Optimization of liquid chromatographic parameters for untargeted metabolomics of dried blood spots

Camilla Elene Arnesen

Master Thesis
Chemistry
60 credits

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Abstract

For untargeted metabolomics of dried blood spots (DBS), the liquid chromatography (LC) parameters of a liquid chromatography-mass spectrometry (LC-MS) method were optimized to achieve separation of as many compounds as possible. This was done by studying how each parameter affects peak capacity (P_C) (i.e. the number of chromatographic peaks that can be separated within the gradient elution time). A dried blod spot (DBS) spiked with nine selected compounds representing the metabolome was used for method development. The parameters tested were reversed phase (RP) analytical column, mobile phase (MP) composition, gradient elution profile, injection volume, column temperature and flow rate.

Five carefully selected RP analytical columns were tested, and a C18-Diphenyl column (250 x 2.0 mm, 3 µm) was chosen because of the high degree of separation, minimal background noise and superior retention of the hydrophilic compounds. Acetonitrile (ACN) and methanol (MeOH) as organic MP were tested and no marked difference was observed. However, MeOH gave slightly better separation of the polar compounds and has the extra advantage of halving the reagent cost. Two different organic additives were tested; 0.1 % formic acid (FA) and 10 mM ammonium acetate (NH_4COOCH_3). 0.1% FA was found to be the best compared to the contamination of the sample cone and higher retention time (t_R) experienced when using 10 mM NH_4COOCH_3. Six different solvent gradients were tested using a 55 minute run time program. The gradient chosen was a step gradient that gave the most even separation time distribution between the polar and the lipophilic compounds. Injection volumes of 2, 10 and 20 µL were tested, and 2 µL was found to give the highest P_C, with no particular tailing. Temperatures of 30, 40, 50 and 55 ˚C were tested, and 30 ˚C gave the best reproducibility. Flow rates of 50, 150 and 300 µL/min were evaluated, and 300 µL/min was chosen since it provided the shortest gradient elution time (t_G) with no significant loss of P_C.

In conclusion, the LC part of a LC-MS method for untargeted metabolomics of DBS has been developed and is ready for validation and subsequent testing in clinically relevant samples. P_C obtained by the final optimized LC-parameters was 100.

Preface

The work presented in this master thesis has been performed at the Department of Medical Biochemistry at Oslo University Hospital Rikshospitalet (OUS-RH) from August 2015 to May 2017.

First of all I would like to give a huge thanks to my supervisors; Associate Professor Steven R. H. Wilson, Ph.D. Katja B. P. Elgstøen and M.Sc. Anja Østeby for their motivation, excellent guidance and constructive feedback throughout my master's degree. One day, I hope to inspire someone else as you have inspired me.

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I also wish to express my appreciation to all co-workers at the Department of Medical Biochemistry and the entire Bioanalytical Chemistry group at the University of Oslo for a wonderful environment. In this regard, I would like to give a special thanks to Hanne B. Skogvold for the cooperation and support throughout this period.

In addition, I would like to thank my lovely family and friends for all the support.

Most of all I would like to thank my dearest Eirik for endless love and support.

Oslo, Norway, May 2017

Camilla Elene Arnesen

Table of content

1 Abbreviations and definitions

1.1 Abbreviations

Abbreviation	Term
2D	Two-dimensional
ACN	Acetonitrile
CV%	Coefficient of variation
DBS	Dried blood spot
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
FA	Formic acid
GC	Gas chromatography
H	Plate height
HPLC	High performance liquid chromatography
I.D.	Inner diameter
IEM	Inborn error of metabolism
k	Retention factor
L	Column length
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MeOH	Methanol
MP	Mobile phase
MS	Mass spectrometry
M_r	Molecular mass

n	Replicate(s)
N	Plate number
N_2	Nitrogen gas
NBS	Newborn screening
OUS-RH	Oslo University Hospital Rikshospitalet
P_C	Peak capacity
PFP	Pentafluorophenyl
PGC	Porous graphitic carbon
Psi	Pound-force per square inch
Q-TOF	Quadrupol-time of flight
R_S	Resolution
RP	Reversed phase
SP	Stationary phase(s)
SD	Standard deviation
t_G	Gradient time
TIC	Total ion chromatogram
t_R	Retention time(s)
UHPLC	Ultra-high performance liquid chromatography
w	Peak width(s)
Å	Ångstrøm
α	Selectivity factor

1.2 Definitions

Term	Definition
Metabolite	A substance essential to the metabolism of a particular organism or to a metabolic process.
Metabolome	The full complement present in a cell, tissue or organism in a particular physiological or developmental state.
Metabolomics	The scientific study and analysis of the metabolites produced by a cell, tissue or organism.
Peak capacity	The theoretical maximum number of peaks that can be separated with a $R_S = 1.0$ within a given time period.
Retention time	The time a compound uses from injection into the chromatographic system until it reaches the detector.

2 Introduction

2.1 Inborn error of metabolism and newborn screening

Inborn errors of metabolism (IEMs) are a large and complex group of diseases [1]. These genetic diseases are characterized by a transport protein or an enzyme, normally present in a cell, that is either not produced, or is produced in a dysfunctional form. This leads to aberrant concentrations of one or more metabolites causing cell damage and symptoms in patients with IEMs [2]. Patients with IEMs may have an aberrant concentration of characteristic metabolites that are not normally present, and these so-called "diagnostic metabolites" are analyzed and quantified in diagnostic labs as their identity points directly to the metabolic error and the diagnose [3].

The varieties of symptoms are wide, and it can be difficult to identify the correct IEM in the patient. The symptoms may come from all organs, but organ systems like the nervous system, liver and heart are most commonly affected. Accumulation of an organic acid occurs in many cases. Some patients develop an abnormal body odor, due to the accumulation of a substance with a characteristic smell. When the defect is in the turnover of exogenous substances, recurrent symptoms may result. For example, a person with fructosemia will be clinically healthy as long as she does not consume fructose. Several of these diseases also give rise to malformations [2].

Today about 1500 different IEMs are known [4]. Each IEM is individually rare, but since there are so many different IEMs, these diseases as a group are relatively common [2]. The purpose of newborn screening (NBS) for IEMs is to detect selected diseases as early as possible before symptoms and irreversible damage occurs [4]. The World Health Organization states several requirements for an IEM to be included in NBS. The disease must pose a major health problem, it must be detectable in newborns, a treatment must be available, and the costs associated with the detection must be properly balanced against the advantage of detecting the disease [4].

NBS in Norway was expanded on March 1st, 2012 from 2 to 23 hereditary diseases. About 1 of 1000 children in Norway is born with one of these 23 diseases. The sample material used is DBS and targeted metabolomics using mass spectrometry (MS) is used to quantify amino

acids and acylcarnitines. For several IEMs symptoms will not appear immediately, and NBS is therefore an important way to detect an IEM before serious disease symptoms develops [5].

