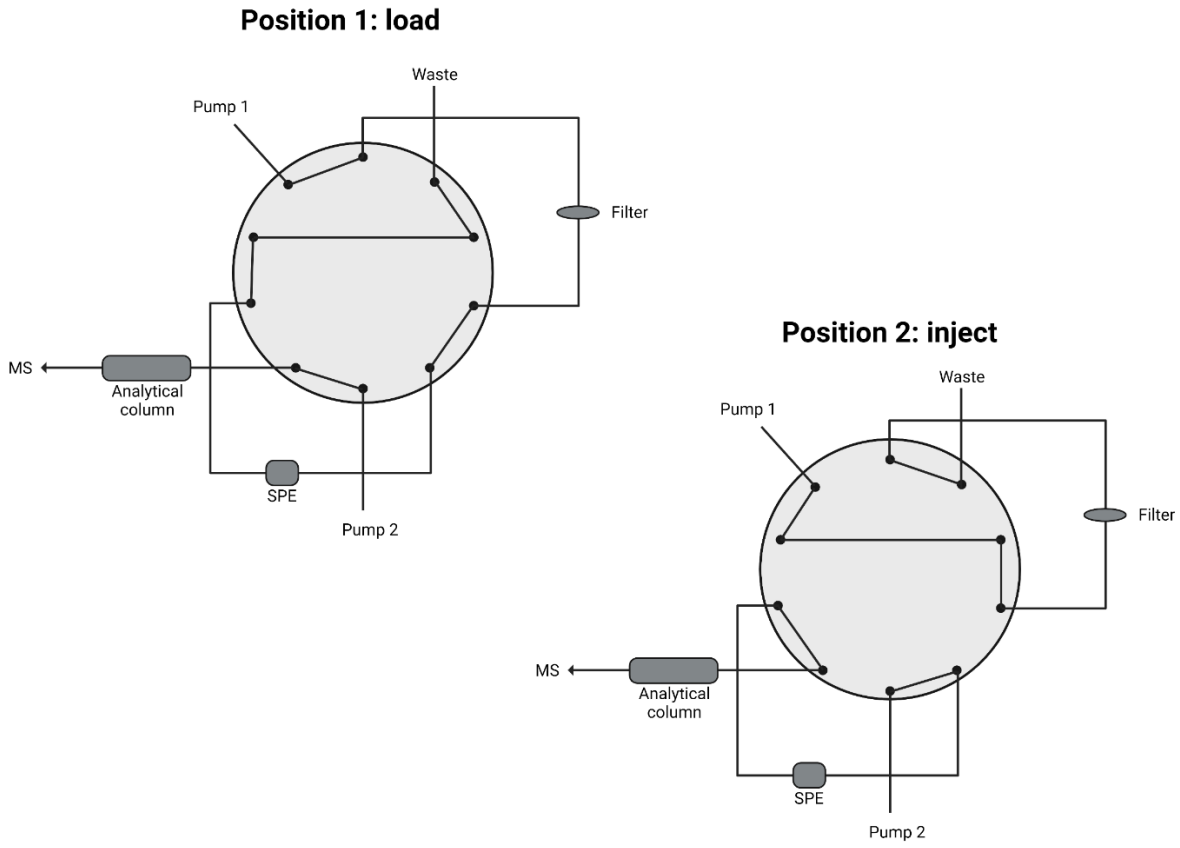

Figure 11: Illustration of a column switching system integrating on-line SPE-LC-MS with AFFL. Adapted from *[38]*.

2 Aim of study

As previously described, organoid technologies seem promising in regard to improving the drug development process, among other things. The development of OiC has been established in order to overcome shortcomings of coupling analytical methods like LC-MS to OoC. However, a problem with OiC is the lack of sample preparation.

Thus, the aim of this study was to expand the use of OiC by developing a robust on-line sample preparation method, cleaning the cell medium, and enriching analytes from OiC experiments for LC-MS analysis of drugs and their metabolites. Moreover, the drugs phenacetin, tolbutamide, and fluoxetine, and their metabolites acetaminophen, 4-hydroxytolbutamide, and norfluoxetine were to be used as model analytes for liver organoid metabolism since the liver organoids are shown to metabolize these drugs.

It was thought that the addition of an AFFL system prior to LC-MS analysis would help achieve sufficient sample clean-up of the organoid cell culture samples from OiC, and so the OiC-AFFL-LC-ESI-MS system (**Figure 12**) was to be developed and examined.

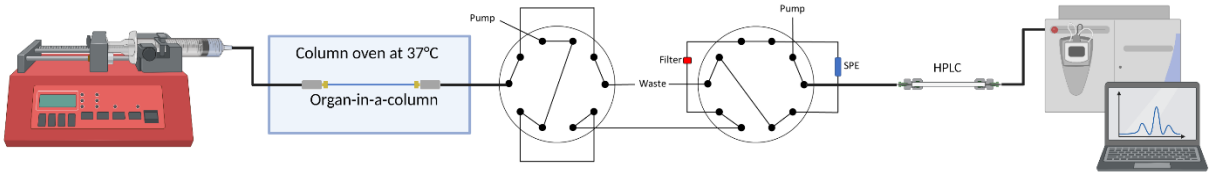

Figure 12: Schematic representation of the OiC-AFFL-LC-ESI-MS system. Made with BioRender.com.

3 Experimental

3.1 Chemicals

HiPerSolv CHROMANORM Water (LC-MS grade), HiPerSolv CHROMANORM Methanol (MeOH, LC-MS grade), HiPerSolv CHROMANORM formic acid (FA, ≥ 99%) and HiPerSolv CHROMANORM Acetonitrile (ACN, LC-MS grade) were purchased from VWR International (Radnor, PA, USA). Type 1 water was acquired from a Milli-Q® Integral 5 water purification system from Merck Millipore (Billerica, MA, USA). Unless stated otherwise, type 1 water will be referred to as water.

Uracil was purchased from Merck (Darmstadt, Germany). Phenacetin (≥ 98%, HPLC), acetaminophen (analytical standard), tolbutamide (analytical standard), 4-hydroxytolbutamide (≥ 98%, HPLC), and fluoxetine hydrochloride (≥ 98%, HPLC) were purchased from Merck Millipore (Burlington, MA, USA). Norfluoxetine hydrochloride (≥ 98%) was purchased from Cayman Chemicals (Ann Arbor, MI, USA).

Nitrogen gas with a purity of 5.0 (99.999%) and argon gas with a purity of 5.0 (99.999%) came from Nippon Gases Norge AS (Oslo, Norway).

3.2 Solutions

The mobile phases (MP) were prepared in 1L graduated laboratory bottles. MP reservoir A (MP A) contained 0.1% FA in LC-MS graded water (*v/v*), and MP reservoir B (MP B) contained 0.1% FA in MeOH (*v/v*). Three different loading solutions were prepared in 1L graduated laboratory bottles, consisting of 5%, 3%, and 0% MeOH (*v/v*) in LC-MS graded water with 0.1% FA (*v/v*), respectively.

Cell medium were provided by Dr. Alexandra Aizenshtadt at Hybrid Technology Hub (Faculty of Medicine, University of Oslo).

1 mg/mL stock solutions of phenacetin, acetaminophen, tolbutamide, 4-hydroxytolbutamide, fluoxetine, and norfluoxetine in acetonitrile (ACN) were prepared by Tonje Monica Erlandsson in 2020 and stored at -20°C before use (except for norfluoxetine which was stored

at -80°C before use). Solutions in the range 5 ng/mL – 50 µg/mL (in water or cell medium) were all made in Safe-Lock tubes from these stock solutions, and stored at 4°C before use.

3.2.1 Preparation of standards in water

For the preparation of 10 µg/mL acetaminophen, phenacetine, and 4-hydroxytolbutamide, 10 µL of 1 mg/mL was transferred to safe-lock tubes and diluted with 990 µL water. These final solutions contained 1% ACN in water.

For the preparation of 40 µg/mL tolbutamide, norfluoxetine, and fluoxetine, 40 µL of 1 mg/mL were transferred to safe-lock tubes, evaporated to dryness, resolved in 10 µL ACN and diluted with 990 µL water. These final solutions contained 1% ACN in water.

For the preparation of 10 ng/mL tolbutamide, 4-hydroxytolbutamide, and fluoxetine, 1 µL of 10 µg/mL was transferred to safe-lock tubes, evaporated to dryness, and resolved in 1000 µL water. For the preparation of 100 ng/mL norfluoxetine, 10 µL of 10 µg/mL was transferred to a safe-lock tube, evaporated to dryness, and resolved in 1000 µL water.

3.2.2 Preparation of standards in cell medium

For the preparation of 20 ng/mL tolbutamide, 4-hydroxytolbutamide, and fluoxetine in cell medium, 1 µL of 10 µg/mL were transferred to safe-lock tubes, evaporated to dryness, and resolved in 500 µL cell medium. For the preparation of 200 ng/mL norfluoxetine in cell medium, 10 µL of 10 µg/mL was transferred to a safe-lock tube, evaporated to dryness, and resolved in 500 µL cell medium.

For the preparation of 15 ng/mL tolbutamide, 4-hydroxytolbutamide, and fluoxetine in cell medium, 1.2 µL of 10 µg/mL were transferred to safe-lock tubes, evaporated to dryness, and resolved in 800 µL cell medium.

For the preparation of 5 ng/mL and 10 ng/mL tolbutamide, 4-hydroxytolbutamide and fluoxetine in cell medium, 133 µL and 267 µL 15 ng/mL of the analytes in cell medium, respectively, were transferred to safe-lock tubes and diluted with 267 µL and 133 µL cell medium, respectively.

For the preparation of 50 ng/mL, 100 ng/mL, and 150 ng/mL norfluoxetine in cell medium, 2 µL, 4 µL, and 6 µL, respectively, of 10 µg/mL norfluoxetine solution were transferred to safe-lock tubes, evaporated to dryness and resolved in 400 µL cell medium.

3.3 Hardware and consumables

Stainless steel (SS) unions, SS reducing unions (1/16" to 1/32"), SS ferrules, and SS nuts (all made for 1/32" tubing), SS tubing (1/32" OD, 0.12 mm, 0.25 mm, 0.50 mm ID), Cheminert® 10 port switching valves, 1/32" SS screens (1 µm pores), internal reducers (1/16" to 1/32") and tubing cutter were purchased from VICI Valco (Schenkon, Switzerland).

Eppendorf Safe-Lock Tubes (1.5 mL) were purchased from Eppendorf (Hamburg, Germany). The 25 µL syringe was from Hamilton (Reno, NV, USA).

For the on-line SPE, two 1.0x5.0 mm HotSep® C18 kromasil columns with 5µm sized particles and 100Å pore size were used. The chromatographic column used was a 1.0x50 mm micro-HotSep® C18 kromasil with 3.5µm sized particles and 100Å pore size. These columns came from G&T Septech (Ski, Norway).

3.4 Instrumentation

The LC-UV system was from Agilent Technologies (Santa Clara, CA, USA) and consisted of an 1100 series G1376A capillary pump, an 1100 series G1379A degasser, and a 1200 series G1365B multiple wavelength detector with a 10 mm flow path.

The LC-MS system was from Thermo Fisher Scientific and consisted of the Dionex Ultimate 3000 UHPLC system and the TSQ Vantage MS with the HESI-II ion source.

An L-7100 pump from Hitachi High-technologies (Tokyo, Japan) was used for loading the sample on to the AFFL system.

The Concentrator plus vacuum concentrator was from Eppendorf.

3.5 Liquid chromatography ultraviolet detection

3.5.1 Analyte breakthrough on solid phase extraction column

For the examination of analyte breakthrough on the SPE columns, the valve system was configured as seen in **figure 13**. The UV detector was positioned so that injected standards had to pass through the filter and SPE column before they reached and could be detected by the multiple wavelength detector. In this set-up, only the first 10-port valve system (V1) switched between positions A and B. The second 10-port valve (V2) was left in position A.

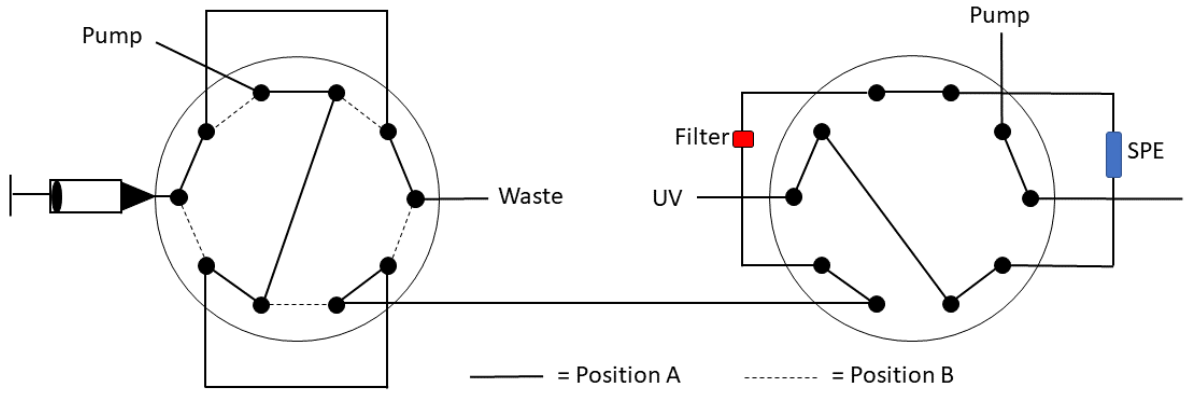

Figure 13: Illustration of the valve system configuration for the examination of breakthrough on the SPE column.