2.2 Laboratory diagnostics of inborn error of metabolism

The Department of Medical Biochemistry at OUS-RH is responsible for laboratory diagnostics of IEMs in Norway. It is part of the Norwegian National Unit for Screening and Diagnostics of Congenital Pediatric Metabolic Disorders that also includes NBS. The NBS program in Norway screens all IEMs simultaneously. If the screening result indicates an IEM or if there are other indications of an IEM, the Department of Medical Biochemistry at Rikshospitalet is responsible for the laboratory diagnostics.

The assessment of IEMs is comprehensive and complicated and puts great demands on clinical, biochemical and analytical technical knowledge, as well as being time-consuming and expensive. IEMs can be suspected based on the clinical presentation, and that applies especially for infants and young children [2]. To specifically identify the exact IEM, however, is usually complicated.

One of the most important diagnostic tools in the laboratory is specialized metabolic analyses where a large number of metabolites are measured to look at the metabolic profile. Diagnosis can also be done on the genetic level or by analyzing the activity of the suspected defective enzyme in a relevant patient sample. Some IEMs can be diagnosed prenatally at a very early stage in amniotic fluid or fetal tissues or cells. For prenatal diagnosis DNA-based diagnostics are preferred, but analyses of metabolites and enzyme activity are also possible [2].

2.3 Dried blood spot

Dried blood spot (DBS) are blood drops spotted onto a filter paper, and dried [6]. The adsorption of blood on the paper that is dried makes the analytes less reactive and more stable than the analytes in liquid whole blood. Only a small blood volume is needed, which is of great advantage when one has a limited amount of whole blood available from newborns. DBS sampling is minimally invasive. For newborns the sample are taken by a small puncture in the heel, and for children and adults the sample are taken by a small puncture in the finger [7]. DBS samples are cost-effective as they are easy to gather, transport and store [8]. The sample is collected between 48-72 hours after the baby is born, and the DBS is sent to The Department of Newborn Screening at OUS-RH.

The DBS cards should be refrigerated/frozen (dependent on storage time period) in an airtight bag with silica. Prior to analysis, frozen cards (at - 20 º C or lower) should be defrosted in the bag with silica at room temperature for at least 30 minutes to prevent condensation [6].

2.4 Metabolomics

Metabolomics is the systematic study of the metabolome in biological samples and tissues, and is a complementarily tool to genomics, proteomics and other –omic-analyses. The metabolome consists of the total metabolite composition in a biological sample [9]. Metabolism is a designation used for all biochemical reactions that occur in living cells. Metabolism is divided into catabolism (degradation of compounds) and anabolism (synthesis). Metabolites are small-molecular intermediates and end products of metabolism, e.g. carbohydrates, amino acids and fatty acids [10]. The metabolome provides information about the physiological state of the analyzed sample material in an organism at the time of sample collection. The change in the metabolome will among other things be affected by diet, disease and age [11].

Metabolomics can be divided into a targeted or untargeted approach [12]. Targeted metabolomics can be used to investigate selected metabolites to validate observed alterations in metabolic profiles and to quantify low-abundance bioactive metabolites. NBS is an example of targeted metabolomics. [11]. Untargeted metabolomics studies the total metabolome in the sample of an organism. At any given time thousands of biochemical processes are taking place in an organism [13], and the number of metabolites is therefore enormous. With untargeted metabolomics one seeks to analyze as many metabolites as possible. This puts great demands on the analytical method used. A typical process for untargeted metabolomics is to examine differences in the metabolic profile between a control and a patient group [14].

Bioinformatics tools are used to compare the metabolomes to determine whether there are differences in the metabolome of the patient compared to the normal-metabolome and if possible, identify relevant metabolites [15].

There is a wide range of analytical techniques available for metabolomics. MS is well suited, due to rapid, selective and sensitive quantitative and qualitative analyses [16].

Analytical techniques which are applied in metabolomics include gas chromatography-mass spectrometry (GC-MS), LC-MS, direct-injection mass spectrometry (DIMS), capillary electrophoresis-mass spectrometry (CE-MS), matrix-assisted laser desorption ionization (MALDI), laser desorption ionization (LDI), direct ionization on silicon (DIOS), fourier transformer infrared spectroscopy (FT-IR) and nuclear magnetic resonance spectroscopy (NMR) [16, 17].

2.5 Liquid chromatography – mass spectrometry

The LC-principle is to pump the analytes through an analytical column and separate the analytes according to their interactions with the stationary phase (SP) and the mobile phase (MP) elution strength. Liquid chromatographic instruments mainly consist of pumps, an auto sampler, an injector, a pre-column, a main-column, a column oven, a degasser and a switching valve [18].

The main-column is the analytical column and consists of a SP that is a solid particulate material. High pressure is needed to pump the MP through the analytical column [19]. The main difference between a LC and UHPLC (ultra-high performance liquid chromatography) system is that UHPLC systems can operate with a higher pressure. With HPLC instrumentation (400 bar) the columns have an inner diameter (I.D.) of 2.1-4.6 mm, 5-25 cm length and 3-5 μm particle size. With UHPLC instrumentation (1100 bar) the columns have 1.0-2.1 mm (I.D.), 5-15 cm length and sub-2 μm particle size [20]. In our case a UHPLC system with a HPLC column is used, and the advantage is that one can use a relative higher pressure (600 bar) with this system compared to a conventional HPLC system [21].

A MS instrument consists of injection port, ion source, mass analyzer and a detector. A MS analyzes ions in gas phase, and various ion sources and ionization techniques exist for different kind of samples and analytes. MS separate ions in gas phase based on different mass-to-charge ratios (*m/z*) [22]. More details about different mass spectrometers are provided in **Appendix**, section 6.1.

The electrospray ionization (ESI) source is used to convert ions to gas phase from a liquid phase, before entering the MS [23]. By using this technique, compounds with moderate to high polarity with a mass range from ~60-10,000 Dalton (Da) are applicable to be ionized [24]. The ESI ionization method is a soft ionization technique and is an advantage when

analyzing biological samples since little or no fragmentation occurs [25]. An ESI ion source coupled to LC-MS is a powerful technique capable of analyzing both small and large molecules of various polarities in a complex biological sample mixture [26, 27]. Illustration of an electrospray is shown in **Figure 1**.

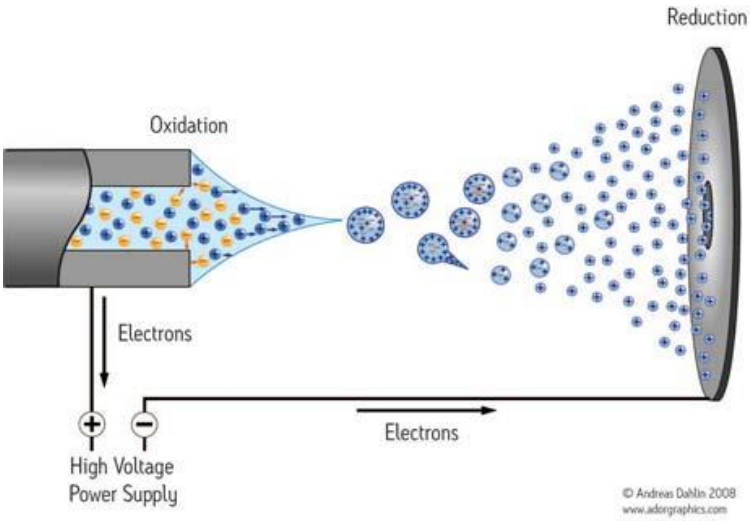


Figure 1 *Illustration of a pneumatically assisted electrospray interface for MS Figure reproduced from Waters [28].*

Figure 1 illustrates the formation of ions in the ESI, MP from chromatography column enters the steel nebulizer capillary along with a coaxial flow of N_2 gas. For positive ionization mode, the nebulizer is held at 0 V and the spray chamber is typically held at large negative potential (e.g. -3500 V) [19]. The strong electric field at the nebulizer outlet combined with the coaxial flow of N_2 gas creates a fine aerosol of charged particles. Positive ions from the aerosol are attracted toward the glass capillary leading into the MS by an even more negative potential of about - 4500 V. For negative ionization mode, all voltages are reversed [19].

2.6 Efficiency in liquid chromatography

After injection, a narrow chromatographic band is broadened during its movement through the analytical column. The sharpness of the chromatographic peak indicates column efficiency [29].

The peak width (w) is an indication of peak sharpness, and thereby acts as an indication of the column efficiency. W is depended on column parameters (diameter, length and particle size), and flow rate. The plate number (N) reflects the columns efficiency and is a measure of the peak dispersion through the analytical column, shown in Equation 1 and 2. The analytical

column is divided into theoretical plates (N), where each plate forms a distance over which the sample compounds reaches equilibrium between the SP and the MP. A theoretical plate is equivalent to the plate height (H), shown in Equation 3 [30]. For a given chromatographic column, the greater the number of theoretical plates (N) the greater is the number of ideal equilibrium stage in the chromatographic system and the more efficient is the separation. H and N vary for a particular column depending on the analyte molecule being separated. A column with a high N is more efficient and will give narrower peaks [31]. N can be calculated at baseline (Equation 1) or at 50% peak height (Equation 2).

$$N = 16\left(\frac{t_R}{w}\right) \quad \text{Eq.1}$$

Where N is the number of theoretical plates, t_R is the retention time, and w is the peak width at baseline (13.4% peak height).

$$N = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2 \quad \text{Eq.2}$$

Where N is the number of theoretical plates, t_R is the retention time, and $w_{0.5}$ is the peak width at 50% of the peak height.

$$H = \frac{L}{N} \quad \text{Eq.3}$$

Where H is the plate height, L is the column length, and N is the number of theoretical plates.

To optimize separation efficiency it is necessary to maximize the numbers of theoretical plates. H must be reduced when maximizing the numbers of plates, and H is related to the flow rate of the MP. For a fixed set of MP, SP, and analytes, the separation efficiency can be maximized by optimizing flow rate as described by the van Deemter equation, shown in Equation 4 [30].

$$H = A + \frac{B}{u_x} + C \times u_x \quad \text{Eq.4}$$

Where H is plate height, A is eddy diffusion, B is longitudinal diffusion, C is resistance to mass transfer, and u_X is average linear velocity.

Band broadening reduces the efficiency of the separation being carried out leading to poor resolution and chromatographic performance. The Van Deemter equation includes the main factors contributing to column band broadening. The first of the factors relating to band broadening is the eddy diffusion (A), which represents the different possible paths an analyte molecule can be taken through the SP. Linear velocity (U_X) and longitudinal diffusion (B) also tends to produce a broader band. The mass transfer (C) is a constant that describes the rate of adsorption and desorption of the analyte to the SP. A, B, and C are constants for a given column and SP [30].

The resolution (R_S) of an elution is a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation. R_S is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks. The R_S equation is shown in Equation 5 [30].

$$R_S = \frac{(t_R)_B - (t_R)_A}{[(w)_A + (w)_B]/2} \qquad \text{Eq.5}$$

Where compound B have longer t_R than compound A, and w is the elution peak width.

If the R_S is greater than one ($R_S > 1$), the peaks can usually be differentiated successfully. An example of high peak R_S is shown in **Figure 2** [30].

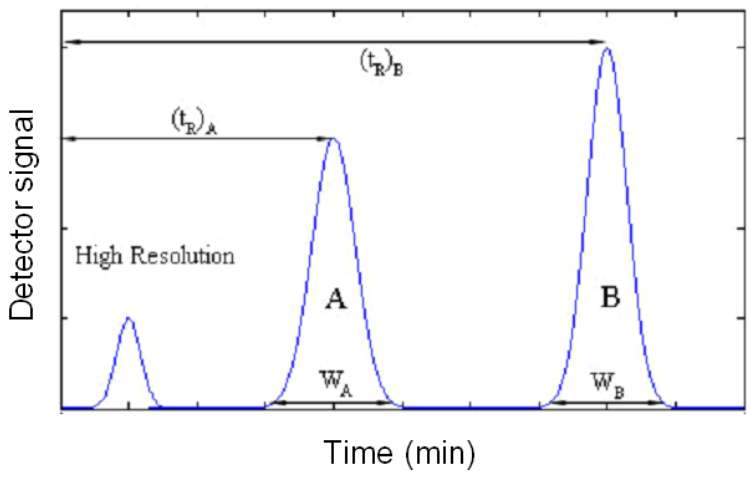


Figure 2 *Example of high resolution between peak A and B, where the factors for each peak used in Equation 5 are shown [30].*

2.6.1 Column dimensions

The majority of LC-MS studies for untargeted metabolite profiling employ RP gradient elution chromatography [32]. Columns used in this master thesis were of RP type and they all have lengths 250 mm except one 150 mm, and I.D. of 2.00 mm and 2.1 mm. The use of a longer (switching from 50 mm to 100 mm) column gives greater retention and selectivity, resulting in better resolution. For any flow rate and column length (L), peak capacity (P_C) varies with gradient time (t_G) in an asymptotic fashion. The efficiency of a column increases as the size of the SP particles decreases, and an efficient column provides increasing P_C [33].