Standards were manually injected with a full 25µL syringe to fill a loop with a fixed volume of 5 µl, followed by subsequently switching the valve from position A to B. Two different constant flow rates were used, 100 µL/min and 50 µL/min. The data acquisition was stopped after 30 min for the flow rate of 100 µL/min and after 60 min for the 50 µL/min flow if no peaks were detected before this time.

Three different load solutions were utilized, consisting of 0 %, 3 %, and 5 % MeOH in LC-MS graded water with 0.1 % FA (*v/v*). Standards of uracil, acetaminophen, phenacetin, 4-hydroxytolbutamide, tolbutamide, norfluoxetine, and fluoxetine with a concentration in the range of 10-50 µg/mL) in 1% ACN (*v/v*) were each injected three times per standard (n=3) for every load solution at both flow rates. The different analytes were detected using different wavelengths, as shown in **table 1**. In addition, the same experiment was performed for an additional SPE column of the same type.

Table 1: Wavelength used for detection of the different analytes in all the experiments with UV detection. The concentration of the analytes used for LC-UV is also presented. U = uracil, A = acetaminophen, P = phenacetin, 4-HT = 4-hydroxytolbutamide, T = tolbutamide, N = norfluoxetine, F = fluoxetine.

Analyte	**U**	**A**	**P**	**4-HT**	**T**	**N**	**F**
Wavelength	250 nm	249 nm	250 nm	229 nm	229 nm	225 nm	225 nm
Concentration (µg/mL)	50	10	10	10	40	40	40

3.5.2 Timing of switch in the AFFL component

A flow rate of 100 µL/min for both the loading solution and the mobile phase was used for the AFFL system (**Figure 14**). The load solution had a composition of 3% MeOH in LC-MS graded water, while the mobile phase consisted of MP B in the range of 30-50%.

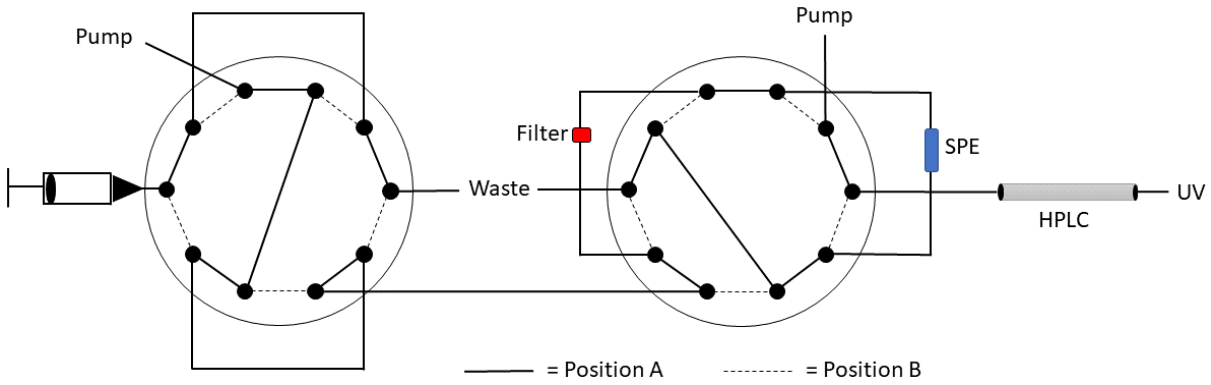

Figure 14: Schematic overview of the LC-UV set-up. The first valve has two sampling loops, and at any given position, one loop can be filled with sample, while the other loop is loaded onto the AFFL system in the second valve.

The second valve (V2) in the AFFL system initially switched from position A to B after 0.65 min, or 36 seconds. Later, this was changed to 1.00 min. The positions for each valve for a given time are presented in **table 2**.

Table 2: Positions for the two different 10-port valve systems, denoted V1 (first, with both sample loops and syringe) and V2 (second, the AFFL system). The difference from initial to subsequent examinations is in the time passed before switching the position from A to B for V2.

Initial switching		
Time (min)	**Position V1**	**Position V2**
Load	A	A
0.00 (start of run)	B	A
0.65	B	B
Subsequently switching		
Time (min)	**Position V1**	**Position V2**
Load	A	A
0.00	B	A
1.00	B	B

3.6 Liquid chromatography with mass spectrometric detection

The MRM transition settings used in all experiments for the MS detection for the analytes tolbutamide, 4-hydroxytolbutamide, fluoxetine, and norfluoxetine are summarized in **table 3**. In addition, the flow rate for the MP solution was set to 100 µL/min for all experiments with LC-MS, and the analytical column temperature was 25 °C. Whenever the AFFL system was used, the second valve (V2, which controls the AFFL) switched from the load position to inject position (from position A to B) after 1.00 min, and the load solution had 3 % MeOH in water + 0.1 % FA.

Table 3: - An overview of the MRM transition settings used for detection in the MS.

Analyte	Monoisotopic mass	Precursor ion (m/z)	Fragment ion (m/z)	MRM collision energy (eV)	S-lens value	Polarity
4-hydroxytolbutamide	286.0987	285.06	185.96 103.96	21 33	83 83	-
Tolbutamide	270.1038	269.11	169.97 106.00	19 33	88 88	-
Norfluoxetine	295.1184	296.13	133.99	5	56	+
Fluoxetine	309.1340	310.14	148.048 43.83	6 13	64 64	+

3.6.1 Optimizing mobile phase composition and determining t_0

A standard of 10 µg/mL 4-hydroxytolbutamide in water was injected three times (n=3) with 95% MeOH in the mobile phase. For optimizing the mobile phase composition, isocratic conditions in the range of 40-70% MP B were examined for the four different analytes. The MS acquisition time was 10 min.

3.6.2 Retention time, signal over time, and carry-over

The following sequence was performed for standards with a concentration of 10 ng/mL in water and 20 ng/mL in cell medium for tolbutamide, 4-hydroxytolbutamide, and fluoxetine, and 100 ng/mL in water and 200 ng/mL in cell medium for norfluoxetine:

- (Injection + blank) x3
- Blank
- (Injection + blank) x3

For tolbutamide and 4-hydroxytolbutamide the MP B = 50 % and for fluoxetine and norfluoxetine the MP B = 60 %.

An additional injection + blank was performed for the three standards containing tolbutamide, 4-hydroxytolbutamide, and norfluoxetine in cell medium.

3.6.3 Calibration experiments with and without AFFL

Cell medium was spiked with the analytes to make standards with concentrations of 5 ng/mL, 10 ng/mL, and 15 ng/mL for tolbutamide, 4-hydroxytolbutamide, and fluoxetine, and 50 ng/mL, 100 ng/mL, and 150 ng/mL for norfluoxetine. For each standard, three replicates (n=3) and three blank injections (alternating standard and blank) were performed. This experiment was done both with and without the use of AFFL.

For tolbutamide and 4-hydroxytolbutamide the MP B = 50 % and for fluoxetine and norfluoxetine the MP B = 60 %.

The following standards, 10 ng/mL 4-hydroxytolbutamide, 5 ng/mL tolbutamide, 50 ng/mL norfluoxetine, and 5 ng/mL fluoxetine all with AFFL and 50 ng/mL norfluoxetine 5 ng/mL without AFFL had an extra injection of standard + blank after the sequence.

3.6.4 Full scan of cell medium

Full scan with a m/z range of 150-1050 were performed on non-spiked cell medium both with and without the use of AFFL, and with 50% and 60 % MP B.

3.7 Statistical calculations

Grubbs' test

For the identification of outliers, Grubbs' test was performed according to **equation 3**:

$$G_{calc} = \frac{Questionable\ data - \bar{x}}{s} \quad (3)$$

The calculated value, G_{calc}, was compared with a critical value, G_{table}, from **table 4** at 95 % confidence, and could be rejected if $G_{clac} > G_{table}$.

Table 4: Critical values of G for rejection of an outlier with 95 % confidence.

Number of observations	**G_{table} (95 % confidence)**
4	1.463
5	1.672
6	1.822
7	1.938

Calculation of LOD and LOQ from calibration curves

The calculation of the limit of detection (LOD) and limit of quantification (LOD) from calibration curves, was done by following *The International Council for Harmonisation of Technical Requirements for Pharmaceutical for Human Use* (ICH) quality guidelines for validations of analytical procedures [43]. This guideline states that the LOD and LOQ may be expressed as (**equation 4-5**):

$$LOD = \frac{3.3\,\sigma}{S} \quad (4)$$

$$LOQ = \frac{10\,\sigma}{S} \quad (5)$$

where σ is the residual standard deviation of a regression line, and S is the slope of the calibration curve. Linear regression in Excel was used to generate a calibration curve. The data analysis tool in Excel was used to establish the residual standard deviation (**Table 12,** in appendix).

4 Results and discussion

The aim of this study was to expand the use of OiC by developing a robust on-line sample preparation method, cleaning the cell medium, and enriching analytes from OiC experiments for LC-MS analysis of drugs and their metabolites. Moreover, the drugs phenacetin, tolbutamide, and fluoxetine, and their metabolites acetaminophen, 4-hydroxytolbutamide, and norfluoxetine were to be used as model analytes for liver organoid metabolism since the liver organoids are shown to metabolize these drugs.

However, due to unforeseen events, organoids were not provided, and the use of OiC was not possible. As an alternative, the focus was therefore on method development for AFFL-LC-ESI-MS with spiked cell-medium.

The results and discussion is divided into two main parts; preliminary method development using liquid chromatography-ultraviolet detection (**section 4.1**) and method development using liquid chromatography-mass spectrometry (**section 4.2**).

4.1 Preliminary method development using liquid chromatography-ultraviolet detection

For the preliminary method development with liquid chromatography ultraviolet detection, standards of the six different analytes in water were used. The different analytes have different wavelengths for maximal absorption and were therefore detected using wavelengths customized for each analyte. Since only one analyte standard was examined at a time, there would be no interferences, and hence UV detection would be more than satisfactory. In addition, no quantification of analytes was to be done with UV, so the need for better sensitivity and selectivity that MS provides was not necessary.

Two different set-ups were used with UV, as described in **section 3.5**. For the examination of analyte breakthrough on SPE columns only the first 10-port valve system (V1) needed to switch between the positions A and B, thus leaving the second pump on V2 in standby. When the AFFL system was added, both valves, V1 and V2, had to switch in a given manner in order to make use of the AFFL system (**section 3.5.2**). The use of these set-ups is discussed in the following sections (**4.1.1-4.1.2**).

4.1.1 De-wetting of the stationary phase leads to severe loss in retention for the solid phase extraction column

SPE as a sample preparation procedure is used to generate a cleaner, more enriched sample with a high degree of recovery. However, during sample loading and subsequent wash steps, analyte breakthroughs can happen, yielding low recoveries. This is the most common cause of low recoveries in SPE in addition to using too weak solvents when analytes are to be eluted [44]. To study analyte breakthrough on the SPE column, the system used was coupled according to **figure 13**, and each standard was injected three times (n=3). The UV detector was positioned so that the injected standards had to pass through the filter and SPE column before they reached and could be detected by the multiple wavelength detector. The analytes were loaded on to the SPE column with a load solution consisting of LC-MS water with the addition of 0.1 % FA and a flow rate set to 100µl/min. This solution is a very weak elution solvent in RP chromatography and SPE due to no organic component that could counteract the hydrophobic interactions between the analyte and the stationary phase. In RP SPE it is also common to use a 100 % aqueous solution for washing since it removes inorganic salts and polar compounds while still having strong retention of the analytes [34]. This solution was then chosen because it was believed that this weak elution strength would prevent analyte breakthrough and thus could achieve sufficient cleaning of samples.

The dead time (t_0) of the system needed to be determined before continuing with the analytes of interest. The compound uracil was chosen for this task for the reason being that it is a well-known compound for determining the dead time of a RP-LC system and is shown to be unretained on a C18 column [45]. With the parameters described above, t_0 was determined to be 0.737 ± 0.003 min. The retention times for the analytes under these conditions are summarized in **table 5** with the addition of their log P value.

Table 5: Retention time (min, n=3), t_R, with standard deviation for the different analytes when testing for analyte breakthrough time on SPE number 1 with 100 µl/min flow rate and LC-MS graded water with 0.1% FA. Their log P value obtained from PubChem is also listed.

Analyte	**t_R ± SD (min)**	**Log P (from PubChem)**
Uracil	0.737 ± 0.003	-1.07
Acetaminophen	0.734 ± 0.002	0.46 0.49

Phenacetin	1.197 ± 0.006	1.58
4-hydroxytolbutamide	1.385 ± 0.002	1.1
Tolbutamide	4.17 ± 0.04	2.34 2.2
Norfluoxetine	1.35 ± 0.05	3.5
Fluoxetine	1.17 ± 0.01	4.05 4.6

All analytes except tolbutamide showed so little retention that their detected peak in the chromatogram overlapped, fully or partially, with the uracil t_0 peak. As a consequence, the analytes would not be well retained on the SPE during an AFFL procedure, yielding bad recoveries or not sufficient sample clean-up. The observed retention was not as expected based on the log P value for the analytes and a low flow rate of 100 µl/min. Moreover, their retention order is also different from other in-house experiments previously performed [22].

To examine if something was wrong with the SPE column that lead to this low retention, the experiment was performed on an additional SPE column. All analytes except acetaminophen had some increase in retention time (**table 7** in appendix, 0%MeOH SPE2). The retention time increased most for tolbutamide, fluoxetine, and norfluoxetine, with 3.38 min, 1.14 min, and 0.89 min, respectively. For 4-hydroxytolbutamide and phenacetine the increase in retention times was 0.24 min and 0.08 min, respectively. Although the retention time increased, the retention was still too low for several of the analytes to provide sufficient sample clean-up.