2.7 Liquid chromatographic parameters

2.7.1 Peak capacity

P_C was first described by Giddings, defined as the theoretical maximum number of peaks that can be separated with R_S = 1.0 within a given time period [34]. P_C is a function of column efficiency, t_G, flow rate and analyte characteristics, and is a useful measure of the comparative separating power of different analytical systems [33]. When using the same column and similar analysis time, one can increase P_C by a factor of two using a gradient method compared to an isocratic method, due to the reduced peak width obtained. An extra advantage of gradient elution is the increased ability to separate compounds with great variation in hydrophobicity [35]. With isocratic elution, the w will increase with $t_{R,}$ in contrast to a gradient elution showing only limited increase in w. To resolve complex samples, gradient elution is necessary, which is the case in this master thesis. To calculate the theoretical P_C in gradient methods one must use advanced formulas which depend on retention parameters, and these may differ for the individual components of a sample. The theoretical P_C in gradient methods for complex samples is estimated by using Equation 6.

$$P_C \approx 2 \times \sqrt{N} \qquad \text{Eq.6}$$

Where P_C is the peak capacity and N is the plate number.

An example of the effects of L and t_G on P_C is shown in **Figure 3** [36].

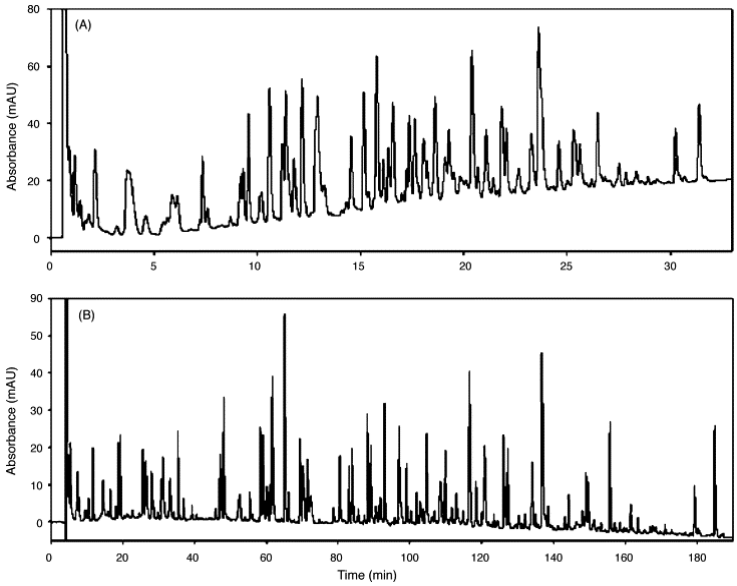


Figure 3 *P_C of the separation in Figure 3A was estimated to be 165, and in Figure 3B the P_C was estimated to be 461. P_C was increased from 165 to 461 by changing to a longer column (7.5 - 60 cm), and by increasing the* t_G *(30 – 240 min) [36].*

P_C is the most common metric of separation power in gradient elution chromatography [35]. In a one-dimensional separation of peptides a typical P_C in the range from 100 to 400 may be obtained. However, Shen et al.[37] reported a P_C of 1500 in a 2000-minute, one-dimensional separating using a 200 cm long capillary column packed with 3 μm particles operated at 20 kilo pound-force per square inch (KPsi) backpressure (14.5 pound-force per square inch (psi) ~ 1 bar).

Different formulas exist for P_C calculation, it is therefore important to know which formula has been used. Calculation of P_C on a gradient LC-system can be estimated using Equation 7 [35].

$$P_C = 1 + \frac{t_G}{\frac{1}{n}\sum_1^n w} \qquad \text{Eq. 7}$$

Where t_G is the gradient elution time, n is number of peaks, and w is the peak width measured at baseline (13.4% peak height) for each peak (n).

Equation 8 is an alternative equation to estimate P_C, where the t_R is taken into account [38].

$$P_C = \frac{t_{R2} - t_{R1}}{\frac{1}{n}\sum_1^n w} \quad \text{Eq. 8}$$

Where t_{R1} is the first eluted solute and t_{R2} is the last eluted solute, n is number of peaks measured, and w is the peak width measured at baseline (13.4% peak height) for each peak (n).

2.7.2 Mobile phase velocity

A study by Wang et al. [39] looked at the effect of different flow rates on P_C, and observed that w varies considerably with the flow rate and gradient time, as shown in **Figure 4**.

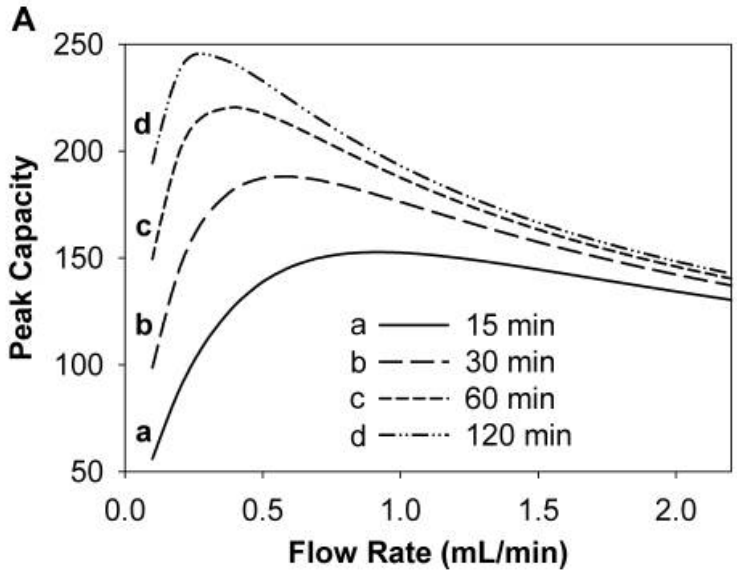


Figure 4 *P_C as a function of flow rate at* t_G*: 15, 30, 60 and 120 min [39].*

The study concludes that P_C is maximized at an intermediate flow rate (0.40 mL/min), and the optimum flow rate varies considerably with the t_G when other factors, such as temperature, are held constant [39]. The study also concludes that P_C is maximized at the longest t_G. When keeping the t_G constant, P_C and w are controlled by the effect of flow rate on the isocratic efficiency. In this case the flow rate is the dominant factor [39].

2.7.3 Gradient elution time

Generally a longer gradient elution time produce a higher P_C although P_C tends to reach a limit. But, the rate of increase in P_C against t_G is much greater at lower flow rates [39]. An illustration showing the coherence between P_C and t_G is shown in **Figure 5**.

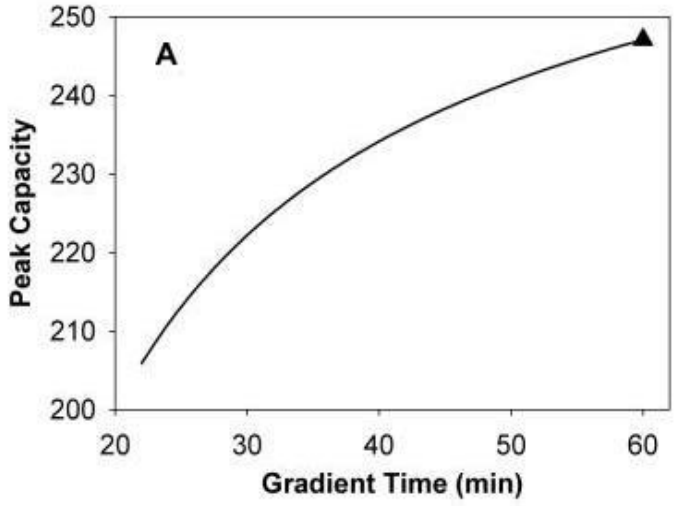


Figure 5 *P_C as a function of t_G with a flow rate of 0.46 mL/min[15].*

A study done by Wang et al. [39] has concluded that there is a strong interaction between flow rate and t_G, a long t_G generally provides higher P_C [39]. A study done by Wren [33] shows how a change in t_G from 4 minutes to 45 minutes affects P_C. The conditions Wren used was a 50 mm column and a flow rate of 0.5 mL/min while using a linear gradient of MP changing from 10- 40% B. By increasing the t_G from 4 min to 45 min the study shows that the P_C increases significantly. The changes in P_C as a function of t_G are shown in **Figure 6** [33].

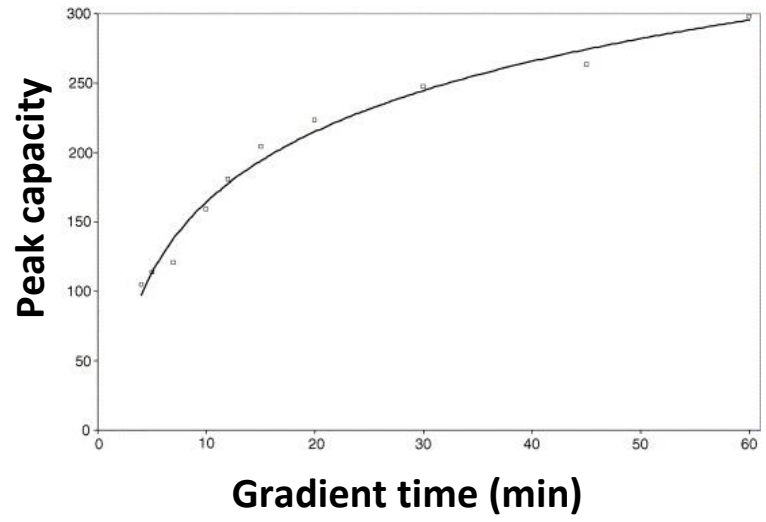


Figure 6 *P_C as a function of t_G for a 50 mm column with a flow rate of 0.5 mL/min [33].*

Figure 6 shows that increase of P_C is not linear, and increases due to increasing t_G. **Figure 6** also shows that P_C flattens out after 30 min [33]. Wren and Wang et al. concluded that use of longer t_G generally provides higher P_C but that the benefits reduce at higher t_G.

2.8 Selectivity in liquid chromatography

The selectivity of the chromatographic separation of two components is generally characterized by the relative retention (α) of the components under fixed operating conditions [11]. The formula for calculating selectivity is shown in Equation 9. The retention of a compound depends on its distribution coefficient (K) between the SP and the MP, where K is proportional to the retention factor (k) (i.e. the t_R of a solute in the SP) for a given column [40], calculation of the retention factor (k) is shown in Equation 10.

$$\alpha = \frac{K_2}{K_1} = \frac{k_2}{k_1} \qquad \text{Eq.9}$$

Where K2 is the distribution factor of the more retained compound, K1 for the less retained compound, and k_1 and k_2 are the retention factors of compounds 1 and 2 respectively.

$$k = K\frac{V_S}{V_M} \qquad \text{Eq.10}$$

Where k is the retention factor, K is the distribution coefficient, V_S is the volume of stationary phase and V_M is the volume of mobile phase.

2.8.1 Effect of gradient slope

Employing either a long t_G and low flow rate, or a short t_G and high flow rate will in both cases provide the same selectivity under fixed operating conditions [41]. An example of decreasing the slope (%/mL) and increasing the flow rate is shown in **Figure 7.**

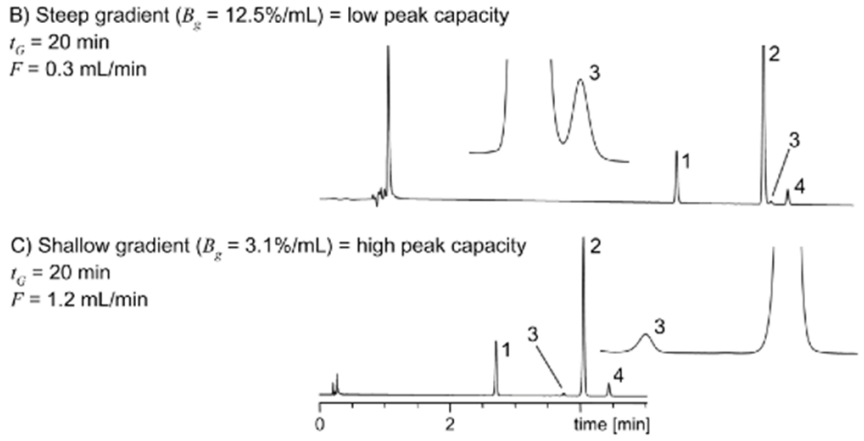


Figure 7 *Chromatogram with the same* t_G*, gradient elution from 20-95 % ACN and temperature at 60 ºC. The different between B) and C) is the flow rate 0.3 mL/min and 1.2 mL/min respectively [41].*

The t_R for peak number 4 in B) and C) in **Figure 7** during the t_G is shown after 7.2 min and 4.4 min respectively. When decreasing the slope (%/mL) the elution order for peak number 2 and 3 change places. When increasing the flow rate for a given t_G as shown in **Figure 7**, the results show a shallower gradient, an increased P_C and shorter run time [41].