An explanation for this observed low retention could be due to a phase collapse of the bonded alkyl chains on the particle surface when using a 100 % aqueous mobile phase [46, 47]. This more compact conformation of the alkyl chains severely reduces the accessibility to the stationary phase for the analytes, causing a severe reduction in retention. However, this explanation for the observed retention-loss may be wrong an can be better described as a de-wetting phenomenon [31, Section 5.3.2.3, 48]. De-wetting is the extrusion of solvent (water in this case) from the pores of the particles in the column due to a pressure difference between water vaper from dissolved micro-bubbles formed in the pores, and the liquid water pressure outside of the particle (known as *Laplace pressure*). This results in analytes no longer being

in contact with the internal surface area on the inside of the pores on the silica particles, consequently leaving them unable to be exposed to most of the stationary phase [48]. Moreover, this will reduce retention and can be a valid explanation for the observed low retention.

Particles with pore sizes below ~200Å are more prone to de-wetting [48, 49]. Choosing a column containing particles with bigger pore sizes may deal with the problem of de-wetting. However, bigger pores come with the disadvantage of less stationary phase material due to less surface area available for the bonded stationary phase, and may therefore not increase the retention as much as desired.

The pressure in the SPE was only about 15 bar when the flow rate was 100 µL/min, and even lower when the flow rate was 50 µL/min (around 8 bar). Since the Laplace pressure between the water vapor inside the pores, and the liquid water around the particle is ~20 bar, the de-wetting would still happen. Keeping the outlet column pressure above 50 bar could therefor minimize the observed retention loss [48]. For this, the use of a restrictor could have been examined.

However, the problem with de-wetting of the stationary phase may be better dealt with by changing the mobile phase, or the load solution in this case, so that it would contain organic modifiers and thus avoid the retention loss. The same experiment was therefore repeated with two different loading solutions containing 3 % and 5 % MeOH in LC-MS water, respectively, with 0.1 % FA. All analytes except acetaminophen had a vast increase in retention compared to the initial 100% aqueous solution (**Figures 15-18**).

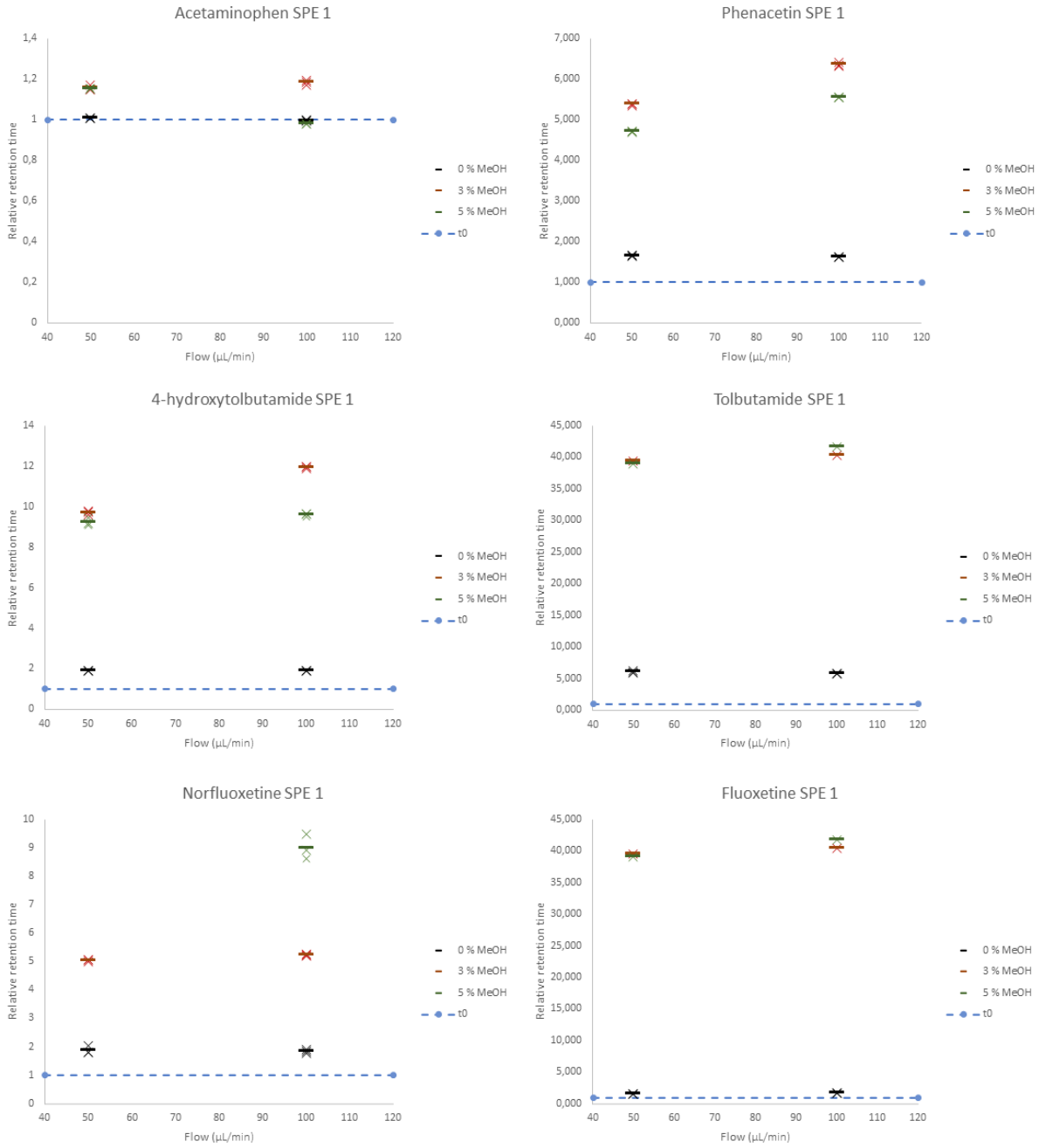

Figure 15: Plots of the relative retention time for the analytes acetaminophen, phenacetin, 4-hydroxytolbutamide, tolbutamide, norfluoxetine, and fluoxetine compared to the t_0 retention determined by uracil for flow rates of 100 µL/min and 50 µL/min for SPE 1. They show a big increase in retention from 100 % aqueous solution to 97 % and 95 % aqueous solution (3 % and 5 % MeOH respectively) for all analytes except acetaminophen.

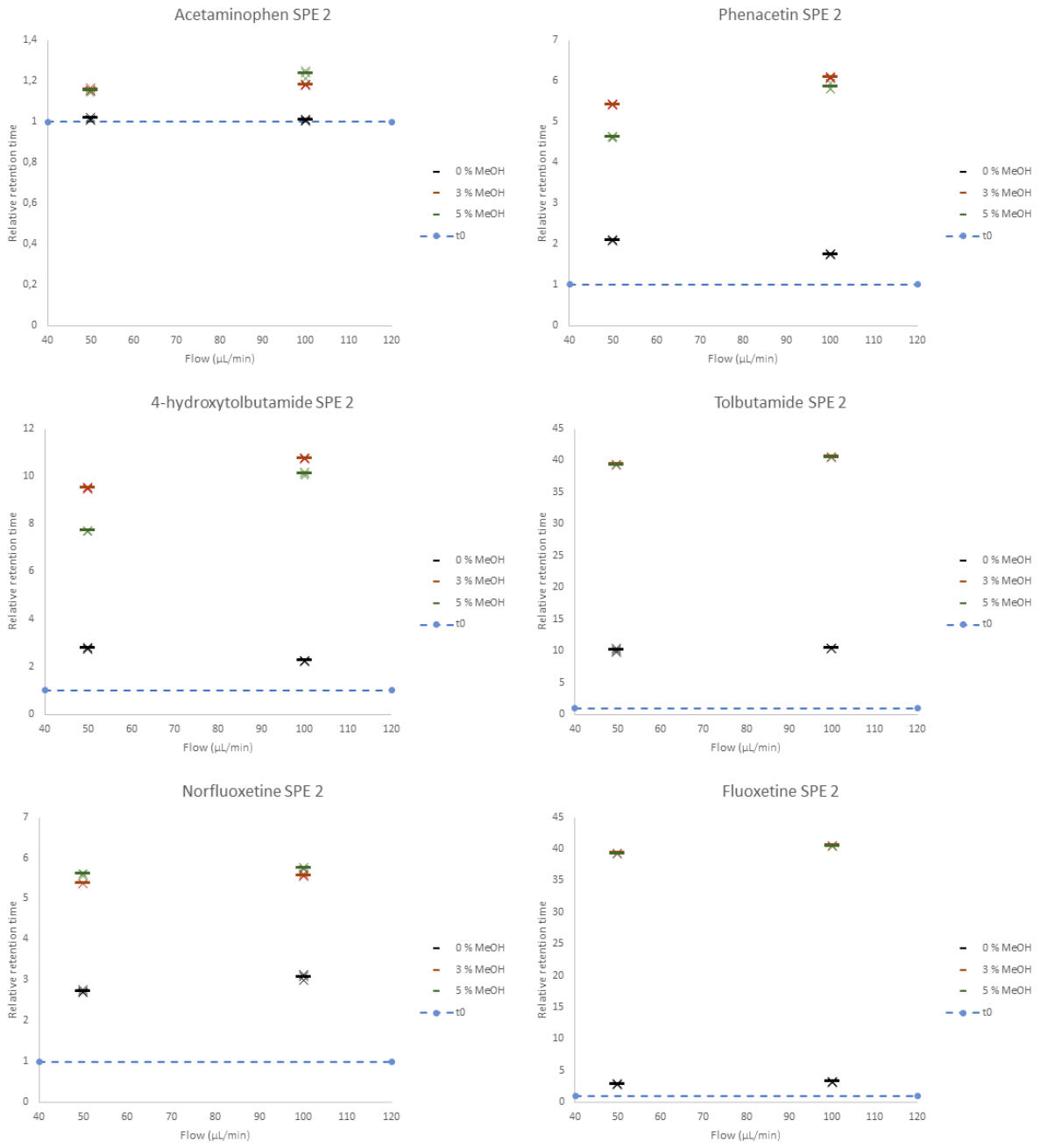

Figure 16: Plots of the relative retention time for the analytes acetaminophen, phenacetin, 4-hydroxytolbutamide, tolbutamide, norfluoxetine, and fluoxetine compared to the t_0 retention determined by uracil for flow rates of 100 µL/min and 50 µL/min for SPE 1. They show a big increase in retention from 100 % aqueous solution to 97 % and 95 % aqueous solution (3 % and 5 % MeOH respectively) for all analytes except acetaminophen.

Although SPE 2 provided better retention for a load solution containing only water, it still had a huge retention loss compared to when the load solution contained an organic modifier. For instance, both tolbutamide and fluoxetine were not detected before 30 min and 60 min had passed with a flow of 100 µL/min and 50 µL/min, respectively (**Figures 17-18**). These results show that de-wetting is a problem with no organic component in the load solution.

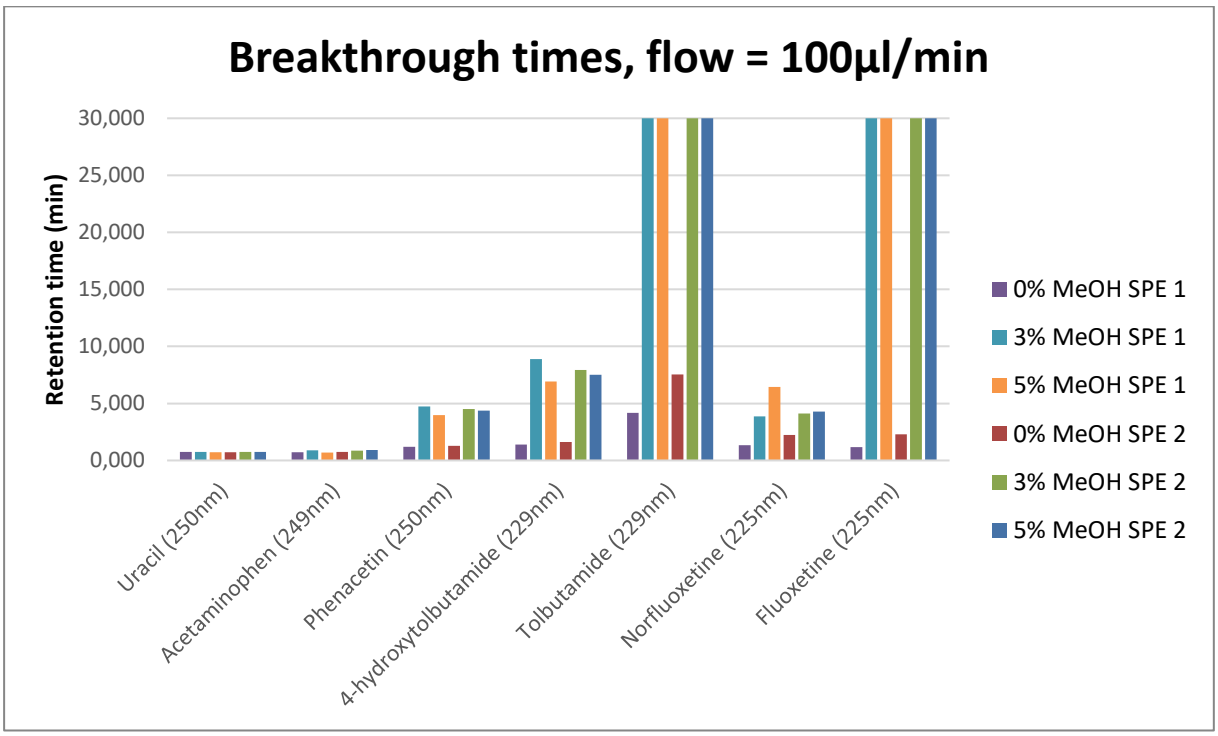

Figure 17: Average retention times observed for the different analytes using two different SPE columns, with a flow rate of 100 µL/min, and three different load solutions containing 0%, 3%, and 5% MeOH in LC-MS graded water with 0.1 % FA. An increase in retention times when adding MeOH to the load solution was observed.