2.8.2 Column temperature

Column temperature has two important roles in RP-LC; control of k and control of α, and can be selected in a manner that optimizes chromatographic resolution [42]. The van Deemter Equation (shown in Equation 3, section 2.6) describes the relationship between flow rate (linear velocity) and plate height (H) (i.e. the column efficiency) [43].

When increasing column temperature, a large mass transfer (C-term) in the van Deemter equation would be expected to be more sensitive to changes in temperature. The C term shows some dependence on the retention factor (k) and the longitudinal diffusion within the stationary phase (B-term) can give rise to changes in N with k [44].

These effects leads to a shift of the minimum of the van Deemter equation toward higher flow rates, and a reduced H at flow rates above the minimum in the van Deemter equation [41]. At flow rates above the minimum, an increased temperature will result in increased P_C, as shown in **Figure 8**.

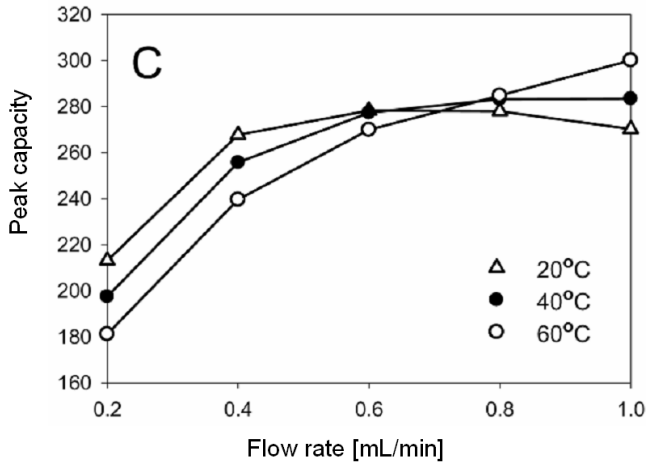


Figure 8 *P_C as a function of flow rate with a t_G of 40 min at different temperatures (20, 40 and 60 ºC) [41].*

The study concludes that for analytes with low k (< 10-20) the P_C increases with increasing flow rate and temperature. As shown in **Figure 8**, a lower flow rate a low temperature

provides the highest P_C, and higher flow rate a high temperature provides the highest peak capacity. P_C is dependent of the coherence between flow rate and temperature [41].

2.8.3 Stationary phases

There are many different stationary phases (SP), such as hydrophilic interaction chromatography (HILIC), porous graphitic carbon (PGC), ion-exchanger chromatography (IEX), RP-type material etc. [7]. Five different analytical RP columns have been tested in this master thesis, Polaris C18-Ether, ACE C18-PFP (pentafluorophenyl), Pursuit XRs C18-Diphenyl, Aeris Peptide XB-C18 and Restek Raptor Biphenyl.

The SPs have different selectivity which means that they have different abilities to distinguish analytes from other species in the sample. Polaris C18-Ether column are endcapped with an ether group to create a more polar surface for selectivity variation. The polar modifications help it avoid poor peak shape and retention issues in low organic conditions [17]. The ACE C18-PFP column is a C18 bonded HPLC column with the extra selectivity of a PFP phase. This phase utilizes a specially developed ligand combining a C18 chain with integral PFP functionality, resulting in a phase that maintains the hydrophobicity, stability and low bleed characteristics [18]. Pursuit XRs Diphenyl has pi-pi selectivity and is often used for aromatic compounds. The lack of hydrophobic space gives diphenyl very unique shape selectivity. Aeris Peptide XB-C18 is recommended for separation of low molecular weight peptides and for peptide mapping. It has small pores optimized for peptide diffusion and has chemistry suited for resolving peptides [45]. Restek Raptor Biphenyl columns have phenyl groups as the stationary phase, biphenyl as the ligand type and superficially porous silica particles. The Biphenyl column is used for drug and metabolite analyses and has heightened selectivity and retention for compounds that are hard to resolve or elute using C18 and other phenyl chemistries [46].

In RP columns, polar compounds such as silanol groups may remain after modification of the silica, and can cause tailing. In more recent times, columns have been modified with plentiful cross links and few silanol groups, so tailing can, to a larger extent than before, be eliminated [19]. The structure of a C18 SP is shown in **Figure 9**.

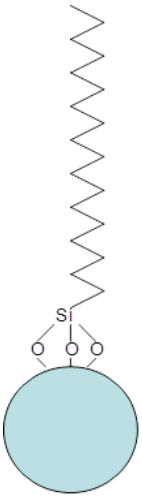


Figure 9 *The structure of a C18 SP where silanol groups are modified through chemical reactions.*

This is a non-polar material which is produced through chemical modification of the surface of silica, shown in **Figure 9**. The silanol groups on the surface of the particle are applied with alkane-chains of type $C_{18}H_{37}$. The Si-O-Si bonds become less available for hydrolysis and cleavage because the long carbon chain provides optimum shielding of the silica surface. The most stable chemical modification of silica is C_{18}. At extreme or pH values the modified phases of silica will be destroyed, so the typical application area is limited to pH 2 to 10 [19].

RP chromatography is suitable for the separation of non-polar to polar compounds, as long as the analytes have sufficient hydrophobic properties that they can bind to the SP. A C_{18}-column is widely used because the long and non-polar carbon chain gives the material high stability and because it binds a broad selection of compounds [19]. Analytes in blood have compounds that are predominantly more or less polar, it is therefore important to select an analytical column with a SP that manages to capture the largest possible number of these analytes.

2.9 Aim of study

The aim of study was to optimize chromatographic parameters of a LC-MS method to achieve separation of as many compounds as possible for untargeted metabolomics of DBS. This was done by studying how each parameter affects P_C. A DBS spiked with nine selected compounds representing the metabolome was used for method development. The parameters tested were RP analytical column, MP composition, gradient elution profile, injection volume, column temperature and flow rate.

3 Experimental

3.1 Chemicals

3.1.1 Solvents

Acetonitrile (ACN) (≥ 99.9% purity), methanol (MeOH) (≥ 99.9% purity) and formic acid (FA) (98-100%) were purchased from Merck (Dramstadt, Germany). Type 1 water (resistivity of 18.2 MΩ•cm at 25 ˚C) was taken from a Milli-Q Integral purification system featuring a Q-POD (0.22 µm filter) dispenser from Millipore (Dramstadt, Germany).

3.1.2 Reagents

Bilirubin and creatinine (Merck), D-(+)-glucose, and uric acid (Sigma), creatine (Nutritional Biochemical Corporation), tobramycin, C2-, C12-, and C16 acylcarnitine (Larodan, Malmö). Ammonium acetate (NH_4COOCH_3) was purchased from VWR International (Radnor, Pennsylvania, USA).

3.1.3 Solutions

A solution was made with the reagents listed above (except bilirubin); 13.940 mg creatinine (1.233 mM), 92.239 mg D-(+)-glucose (5.12 mM), 5.931 mg uric acid (0.353 mM), 1.831 mg creatine (0.1397 mM), 1 mL 69.4 mg/L tobramycin, 1 mL 0.303 mM acylcarnitine C2, 1 mL 0.053 mM acylcarnitine C12 and 1 mL 0.045 mM acylcarnitine C16 were dissolved to a final volume of 100 mL. This solution was called the "standard mix". A 10 mM aqueous NH_4COOCH_3 buffer was made with 0.7706 g NH_4COOCH_3 mixed with type 1 water to a final volume of 1000 mL. A 10 mM organic NH_4COOCH_3 buffer was made with 0.3882 g NH_4COOCH_3 mixed with MeOH to a final volume of 500 mL. A 10 mM organic NH_4COOCH_3 buffer dissolved with ACN was made with 0.3933 g NH_4COOCH_3 dissolved in 5 mL type 1 water and mixed with 495 mL ACN. Aqueous MPs consisted of water/FA (*99.9/0.1, v/v*) and water/NH_4COOCH_3 (*99.9/10 mM, v/v*). Organic MPs consisted of MeOH/FA (*99.9/0.1, v/v*), MeOH/NH_4COOCH_3 (*99.9/10 mM, v/v*), ACN/FA (*99.9/0.1, v/v*) and ACN/NH_4COOCH_3 (*99.9/10 mM, v/v*).

Extraction solution (used for DBS sample preparation, see below) was MeOH/water/FA (*80/20/0.1, v/v/v*). A 10 mM NH_4COOCH_3 re-extraction solution (used for DBS sample preparation, see below) was made with 0.0823 g NH_4COOCH_3 mixed with 95 mL type 1

water and 5 mL MeOH. A 10 mM NH_4COOCH_3 re-extraction solution (used for DBS sample preparation, see below) was made with 0.0822 g NH_4COOCH_3 mixed with 95 mL type 1 water and 5 mL ACN. Re-extraction solutions (used for DBS sample preparation) included water/MeOH/FA (*95/5/0.1, v/v/v*), water/ACN/FA (*95/5/0.1, v/v/v*), water/MeOH/NH_4COOCH_3 (*95/5/10 mM, v/v/v*), water/ACN/NH_4COOCH_3 (*95/5/10 mM, v/v/v*), water/MeOH/FA (*98/2/0.1, v/v/v*) and water/ACN/FA (*98/2/10 mM, v/v/v*).

Calibration solutions were Pierce LTQ Velos ESI Positive Ion Calibration solution and Pierce ESI Negative Ion Calibration solution purchased from Thermo Fisher Scientific (Waltham, Massachusetts, 02451, USA).

3.2 Materials and equipment

Whatman 903 Protein Saver filter paper cards were from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, United Kingdom). A manual puncher (size 3.2 mm) was from Perkin Elmer (Waltham, Massachusetts, USA). Eppendorf tubes (1.5 mL) were from Sarstedt AG & Co. (Nümbrecht, Germany). PIPETMAN Neo P200N and P1000N pipettes were purchased from Gilson, Inc. (Middleton, Wisconsin, USA). The Comfort thermo mixer was from Eppendorf (Hamburg, Germany). Evaporation glasses were from VWR International. An evaporator (TurboVap LV) was from Caliper Life Sciences (Waltham, Massachusetts, USA). A vortex mixer (Vortex L24) was from Labinco Beheer B.V. (Breda, Netherlands). HPLC vials (32x11.6 mm) with insert glass (200 µL) and caps were from Matriks AS (Oslo, Norway).

The analytical columns chosen for comparison were Polaris C18-Ether and Pursuit XRs C18-Diphenyl (Matriks AS), C18 Pentafluorophenyl (PFP) from ACE Technologies (Aberdeen, Scotland), Aeris Peptide XB-C18 borrowed from Phenomenex (Torrance, California, USA) and Raptor Biphenyl borrowed from Restek (Bellefonte, Pennsylvania, USA). The specifications of each column are shown in **Table 1**.

Table 1 *Specifications of analytical RP columns examined during method development.*

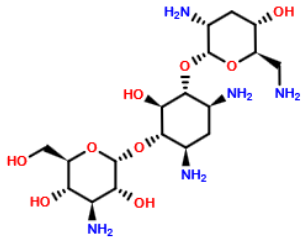
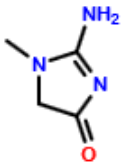
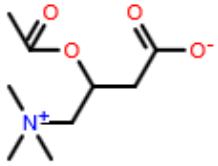
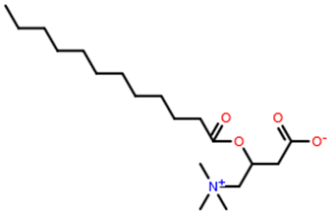
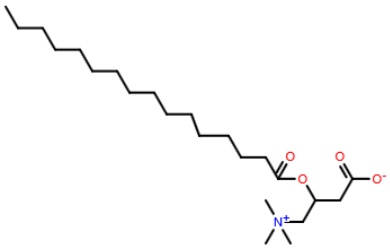
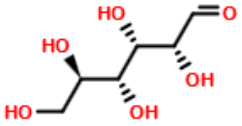
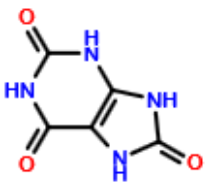
Column	Column length (mm)	Column diameter (mm)	Particle size (µm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)
Polaris C18-Ether	250	2.0	3	180	200	12.1
Pursuit XRs C18-Diphenyl	250	2.0	3	100	440	14.6
ACE C18-PFP	250	2.1	3	100	300	14.3
Aeris Peptide XB-C18	250	2.1	3.6	100	200	10
Raptor Biphenyl	150	2.1	2.7	90	150	7

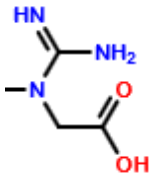
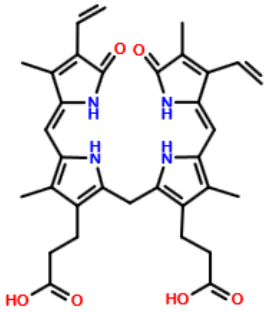
3.3 Sample preparation

3.3.1 Standards and samples used for method development

Blood samples from a healthy volunteer were drawn into an ethylendiaminetetraacetic acid (EDTA) filled tube to prevent the risk of contamination and coagulation, and then mixed with the standard mix solution. DBS were made as a control and are called "control DBS". The standard mix solution was used to make spots on filter paper cards (same type of card used to make DBS) and are called "spiked spots". The standard mix solution was also mixed (50:50) with blood from the same healthy volunteer. The mix of blood and standard mix (50:50) was used to make spots on filter paper card and are called "spiked DBS". The standard mix consisted of nine carefully selected compounds. These compounds cover a wide range of polarity structure and molecular mass. In addition they can be quantified in Standard Clinical Chemistry lab at OUS given the extra advantage of suitability for qualitative method comparison. Molecular masses, given color codes, formulas and structures of compounds in the standard mix are shown in **Table 2**.