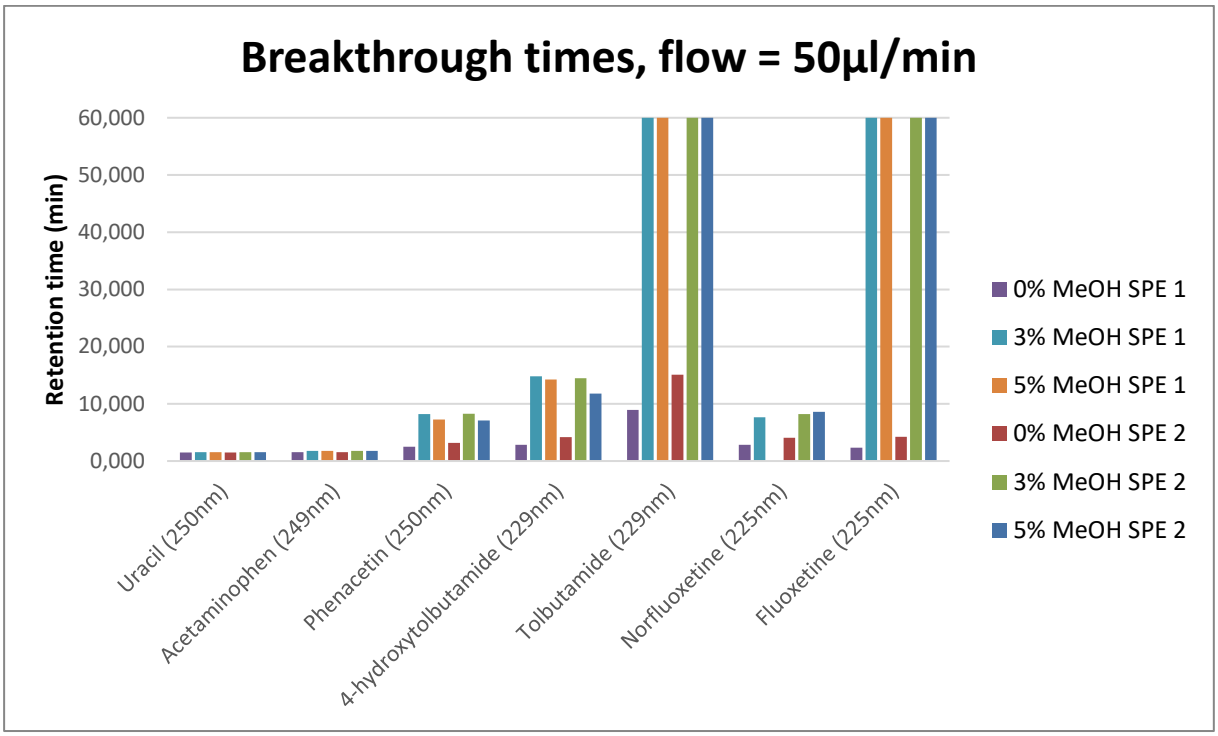

Figure 18: Average retention times observed for the different analytes using two different SPE columns, with a flow rate of 50 µL/min, and three different load solutions containing 0%, 3%, and 5% MeOH in LC-MS graded water with 0.1 % FA. An increase in retention times when adding MeOH to the load solution was observed.

The analyte breakthrough time with three different load solutions containing 0 %, 3 %, and 5 % MeOH in water with 0.1 % FA and two flow rates of 100 µL/min and 50 µL/min were examined. When only water was used as the load solution, the stationary phase was de-wetted leading to retention loss. All analytes had an increase in retention time when the load solution contained 3 or 5 % organic modifier compared to 0 %.

4.1.2 Acetaminophen is not compatible with on-line SPE system due to little retention

With an estimated internal tubing/dead volume of approximately 40 µL before the SPE column, the initial switch time was set to 0.65min (or 36 seconds). With a flow rate of 100 µL/min, 65 µL would flow through the column. With this volume the injected standards had time to reach the SPE column (estimated 40 µL used) and load the sample (5 µL), followed by a little over one column volume (15.7 µL) with washing to clean up the sample before transferring to the HPLC column.

From early testing of the system, there were detected peaks for every analyte standard injected. However, when examinations of breakthrough were performed it was shown that acetaminophen had very little to no retention on the SPE column used. Since acetaminophen had breakthrough, it should have flushed through the SPE leaving the column "blank". Following with a valve switch, no analyte should have been transferred to the LC-UV part and thus no peak should have been detected.

These results led to the assumption that the internal volume probably was more than 40 µL and that the initial detection of acetaminophen could be explained by the timing of the switch for the second 10-port valve. After 0.65 min the analyte band (and also the mobile phase front) have not had enough time to completely elute from (or pass through) the SPE; When the switch then is performed, the remaining parts of the analyte band are transferred to the HPLC-column and further to detection in the UV.

This was later confirmed by delaying the V2 switch to 1 min, which then did not give any detection of acetaminophen. After 1 min, still with a flow rate of 100 µL/min, the SPE had more time to be thoroughly washed. The entire analyte band has now had time to completely pass through the SPE and go to waste. Therefore, when the switch was performed, no acetaminophen was left in the system and nothing was transferred to the HPLC-column and UV for detection. This led to the conclusion that a switch time of more than 0.65 min had to be used. It also confirmed that acetaminophen had breakthrough on the SPE.

In despite of this, why can't a switch time of 0.65 min be used if it provides a detectable peak from acetaminophen? If a peak from a non-retained compound is detected when using this switch-time, then the SPE won't serve its purpose in the AFFL system, i.e. to perform a clean-up of the injected samples by retaining the analyte compounds and wash away undesired compounds to reduce the complexity of the sample and achieve better selectivity and sensitivity (because SPE can allow more injected analyte).

The goal of this study was to use these analytes as model analytes for liver organoid metabolism. Since acetaminophen was not compatible with the AFFL system due to low retention on the SPE, it could be discarded from the rest of the experiments, together with its parent drug phenacetine.

4.2 Method development using liquid chromatography-mass spectrometry

Upon switching from LC-UV to LC-ESI-MS, experiments were performed without the use of phenacetin and acetaminophen (see section **4.1.2**). When using the MRM mode for the detection of the analytes, different transition settings for the analytes tolbutamide, 4-hydroxytolbutamide, fluoxetine and norfluoxetine were used. These are summarized in **table 3** (section 3.6). The MRM transitions were based on a former student's optimization on the same MS instrument as used in this thesis [22].

4.2.1 Determining t_0

To make sure that the analytes had retention with the MP composition used, t_0 had to be determined. Since uracil is not compatible with ESI-MS detection, t_0 had to be determined in another manner. This was done by utilizing 95 % MP B and injecting 10 µg/mL 4-hydroxytolbutamide in water, the analyte with the lowest retention of the four used for LC-MS. By having nearly solely organic solvent in the mobile phase, the analyte would have close to none (or at least very weak) interactions with the stationary phase. This would then lead to the analyte eluting with the mobile phase front and being detected by the MS. In retrospect, utilizing phenacetin may be a more suitable alternative due to its comparatively weaker retention while still maintaining compatibility with AFFL.

Three replicates of 4-hydroxytolbutamide were made, and with this method, the t_0 was determined to be 1.57 ± 0.006 min. This showed that the analytes had enough retention to be resolved from the t_0 peak, and the optimization for MP composition could be used (section 4.2.2).

With 95 % MP B, 4-hydroxytolbutamide was used to determine t_0, as an alternative to uracil.

4.2.2 Optimization of mobile phase composition

The most crucial factors during optimization were separating one drug from its metabolite, i.e. separating tolbutamide from 4-hydroxytolbutamide and fluoxetine from norfluoxetine. For the optimization of the mobile phase composition, MP B from 40 % to 60 % was utilized for tolbutamide and 4-hydroxytolbutamide, and MP B from 40 % to 70 % for fluoxetine and

norfluoxetine. All standard solutions for the different analytes were injected individually and had a concentration of 10 µg/mL in water. The acquisition times were 10 minutes.

With 40 % MP B, 4-hydroxytolbutamide had a retention time of 2.77 min, tolbutamide had a retention time of 8.80 min and the peaks were well separated. However, the peak shape for tolbutamide exhibited suboptimal characteristics, without a symmetric Gaussian shape. Continuing with 50 % MP B, the peaks were still well separated with retention times of 2.02 min and 3.80 min. Moreover, the peak intensity for tolbutamide improved a lot, as seen in **figure 19**. With global normalization used in the chromatograms, the peak for tolbutamide with 40% MP B was so little it cannot be seen without zooming in. The increase in signal intensity also improves the sensitivity, which is advantageous. With 60 % MP B, the retention for 4-hydroxytolbutamide was at the point it would overlap partially with the t_0 peak and not be resolved as well as possible, with an estimated resolution of 0.93 based on calculations using **equation 2** with values from inspection of chromatograms. Such a low resolution is not desirable, and 50 % MeOH was chosen as the optimal MP B composition.

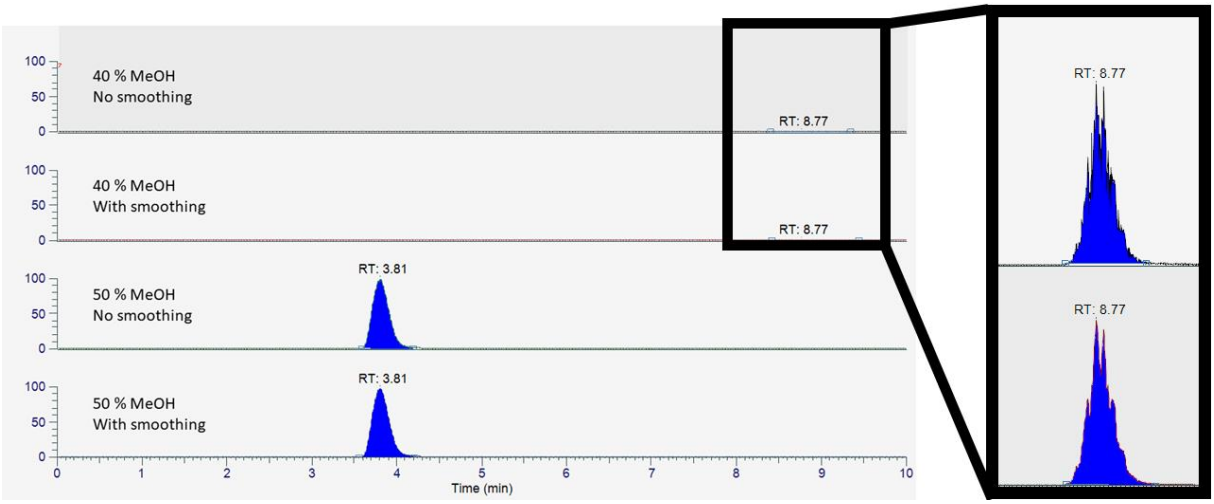

Figure 19: Chromatograms of 10 µg/mL tolbutamide with a mobile phase of 40 % MeOH and 50 % MeOH. A big difference in retention is seen, and the intensity of the peak was higher with 50 % MeOH. In addition, the difference between applied smoothing (Gaussian 7) and no smoothing can also be seen. MRM transitions were m/z 269.11 → 169.97 and 269.11 → 106.00.

When MP B was 40 % for fluoxetine and norfluoxetine, no peaks were detected for either of the compounds after the 10 min of acquisition time used in the MS method. However, when a blank injection was performed afterward, a peak with a lot of tailing was detected for both of the analytes. A new injection of the standard followed by a blank was made. The same happened, but with arbitrary retention times, due to different times between each injection.

After increasing the MP B to 50 %, both analytes eluted after approximately 4 minutes, but still with a lot of tailing in the peak shape. Further increasing to 60% MP B lead to less tailing. As expected, no separation of the two compounds was observed when increasing the organic component of the MP. 70% MP B provided even better peak shapes, but here the retention became so low that the resolution between these analytes and the t_0 peak was not satisfactory with a value of ~0.75 for both analytes.

As a result of mobile phase optimizations, separating fluoxetine and norfluoxetine chromatographically was not possible with the applied columns. Different stationary phases should be assessed if the goal is to separate these compounds chromatographically. However, since fluoxetine and norfluoxetine have different masses, they are easily separated in MS.

The MP composition was only modified by 10 % each time a different MP was examined. To achieve the best optimization possible, several different MP's could have been used, both different percentages and different organic modifiers. However, this was not necessary when good separation and peak shape were seen for tolbutamide and 4-hydroxytolbutamide. For norfluoxetine, ACN instead of MeOH as the organic modifier lead to problems for a former student [22], and thus MeOH was used to save time.

Mobile phase compositions were optimized in order to provide the best separation and peak shape for each drug and its metabolite. 50 % MP B proved to be best for tolbutamide and 4-hydroxytolbutamide. Fluoxetine and norfluoxetine could not be separated chromatographically, and 60 % MP B was chosen.

4.2.3 Observation of carry-over in the system

Based on the results from the optimization, and that only one drug and its metabolite are going to be examined simultaneously, 50 % MeOH for tolbutamide and 4-hydroxytolbutamide, and 60 % MeOH for fluoxetine and norfluoxetine were used as the MP.

A concentration of 10ng/mL in water was used to analyze the analytes tolbutamide, 4-hydroxytolbutamide, and fluoxetine, and a 100 ng/mL concentration for norfluoxetine. For the standards in cell medium, a concentration of 20 ng/mL of the analytes tolbutamide, 4-hydroxytolbutamide, and fluoxetine was used, and 200 ng/mL for norfluoxetine. Ideally, this concentration would have been 10 ng/mL (and 100 ng/mL for norfluoxetine), the same as for

the water standards. However, this double concentration was used for two reasons. The first and most important reason was due to some shortage of cell medium, and by not having to dilute the standards extra, less cell medium could be used. The other reason was due to expected ion suppression, and a higher concentration would then give better signal intensity.

In total, six replicates (n=6), each followed by a blank, were made for every analyte standard. After the third replicate and blank, one extra blank injection was performed in order to switch sample loops. Therefore, three replicates of standards per loop were performed. An additional injection for tolbutamide, 4-hydroxytolbutamide, and norfluoxetine standards in cell medium was made after the sequence, due to no signal detected for the first replicate. Abnormal signal intensities were also detected for fluoxetine in cell medium and norfluoxetine in water (**Figure 20**), but additional injections of these standards were not performed because it was not perceived until the data processing was performed.

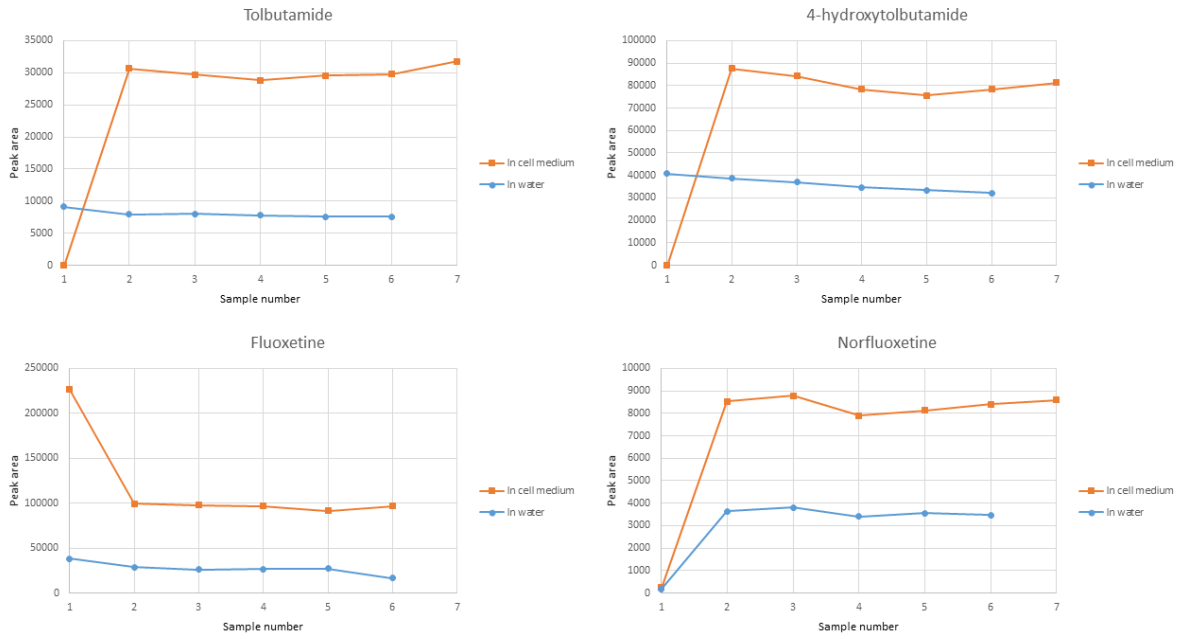

Figure 20: Progression of peak area from the different analytes in both water and cell medium. Note that the concentrations of the cell medium samples are double the concentrations of the water samples.

By performing a Grubbs' test on the irregular values (section 3.7) it can be shown that these suspect values are significantly different from the others, and can therefore be discarded (**table 11**, in appendix). From the peak area of the other replicates, the signal intensities varied with a relative standard deviation (RSD) in the range of 4.2 % - 25.2 % for standards in water,

and 3.0 % - 5.4 % in cell medium. Since the RSD of the responses from each analyte standard is lower in cell medium, the precision is better and thus provides more certain measurements.

From **figure 20** it seems as if the cell medium standards have a much higher peak area than that of the water standards. An important note here is to remember that the cell medium standards have a concentration of 20 ng/mL while the water standards have a concentration of 10 ng/mL, and the difference in peak area is therefore in fact much smaller. Given that the standards in this concentration range exhibit a linear response, the water standards would still have a peak area somewhat lower. A higher signal for the cell medium standards can result in lower LOD and LOQ, which is an advantage when it comes to experiments using organoid cultures. However, the higher signal can possibly be due to the presence of matrix compounds which may potentially cause ion enhancement, and, in turn, could lead to adverse effects on the obtained results. At the same time, ion enhancement can be beneficial if better understood because of improved sensitivity [50].

When preparing the solutions used for examination of carry-over and calibration curves, a small volume (e.g. 1 µL) was measured and transferred to safe-lock tubes, before being evaporated and redissolved in a bigger volume. This was done to get rid of the organic solvent present in the initial standards because it was believed that when it organic solvent would be used on OiC, the organoids could be destroyed or not tolerate those conditions. However, when diluted this much, the resulting organic concentration would be about 0.001-0.002%. This low concentration of organic solvent would likely be a negligible amount. By preparing the solutions in this way, a high degree of uncertainty may occur, because it is difficult to measure 1 µL with high precision and accuracy. This leads to a lot of uncertainty in the results derived from detected signals, like the examination of carry-over (section 4.2.3) and calibration curves (section 4.2.4).

All analytes showed stable retention times (RSD in the range 0.0-0.7 %, n=6) for all replicates, independent of if it was water or cell medium standards (**Figure 21**). These stable retention times would make it easy to detect and quantify the analytes in samples from OiC experiments. As expected from the optimization of the MP composition, the retention times show that tolbutamide and 4-hydroxytolbutamide are well separated, while fluoxetine and norfluoxetine are not separated at all. The separation of the last to compounds is therefore dependent on separation in the MS.

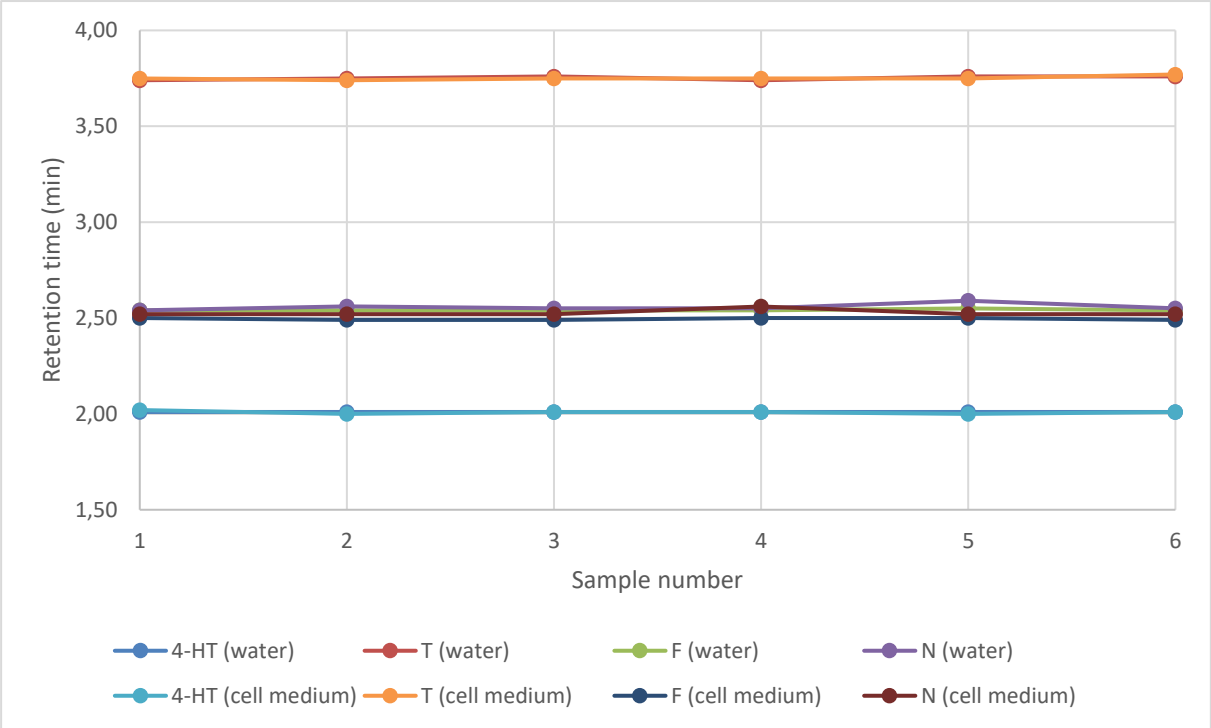

Figure 21: Plotted retention times (min) for the analytes in both water and cell medium. 4-HT = 4-hydroxytolbutamide, t = tolbutamide, F = fluoxetine, N = norfluoxetine. Note that the mobile phase is different for 4-HT and T compared to F and N, with MP B being 50 % and 60 %, respectively.

Since a blank injection was performed after each injection of analyte, the amount of carry over in the system could be assessed (**Figure 22)**. There was little carry-over detected for tolbutamide and its metabolite, with tolbutamide having the least carry-over up to about 0.1 %, and 4-hydroxytolbutamide up to 0.2 %. This amount of carry-over for tolbutamide and 4-hydroxytolbutamide was so little that it was difficult to detect and quantify. Based on visual evaluations, the carry-over was below the LOQ. Fluoxetine and its metabolite norfluoxetine had more carry-over, up to around 3 % at most for fluoxetine in water, and up to 1.5 % for norfluoxetine. Many would find these values for fluoxetine and norfluoxetine unacceptable since the carry-over should be as low as possible and ideally nothing. Moreover, from method validation guidance, carry-over should be, at maximum, 20% of the analyte response at LOQ in the first blank following the highest calibration standard [51]. Validations using calibration curves were not performed for standards of analytes in water and the concentrations used here for the examination of carry-over were above the examined concentration range. Hence, it is difficult to evaluate if these measured carry-over values are too large. However, adding a

washing step in the method, i.e. increasing the organic MP content to a high percentage (80-100 %), could help reduce the carry-over, and should therefore be included.

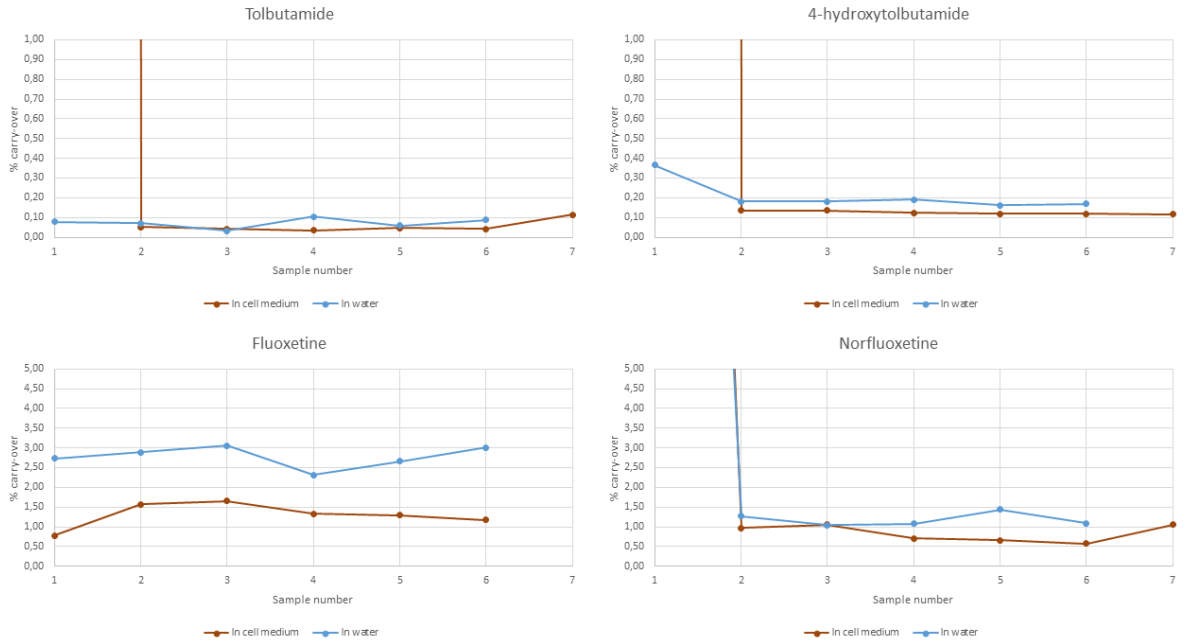

Figure 22: The progression of % carry-over detected in the system for 6-7 replicates of the different analytes in both water and cell medium.

The big carry-over for the first replicate for some of the analytes (up to about 2000 % for 4-hydroxytolbutamide) is a result of the first replicate not having any signal, and the following blank had a detectable signal. Uncommon results from many of the first replicates were seen in other experiments as well. As a conclusion, it should be taken into account that the first replicate is not valid and should be just overlooked.

The carry-over was notably lower in the cell medium standards compared to the water standards. This was particularly the case for fluoxetine, with an average carry-over of about 2.8 % and 1.3 % in water and cell medium, respectively, and norfluoxetine to some extent, with an average carry-over of about 1.2 % and 0.8 % in water and cell medium, respectively (**Figure 23**). The little difference in carry-over for the cell medium and the water standards of tolbutamide and 4-hydroxytolbutamide that can be seen from **figures 22-23**, is practically no difference since it was below the LOQ (based on visual evaluations).

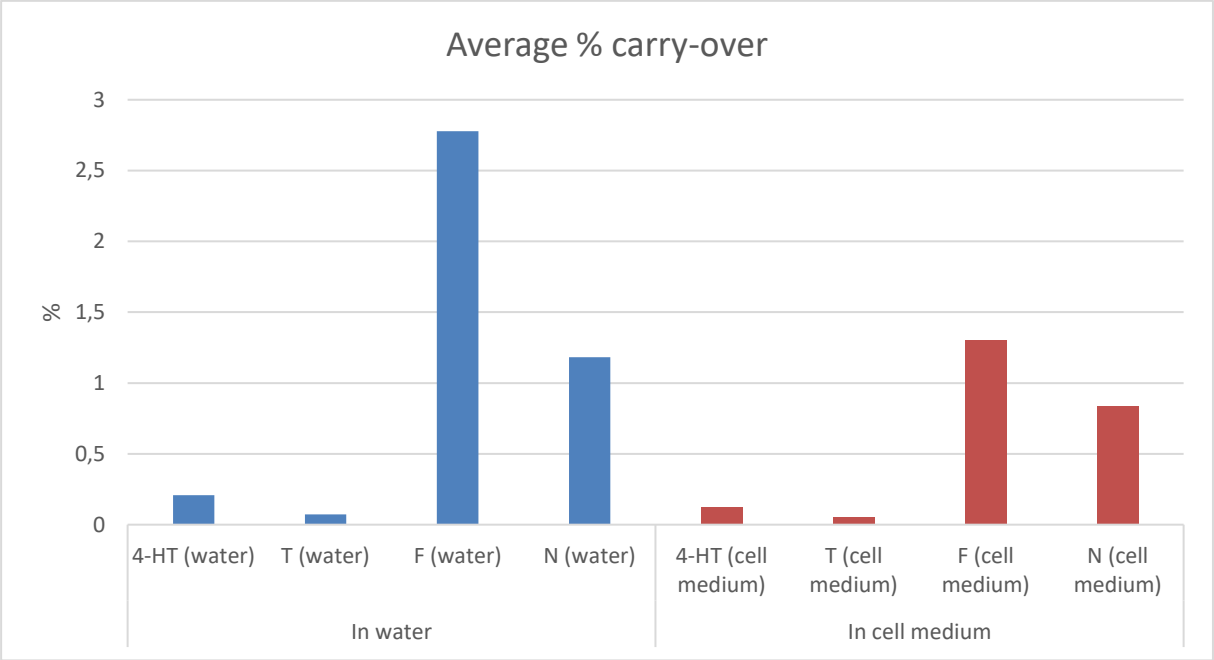

Figure 23: Comparison of the average carry-over detected between analytes in water and cell medium. The big carry-over observed for several of the first injections (**Figure 22**) is not used to calculate the average. 4-HT = 4-hydroxytolbutamide, T = tolbutamide, F = fluoxetine, N = norfluoxetine.

All analytes showed stable retention times in both water and cell medium standards with RSD in the range of 0.0-0.7 % (n=6). The first replicate for several standards was often considered an outlier, and by performing Grubbs' test, these replicates could be rejected. When these replicates were rejected, signal from the standards in cell medium provided better precision than the standards in water. Carry-over values up to 3 % were detected, and the carry-over was lower for the cell medium standards compared to the water standards. Furthermore, tolbutamide and 4-hydroxytolbutamide had carry-over below LOQ, while fluoxetine and norfluoxetine had the highest amount of carry-over. To help reduce the carry-over, the addition of a wash step in the method should be added.

4.2.4 The addition of AFFL enhances signal intensity due to cleaner samples

To examine the response of background ions in cell medium, a full scan of non-spiked cell medium was performed with an m/z range of 150-1050. From a full scan, it can be possible to get a view of whether there are other compounds eluting at the same time as the analytes and thus leading to possible ion suppression.

Full scan chromatograms of non-spiked cell medium both with and without the use of AFFL were performed (**Figure 24**). The deviation in retention time seen in the chromatograms is due to the AFFL system using 1 min extra compared to directly injecting samples into the analytical column. The intensity for the chromatograms is normalized to the highest peak of the two. From the figure, it can easily be seen that the signal of background ions is much lower when AFFL is used. In fact, the peak area for the full scan with AFFL amounts to only about 10 % of the peak without the use of AFFL. This shows that the use of the AFFL system effectively cleans the injected cell medium. The lower signal could be the result of salts and other compounds from the cell medium being removed by elution from the SPE and not being retained.

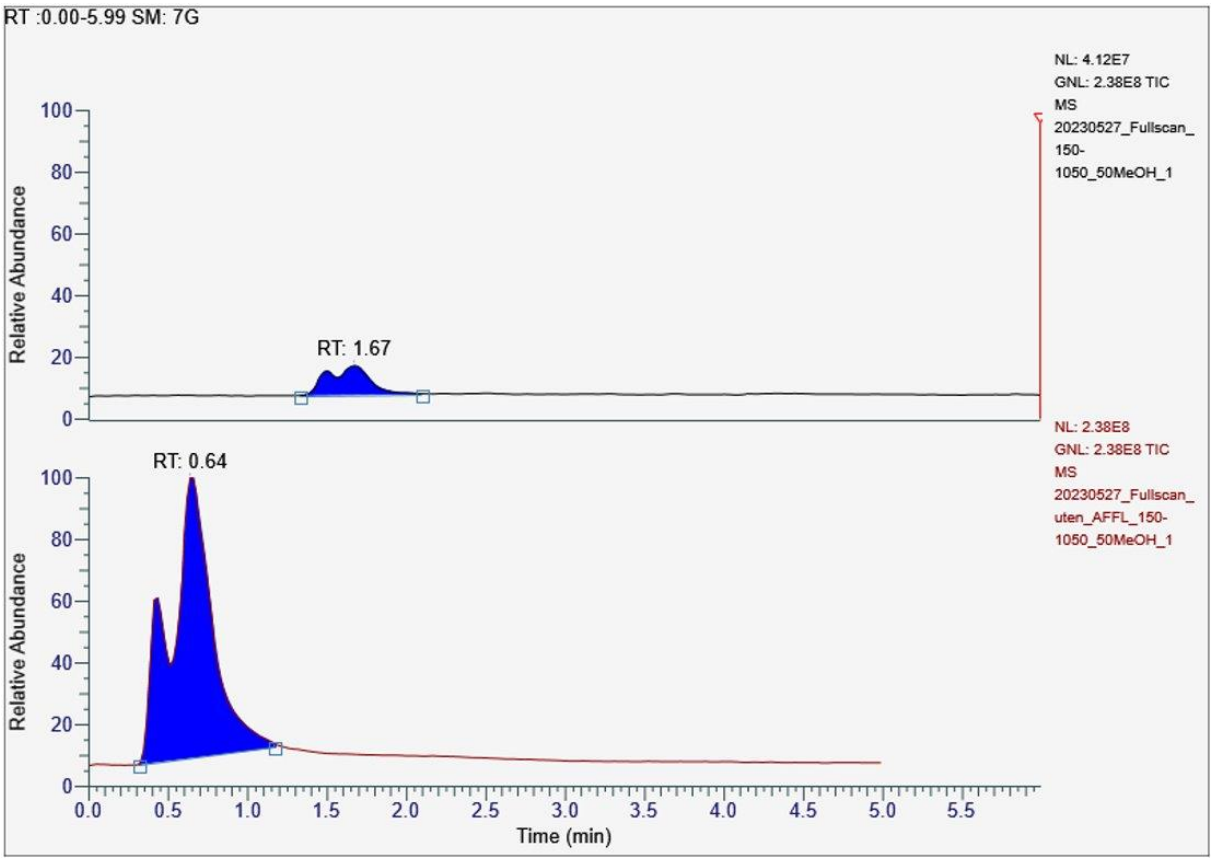

Figure 24: Full scan chromatogram of non-spiked cell medium. The top chromatogram is with the AFFL system, and the bottom chromatogram is without AFFL. The difference in retention time is due to 1 min delay when using AFFL compared to not using it.

Since it is difficult to know how much metabolization of tolbutamide and fluoxetine would occur in the organoid that would have been used for OiC, and in what concentration range

their metabolites would be present, it was not easy to determine the concentrations used for the calibration standards. Concentrations with 5 ng/mL, 10 ng/mL, and 15 ng/mL were used for tolbutamide, 4-hydroxytolbutamide, and fluoxetine, while the concentrations used for norfluoxetine were 50 ng/mL, 100 ng/mL, and 150 ng/mL. Three replicates, each followed by a blank, were injected for all the standards. The average peak area was plotted, and a linear regression was applied to make the calibration curves (**Figure 25**). As can be seen from the slope of the calibration curves, the use of AFFL provides higher sensitivity for the signal for all analytes except fluoxetine. The sensitivity increased by 133 %, 32 %, and 17 % for 4-hydroxytolbutamide, tolbutamide and norfluoxetine, respectively, when AFFL was utilized. This indicates that the use of AFFL sufficiently cleans spiked cell-medium standards containing these analytes. However, for fluoxetine, the sensitivity decreased by 17 % with AFFL utilized which could be explained due to bigger observed errors in the measurements.

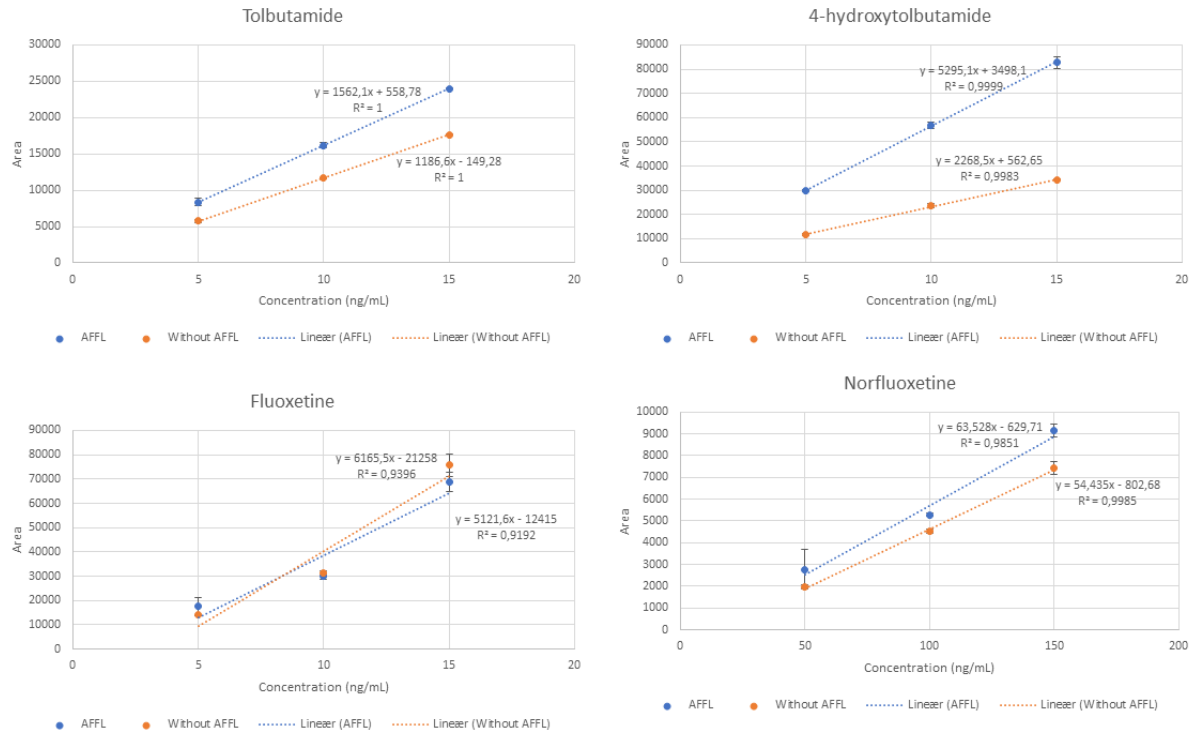

Figure 25: Calibration curves with linear regression analysis for the different analytes in cell medium with and without the use of AFFL. Standard deviations for all data point is represented with error bars (n=3).

The big error observed for 5 ng/mL fluoxetine and for 50 ng/mL norfluoxetine in cell medium with AFFL (RSD = 22 % and 33 %, respectively) can be explained by the first replicate being unusual (as described earlier). Because of this, an additional replicate was injected after the 15 ng/mL and 150 ng/mL standards and was used for calculations instead of the first replicate. These extra replicates had a 46 % and 67 % increased signal for fluoxetine and norfluoxetine,

respectively, compared to the previous replicate for the same concentration. The carry-over from the 15 ng/mL and 150 ng/mL standards could influence the signal detected for the last injection and may be the reason for the increased signal detected. This will further increase the uncertainty in the calculations of LOD and LOQ.

From these calibration curves, the LOD and LOQ (**Table 6**) were calculated following the ICH quality guidelines for analytical validation of analytical procedures (see experimental section 3.7) [43]. The calculated values show that the LOD for tolbutamide and 4-hydroxytolbutamide is lower when AFFL is applied, 0.013 ng/mL (tolbutamide) and 0.24 ng/mL (4-hydroxytolbutamide), compared to no AFFL, 0.15 ng/mL (tolbutamide), and 0.98 ng/mL (4-hydroxytolbutamide). However, the opposite is the case for fluoxetine and norfluoxetine. Here the LOD was 6.9 ng/mL (AFFL) and 5.9 ng/mL (no AFFL) for fluoxetine and 29 ng/mL (AFFL) and 8.9 ng/mL (no AFFL) for norfluoxetine. The values for LOQ are approximately 3 times higher than these values for LOD (**Table 6**).

The ICH guidelines also state that "A specific calibration curve should be studied using samples containing an analyte in the range of DL", (or QL) [43]. Here, DL and QL are the detection limit and quantitation limit, respectively, and are the same as the LOD and LOQ. From visual inspection of the chromatograms, it can be seen that the concentration used in this experiment is way higher than the range of LOD and LOQ. The results may therefore have a lot of uncertainty involved, and hence lower concentrations should be assessed for the determination of LOD and LOQ with less uncertainty.

Table 6: Calculated LOD and LOQ (ng/mL) for the different analytes in cell medium both with and without the use of AFFL.

Analyte	Calculated LOD (ng/mL)	Calculated LOQ (ng/mL)
4-hydroxytolbutamide (AFFL)	0.24	0.71
4-hydroxytolbutamide	0.98	2.96
Tolbutamide (AFFL)	0.013	0.039
Tolbutamide	0.15	0.46
Norfluoxetine (AFFL)	29	87
Norfluoxetine	8.9	27.1
Fluoxetine (AFFL)	6.9	21.0
Fluoxetine	5.9	17.9

Full scan analysis of non-spiked cell medium shows that the response of background ions when AFFL was used, was only 10 % compared to not using AFFL. This indicates that the use of the AFFL system effectively cleans the injected cell medium. In addition, higher sensitivity was obtained with the use of AFFL. Regardless of this, the LOD and LOQ for norfluoxetine were lower with AFFL, due to a lot of uncertainty in the method for calculating LOD and LOQ. The calculated LOD for standards of the analytes in cell medium with AFFL utilized were 0.24 ng/mL, 0.013 ng/mL, 29 ng/mL, and 6.9 ng/mL for 4-hydroxytolbutamide, tolbutamide, norfluoxetine, and fluoxetine, respectively.

5 Concluding remarks

This study aimed to expand the use of OiC by developing a robust on-line sample preparation method for OiC experiments for LC-MS analysis of drugs and their metabolites. Liver organoids were to be used for the OiC, however, these organoids were not provided due to unforeseen events. Therefore, in this study, an on-line sample clean-up system that can be coupled together with OiC has been developed. The system features a valve system for sampling containing two loops, an automatic filtration/filter (back)flushing (AFFL) system for cleaning injected samples, and liquid chromatography (LC) analysis with electrospray ionization mass spectrometry (ESI-MS) detection.

Cell medium spiked with liver organoid metabolized drugs and their metabolites was utilized. The three drugs phenacetin, tolbutamide, and fluoxetine were initially examined with their metabolites acetaminophen, 4-hydroxytolbutamide, and norfluoxetine. Acetaminophen was not compatible with the AFFL system, thus the other two drugs and metabolites were assessed. Each compound needs optimization, and optimization of tolbutamide, 4-hydroxytolbutamide, fluoxetine, and norfluoxetine was performed.

The method provided carry-over up to at most 1.2 % for the standard of fluoxetine in cell medium. However, the carry-over may be reduced by including a wash step in the method. The use of AFFL was shown to (1) increase the sensitivity of the method compared to not utilizing AFFL and (2) reduce the signal intensity of background ions from cell medium, both indicating sample clean-up and enrichment of injected samples.

LOD and LOQ were calculated and were determined to be in the range of 0.013 ng/mL – 29 ng/mL (LOD) and 0.039 ng/mL – 87 ng/mL (LOQ). However, these values come with high uncertainty due to two main factors: how the standards were prepared and the calculation from calibration curves.

Further work

The system developed should be coupled together with an organoid column (OiC) packed with liver organoids, and the drug metabolism of tolbutamide and fluoxetine should be examined. Moreover, investigating the possibility of utilizing different types of organoids in combination with liver organoids should be explored.

During the writing of this thesis, the developed system was used by PhD candidate Stian Kogler together with the OiC. Promising results are seen, with 4-hydroxytolbutamide being detected after exposure of tolbutamide to liver organoids in the OiC.

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Appendix

Raw data from LC-UV

Raw data from experiments examining analyte breakthrough on SPE columns is shown in **table 7**.

Table 7: Retention time raw data for analyte breakthrough on two different SPE columns, with flow rates of 100 µL/min and 50 µL/min, and three different load solutions containing 0%, 3% and 5% MeOH in LC-MS graded water. The retention times for each replicate, and calculates mean and standard deviation (STD) is listed.

SPE 1, 100 µl/min flow, 0% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	0,737	0,734	0,739	0,737	0,002517
Acetaminophen	0,735	0,735	0,732	0,734	0,001732
Phenacetin	1,196	1,203	1,192	1,197	0,005568
4-hydroxytolbutamide	1,388	1,384	1,384	1,385	0,002309
Tolbutamide	4,194	4,193	4,125	4,171	0,039552
Norfluoxetine	1,392	1,354	1,300	1,349	0,046231
Fluoxetine	1,163	1,157	1,176	1,165	0,009713
SPE 1, 100 µl/min flow, 3% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	0,744	0,746	0,743	0,744	0,001528
Acetaminophen	0,889	0,880	0,873	0,881	0,008021
Phenacetin	4,777	4,724	4,705	4,735	0,037314
4-hydroxytolbutamide	8,908	8,858	8,914	8,893	0,030746
Tolbutamide	No peak before 30 min			-	-
Norfluoxetine	3,896	3,842	3,885	3,874	0,028537
Fluoxetine	No peak before 30 min			-	-
SPE 1, 100 µl/min flow, 5% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	0,714	0,721	0,722	0,719	0,004359
Acetaminophen	0,704	0,706	0,704	0,705	0,001155
Phenacetin	3,987	3,990	3,983	3,987	0,003512
4-hydroxytolbutamide	6,931	6,955	6,846	6,911	0,057274
Tolbutamide	No peak before 30 min			-	-
Norfluoxetine	6,791	6,386	6,173	6,450	0,313932
Fluoxetine	No peak before 30 min			-	-
SPE 1, 50 µl/min flow, 0% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	1,519	1,508	1,504	1,510	0,007767
Acetaminophen	1,524	1,521	1,523	1,523	0,001528

Phenacetin	2,500	2,509	2,507	2,505	0,004726
4-hydroxytolbutamide	2,859	2,846	2,821	2,842	0,019313
Tolbutamide	9,131	8,882	8,774	8,929	0,183082
Norfluoxetine	2,728	3,063	2,703	2,831	0,201018
Fluoxetine	2,336	2,326	2,295	2,319	0,021378
SPE 1, 50 µl/min flow, 3% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	1,525	1,525	1,523	1,524	0,001155
Acetaminophen	1,782	1,755	1,747	1,761	0,018339
Phenacetin	8,226	8,231	8,143	8,200	0,049427
4-hydroxytolbutamide	14,841	14,945	14,663	14,816	0,142609
Tolbutamide	No peak before 60 min			-	-
Norfluoxetine	7,711	7,668	7,602	7,660	0,054903
Fluoxetine	No peak before 60 min			-	-
SPE 1, 50 µl/min flow, 5% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	1,539	1,536	1,542	1,539	0,003
Acetaminophen	1,771	1,773	1,781	1,775	0,005292
Phenacetin	7,251	7,227	7,264	7,247	0,018771
4-hydroxytolbutamide	14,543	14,141	14,024	14,236	0,27223
Tolbutamide	No peak before 60 min			-	-
Norfluoxetine	-	-	-	-	-
Fluoxetine	No peak before 60 min			-	-
SPE 2, 100 µl/min flow, 0% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	0,734	0,729	0,735	0,733	0,003215
Acetaminophen	0,743	0,736	0,739	0,739	0,003512
Phenacetin	1,295	1,268	1,286	1,283	0,013748
4-hydroxytolbutamide	1,642	1,627	1,616	1,628	0,013051
Tolbutamide	7,579	7,540	7,541	7,553	0,022234
Norfluoxetine	2,288	2,248	2,191	2,242	0,048748
Fluoxetine	2,381	2,245	2,289	2,305	0,069397
SPE 2, 100 µl/min flow, 3% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	0,744	0,734	0,745	0,741	0,006083
Acetaminophen	0,875	0,878	0,873	0,875	0,002517
Phenacetin	4,519	4,516	4,489	4,508	0,016523
4-hydroxytolbutamide	7,960	7,947	7,929	7,945	0,015567
Tolbutamide	No peak before 30 min			-	-
Norfluoxetine	4,107	4,146	4,103	4,119	0,023756
Fluoxetine	No peak before 30 min			-	-
SPE 2, 100 µl/min flow, 5% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)

Uracil	0,748	0,743	0,746	0,746	0,002517
Acetaminophen	0,933	0,923	0,903	0,920	0,015275
Phenacetin	4,429	4,334	4,332	4,365	0,055435
4-hydroxytolbutamide	7,557	7,509	7,447	7,504	0,055148
Tolbutamide	No peak before 30 min			-	-
Norfluoxetine	4,265	4,295	4,279	4,280	0,015011
Fluoxetine	No peak before 30 min			-	-
SPE 2, 50 µl/min flow, 0% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	1,512	1,508	1,505	1,508	0,003512
Acetaminophen	1,544	1,528	1,522	1,531	0,011372
Phenacetin	3,175	3,155	3,137	3,156	0,019009
4-hydroxytolbutamide	4,195	4,166	4,097	4,153	0,050342
Tolbutamide	15,078	15,449	14,712	15,080	0,368503
Norfluoxetine	4,135	4,039	4,093	4,089	0,048125
Fluoxetine	4,380	4,146	4,122	4,216	0,142534
SPE 2, 50 µl/min flow, 3% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	1,525	1,535	1,525	1,528	0,005774
Acetaminophen	1,779	1,759	1,759	1,766	0,011547
Phenacetin	8,292	8,287	8,279	8,286	0,006557
4-hydroxytolbutamide	14,531	14,455	14,427	14,471	0,053814
Tolbutamide	No peak before 60 min			-	-
Norfluoxetine	8,206	-	-	8,206	-
Fluoxetine	No peak before 60 min			-	-
SPE 2, 50 µl/min flow, 5% MeOH					
	Retention time (min)				
Analyte	Rep. 1	Rep. 2	Rep. 3	Mean	STD (min)
Uracil	1,539	1,533	1,531	1,534	0,004163
Acetaminophen	1,772	1,769	1,756	1,766	0,008505
Phenacetin	7,111	7,079	7,070	7,087	0,021548
4-hydroxytolbutamide	11,775	11,776	11,739	11,763	0,021079
Tolbutamide	No peak before 60 min			-	-
Norfluoxetine	8,511	8,599	8,621	8,577	0,058207
Fluoxetine	No peak before 60 min			-	-

Raw data from LC-MS

Raw data from the observation of carry-over in the system is shown in **table 8**.

Table 8: Raw data from the observation of carry-over in the system for standards of the analytes 4-hydroxytolbutamide (4-HT), tolbutamide, fluoxetine and norfluoxetine in water and cell medium.

10 ng/ml 4-HT (50% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
4-HT 1	40841,34		2,01
blank 1	149,63	0,366369	2,02
4-HT 2	38777,16		2,01
blank 2	70,4	0,18155	2,00
4-HT 3	36977,07		2,01
blank 3	66,96	0,181085	1,99
blank 4	60,13	89,79988	2,01
4-HT 4	34691,71		2,01
blank 5	65,96	0,190132	2,02
4-HT 5	33437,72		2,01
blank 6	54,27	0,162302	2,01
4-HT 6	32179,79		2,01
blank 7	54,29	0,168708	1,99
10 ng/ml Tolbutamide (50% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
Tolbutamide 1	9108,54		3,74
blank 1	7,05	0,0774	3,74
Tolbutamide 2	7954,11		3,75
blank 2	5,55	0,069775	3,73
Tolbutamide 3	8027,53		3,76
blank 3	2,61	0,032513	3,75
blank 4	4,4	168,5824	3,74
Tolbutamide 4	7825,26		3,74
Blank 5	8,23	0,105172	3,76
Tolbutamide 5	7610,23		3,76
blank 6	4,5	0,059131	3,69
Tolbutamide 6	7609,47		3,76
blank 7	6,59	0,086603	3,75
10 ng/ml fluoxetine (60% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
Fluoxetine 1	38449,87		2,54
blank 1	1051,04	2,733533	2,54
Fluoxetine 2	28983,29		2,54
blank 2	837,81	2,890666	2,54
Fluoxetine 3	26208,77		2,54
blank 3	801,52	3,058213	2,54
blank 4	8,71	1,086685	2,54

Fluoxetine 4	27006,06		2,54
Blank 5	625,59	2,31648	2,54
Fluoxetine 5	27411,89		2,55
blank 6	727,82	2,655125	2,56
Fluoxetine 6	16739,16		2,54
blank 7	504,24	3,012338	2,54
100 ng/ml norfluoxetine (60% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
Norfluoxetine 1	186,37		2,54
blank 1	80,61	43,25267	2,58
Norfluoxetine 2	3637,76		2,56
blank 2	46,23	1,270837	2,57
Norfluoxetine 3	3799,98		2,55
blank 3	39,49	1,039216	2,55
blank 4	2,73	6,913143	2,52
Norfluoxetine 4	3417,69		2,55
Blank 5	36,77	1,075873	2,55
Norfluoxetine 5	3556,7		2,59
blank 6	51,01	1,434195	2,55
Norfluoxetine 6	3463,48		2,55
blank 7	37,6	1,085613	2,59
20 ng/ml 4-hydroxytolbutamide in cell medium (50% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
4-HT 1	3,72		1,99
blank 1	74,76	2009,677	1,98
4-HT 2	81175,22		2,02
blank 2	94,61	0,11655	2,02
4-HT 3	87505,15		2,00
blank 3	118,82	0,135786	2,01
4-HT 4	84241,9		2,01
blank 4	114,43	0,135835	1,98
blank 5	77,35	67,59591	1,99
4-HT 5	78234,9		2,01
Blank 6	97,29	0,124356	2,02
4-HT 6	75630,91		2,00
blank 7	90,56	0,119739	2,03
4-HT 7	78294,21		2,01
blank 8	93,51	0,119434	2,01
20 ng/ml tolbutamide in cell medium (50% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
T 1	3,03	Only noise	3,79
blank 1	9,58	316,1716	3,76
Tolbutamide 2	31756,74		3,75
blank 2	35,95	0,113204	3,79
Tolbutamide 3	30632,61		3,74
blank 3	15,89	0,051873	3,75

Tolbutamide 4	29692,73		3,75
blank 4	12,3	0,041424	3,74
blank 5	3,6	29,26829	3,75
Tolbutamide 5	28803,56		3,75
Blank 6	9,8	0,034024	3,77
Tolbutamide 6	29534,84		3,75
blank 7	13,86	0,046928	3,76
Tolbutamide 7	29770,36		3,77
blank 8	12,4	0,041652	3,75
20 ng/ml fluoxetine in cell medium (60% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
Fluoxetine 1	226365,5		2,50
blank 1	1758,51	0,776845	2,50
Fluoxetine 2	99326,39		2,49
blank 2	1553,32	1,563854	2,51
Fluoxetine 3	97949,3		2,49
blank 3	1618,05	1,651926	2,49
blank 4	1564,69	96,7022	2,51
Fluoxetine 4	96791,59		2,50
Blank 5	1290,56	1,333339	2,49
Fluoxetine 5	91703,84		2,50
blank 6	1191,96	1,299793	2,51
Fluoxetine 6	97014,23		2,49
blank 7	1134,88	1,169808	2,51
200 ng/ml norfluoxetine in cell medium (60% MeOH)			
	Peak area	**% carry-over for blank**	**RT (min)**
Norfluoxetine 1	247,16		2,55
blank 1	127,84	51,72358	2,53
Norfluoxetine 2	8591,63		2,52
blank 2	90,78	1,05661	2,54
Norfluoxetine 3	8520,58		2,52
blank 3	82,96	0,973643	2,51
Norfluoxetine 4	8772,73		2,52
blank 4	93,02	1,060331	2,55
blank 5	85,46	91,87272	2,52
Norfluoxetine 5	7885,25		2,56
Blank 6	55,68	0,706129	2,50
Norfluoxetine 6	8137,93		2,52
blank 7	53,37	0,655818	2,52
Norfluoxetine 7	8407,34		2,52
blank 8	48,3	0,574498	2,57

Raw data for calibration of the analytes 4-hydroxytolbutamide (4-HT), tolbutamide (T), fluoxetine (F) and norfluoxetine (N) in cell medium with the use of AFFL, and without AFFL is shown in **table 9** and **table 10**, respectively.

Table 9: Raw data for calibration of the analytes 4-hydroxytolbutamide (4-HT), tolbutamide (T), fluoxetine (F) and norfluoxetine (N) in cell medium with the use of AFFL.

5 ng/mL 4-HT in cell medium with AFFL (50% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
4-HT 1	2,01	29537,07		
Blank 1	2,09	3,24	0,010969	Only noise
4-HT 2	2,01	30240,67		
Blank 2	2,02	6,93	0,022916	Only noise
4-HT 3	2,01	29681,17		
Blank 3	2,04	3,97	0,013375	Only noise
10 ng/mL 4-HT in cell medium with AFFL (50% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
4-HT 1	2,05	2,39		Only noise
Blank 1	2,01	14,87	622,1757	
4-HT 2	2,02	58249,25		
Blank 2	2,04	21,32	0,036601	
4-HT 3	2,01	56404,09		
Blank 3	2,00	15,23	0,027002	
4-HT 4	2,02	55619,83		after 15ng/mL
Blank 4	2,01	34,12	0,061345	
15 ng/mL 4-HT in cell medium with AFFL (50% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
4-HT 1	2,02	83686,42		
Blank 1	2,02	30,03	0,035884	
4-HT 2	2,02	84726,56		
Blank 2	2,03	59,17	0,069836	
4-HT 3	2,02	79899,81		
Blank 3	2,02	59,29	0,074205	
5 ng/mL tolbutamide in cell medium with AFFL (50% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
T 1	No peak			
Blank 1	3,79	1,56	-	Only noise
T 2	3,75	8156,63		
Blank 2	3,79	1,75	0,021455	
T 3	3,75	8012,05		
Blank 3	No peak		0	
T 4	3,76	8931,66		after 15ng/mL
Blank 4	3,78	2,17	0,024296	
10 ng/mL tolbutamide in cell medium with AFFL (50% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**

T 1	3,75	16554,91		
Blank 1	3,84	2,3	0,013893	
T 2	3,75	16137,25		
Blank 2	No peak		0	
T 3	3,74	15862,15		
Blank 3	3,83	1,85	0,011663	
15 ng/mL tolbutamide in cell medium with AFFL (50% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
T 1	3,76	24106,55		
Blank 1	3,78	7,3	0,030282	
T 2	3,77	23931,19		
Blank 2	3,73	7,46	0,031173	
T 3	3,75	23925,58		
Blank 3	No peak		0	
T 4				
Blank 4			#DIV/0!	
50 ng/mL norfluoxetine in cell medium with AFFL (60% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
N 1	2,6	31,26		
Blank 1	2,52	9,25	29,59053	
N 2	2,6	2195,92		
Blank 2	2,61	5,9	0,26868	
N 3	2,58	2289,96		
Blank 3	2,63	12,2	0,53276	
N 4	2,61	3831,93		After 150ng/mL
Blank 4	2,58	51,31	1,339012	
100 ng/mL norfluoxetine in cell medium with AFFL (60% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
N 1	2,6	5239,03		
Blank 1	2,65	27,51	0,525097	
N 2	2,6	5352,19		
Blank 2	2,57	50,09	0,935879	
N 3	2,61	5222,77		
Blank 3	2,61	53,45	1,023403	
150 ng/mL norfluoxetine in cell medium with AFFL (60% MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
N 1	2,59	8982,25		
Blank 1	2,61	81,3	0,905118	
N 2	2,6	8931,9		
Blank 2	2,59	100,4	1,124061	
N 3	2,6	9462,14		
Blank 3	2,62	110,63	1,169186	
5 ng/mL fluoxetine in cell medium with AFFL (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
F 1	2,59	45563,36		
Blank 1	2,59	157,36	0,345365	

F 2	2,59	15688,07		
Blank 2	2,59	218,09	1,390165	
F 3	2,57	15033,19		
Blank 3	2,59	223,89	1,489305	
F 4	2,58	22005,33		
Blank 4	2,58	675,79	3,071029	after 15ng/mL
10 ng/mL fluoxetine in cell medium with AFFL (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
F 1	2,59	29086,95		
Blank 1	2,55	382,14	1,313785	
F 2	2,57	29705,77		
Blank 2	2,56	437,36	1,472307	
F 3	2,57	31315,66		
Blank 3	2,61	486,82	1,554558	
15 ng/mL fluoxetine in cell medium with AFFL (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
F 1	2,59	65197,49		
Blank 1	2,62	918,16	1,408275	
F 2	2,58	68310,04		
Blank 2	2,59	1022,47	1,496808	
F 3	2,59	72868,07		
Blank 3	2,57	1112,96	1,527363	

Table 10: Raw data for calibration of the analytes 4-hydroxytolbutamide (4-HT), tolbutamide (T), fluoxetine (F) and norfluoxetine (N) in cell medium without the use of AFFL.

5 ng/mL 4-HT in cell medium (50 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
4-HT 1	1,02	11238,81		
Blank 1	0,91	103,42	0,920204	
4-HT 2	1,02	11684,18		
Blank 2	1,02	2,53	0,021653	
4-HT 3	1,02	11970,37		
Blank 3	0,98	32,83	0,274261	
10 ng/mL 4-HT in cell medium (50 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
4-HT 1	1,03	24358,54		
Blank 1	0,98	91,96	0,377527	
4-HT 2	1,03	24240,52		
Blank 2	0,98	103,14	0,425486	
4-HT 3	1,02	22788,06		
Blank 3	0,97	105,09	0,461163	
15 ng/mL 4-HT in cell medium (50 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
4-HT 1	1,03	34509,65		

Blank 1	0,97	135,11	0,391514	
4-HT 2	1,04	34267,43		
Blank 2	1,02	177,7	0,518568	
4-HT 3	1,04	34171,29		
Blank 3	0,97	160,06	0,468405	
5 ng/mL tolbutamide in cell medium (50 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
T 1	2,63	5586,12		
Blank 1	2,61	3,06	0,054779	
T 2	2,63	5787,83		
Blank 2	2,64	6,01	0,103839	
T 3	2,63	6044,67		
Blank 3	2,56	6,9	0,11415	
10 ng/mL tolbutamide in cell medium (50 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
T 1	2,63	11665,32		
Blank 1	2,6	7,77	0,066608	
T 2	2,64	11582,47		
Blank 2	2,52	115,51	0,997283	
T 3	2,63	11770,14		
Blank 3	2,49	22,61	0,192096	
15 ng/mL tolbutamide in cell medium (50 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
T 1	2,63	17566,19		
Blank 1	2,54	16,08	0,091539	
T 2	2,63	17637,42		
Blank 2	2,54	18,6	0,105458	
T 3	2,63	17814,36		
Blank 3	2,59	28	0,157177	
50 ng/mL norfluoxetine in cell medium (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
N 1	1,66	8,57		
Blank 1	1,67	1,97	22,98716	Only noise
N 2	1,61	1901,01		
Blank 2	No peak	0	0	Only noise
N 3	1,62	2011,3		
Blank 3	No peak	0	0	Only noise
N 4	1,62	2025,43		
Blank 4	No peak	0	0	Only noise
100 ng/mL norfluoxetine in cell medium (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
N 1	1,61	4499,47		
Blank 1	No peak	0	0	Only noise
N 2	1,61	4419,79		
Blank 2	No peak	0	0	Only noise
N 3	1,6	4641,88		

Blank 3	No peak	0	0	Only noise
150 ng/mL norfluoxetine in cell medium (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
N 1	1,62	7121,15		
Blank 1	No peak	0	0	Only noise
N 2	1,62	7451,93		
Blank 2	No peak	0	0	Only noise
N 3	1,61	7695,05		
Blank 3	No peak	0	0	Only noise
5 ng/mL fluoxetine in cell medium (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
F 1	1,61	28567,85		
Blank 1	1,44	59,43	0,208031	
F 2	1,62	14219,72		
Blank 2	1,44	87,73	0,61696	
F 3	1,6	14286,91		
Blank 3	1,43	89,73	0,628057	
F 4	1,61	13741,85		
Blank 4	1,43	103,93	0,756303	
10 ng/mL fluoxetine in cell medium (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
F 1	1,6	31876,98		
Blank 1	1,43	126,65	0,397309	
F 2	1,61	30344,61		
Blank 2	1,42	127,73	0,420931	
F 3	1,6	31888,33		
Blank 3	1,52	108,59	0,340532	
15 ng/mL fluoxetine in cell medium (60 % MeOH)				
	RT (min)	**Peak area**	**% carry-over for blank**	**Comment**
F 1	1,6	80994		
Blank 1	1,4	612,24	0,755908	
F 2	1,6	72682,44		
Blank 2	1,53	310,25	0,426857	
F 3	1,6	73536,76		
Blank 3	1,5	303,24	0,412365	

Table 11 shows the values used for Grubbs' test of the outliers.

Table 11: Values used when applying the Grubbs' test on questionable data points that were suspected to be outliers. 4-HT = 4-hydroxytolbutamide, T = tolbutamide, N = norfluoxetine, F = fluoxetine.

Grubbs' test (95 % confidence)					
Analyte	**T (cell medium, n=7)**	**4-HT (cell medium, n=7)**	**F (cell medium, n=6)**	**N (cell medium, n=7)**	**N (water, n=6)**
Mean	25741,98	69298,00	118191,81	7223,23	3010,33
STD	11388,48	30817,99	53057,11	3090,286	1390,092
Questionable data	3,03	3,72	226365,51	247,16	186,37
G (calculated)	2,260	2,249	2,039	2,257	2,031
G (table)	1.938	1.938	1.822	1.938	1.822

Values used for calculation of LOD and LOQ in accordance with ICH guidelines for validation of analytical procedures is shown in **table 12**.

Table 12: Values used for calculation of LOD from calibration curves utilizing **equations 4-5** (section 3.7). 4-HT = 4-hydroxytolbutamide, T = tolbutamide, N = norfluoxetine, F = fluoxetine.

Analyte	**Slope of calibration curve**	**Standard error/ residual standard deviation**	**Calculated LOD (ng/mL)**	**Calculated LOQ (ng/mL)**
4-HT (with AFFL)	5295,129	377,5807	0,235314	0,713071684
4-HT	2268,5	671,2296	0,976441	2,958913348
T (with AFFL)	1562,099	6,118281	0,012925	0,039167042
T	1186,645	54,53245	0,151652	0,459551473
N (with AFFL)	63,52827	553,3288	28,74288	87,09962908
N	54,43463	147,4579	8,939367	27,08898961
F (with AFFL)	5121,634	10734,95	6,916802	20,96000615
F	6165,491	11055,61	5,917376	17,93144244