Table 2 *Molecular mass (M_r), formula and structure of color-coded compounds added in the standard mix.*

Compounds in standard mix	Color code	M_r	Formula	Structure
Tobramycin		467.25913	$C_{18}H_{37}N_5O_9$	
Creatinine		113.05891	$C_4H_7N_3O$	
Acylcarnitine C2		203.11576	$C_9H_{17}NO_4$	
Acylcarnitine C12		343.27226	$C_{19}H_{37}NO_4$	
Acylcarnitine C16		399.33486	$C_{23}H_{45}NO_4$	
D-(+)-Glucose		180.15614	$C_6H_{12}O_6$	
Uric acid		168.02834	$C_5H_4N_4O_3$	

Creatine		131.06948	$C_4H_9N_3O_2$	
Bilirubin		584.26349	$C_{33}H_{36}N_4O_6$	

3.3.2 Sample preparation of dried blood spot

The following procedure was optimized during the bachelor thesis [47]. A punch from one DBS was taken and transferred to an Eppendorf tube (each punch is 3.2 mm in diameter and corresponds to approximately 3 µL whole blood). 100 µL extraction solution was added to the Eppendorf tube. The sample solution was extracted by heating and shaking in a Thermo mixer for 45 minutes at 45 ºC and 700 rpm. The sample solution was transferred to an evaporation glass and dried at 40 ºC and 5-10 Psi. Subsequently, 100 µL re-extraction solution consisting of water/MeOH/FA (*98/2/0.1, v/v/v*) was added to the evaporation glass. The sample solution was mixed on a table vortex and transferred to an HPLC-vial with insert for analysis. The card was laid flat to dry for at least 30 minutes prior to analysis. DBS samples were analyzed either after drying or stored in a sealed bag with silica at -80 ˚C.

3.4 LC-MS Instrumentation

3.4.1 MS detection

A Q-Exactive MS (quadrupole-Orbitrap) equipped with an ESI source from Thermo Fisher Scientific was used. Each sample was injected and analyzed in respective positive and negative ionization mode and set to full scan mode with a scan range of 50 – 1700 m/z. The MS settings were held constant and are shown in **Table 3**.

Table 3 *MS-settings when optimizing LC-parameters*

Resolution	70 000
Automatic gain control target (ions)	3e6
Maximum inject time (ms)	200
Sheat gas flow rate (arbitrary unit)	40
Aux gas flow rate (arbitrary unit)	10
Sweep gas flow rate (arbitrary unit)	2
Spray voltage (kV)	3.5
Capillary temperature (˚C)	250
S-lens RF level	50.0
Aux-gas heater temperature (˚C)	300

3.4.2 Computer software

All computer software was from Thermo Fisher Scientific. Xcalibur™ Software version 3.0.63 was used to obtain total ion chromatograms (TICs), extracted ion chromatograms (EICs) and two-dimensional (2D) plots. Other software used was Thermo Q Exactive version 2.5 Build 2042 and Chromeleon Xpress version 6.80.

3.4.3 Pump/autosampler

A Dionex UltiMate 3000 instrumentation with LPG-3400RS LC-pump, TCC-3000RS Column Compartment and a WPS-3000TRS autosampler from Thermo Fisher Scientific were used.

3.4.4 Connections

The LPG-3400RS pump, with outlet tubing Viper SST with dimension of 0.18 x 750 mm (IDxL) was coupled to a switching valve purchased from IDEX Health and Science (Oak Harbour, WA, USA). Viper SST tubing with dimension of 0.13 x 450 mm (IDxL) was then used to connect the valve to the analytical column. The end of the analytical column had an outlet of nanoviper tubing with dimensions 75 µm x 650 mm (IDxL) coupled to a switching valve purchased from IDEX Health and Science and Viper SST tubing with dimension 0.13 x 350 mm (IDxL) was coupled from the valve to the ESI.

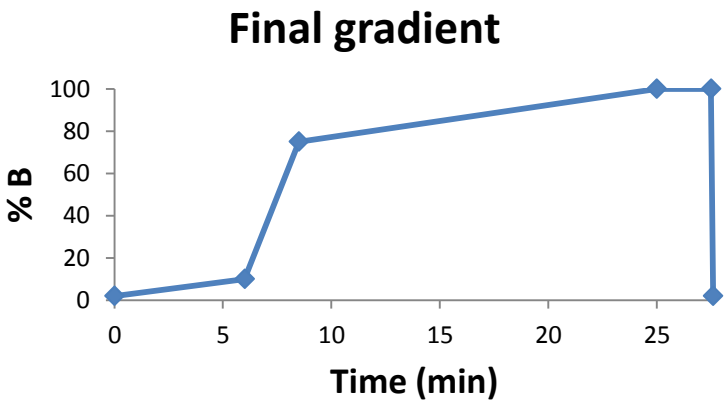
3.4.5 Final liquid chromatography system settings

The LC flow rate was set to 300 µL/min using a Pursuit XRs C18-Diphenyl column (250 x 2.0 mm, 3 µm). The injection volume was set to 2 µL (sample loop was 100 µL), a constant column temperature was set to 30 ˚C and the sample tray was set to a constant temperature at

4 ˚C. A 27.6 minute solvent gradient elution was employed with aqueous MP consisting of water/FA (*99.9/0.1, v/v*) (solvent A) and organic MP consisting of MeOH/FA (*99.9/0.1, v/v*) (solvent B). The gradient elution was performed according to **Table 4**. A 10 minute re-equilibration time with 2% solvent B was employed, giving a total cycle time of 37.6 minutes.

Table 4 *Final gradient elution program (left): time and percentage solvent B (MeOH/FA (99.9/0.1, v/v)), and a schematic profile (right) illustrating the percentage solvent B during* t_G.

Time (min)	Solvent B (%)
0.0	2
6.0	10
8.5	75
25.0	100
27.5	100
27.6	2



4 Results and discussion

4.1 Method development

The LC parameters of a LC-MS method were optimized to achieve separation of as many compounds as possible with the aim of performing untargeted metabolomics of DBS. This was done by evaluating how each parameter affects P_C. A spiked DBS with standards chosen to minimally represent the metabolome was used for method development. The parameters tested were RP analytical column, MP composition, gradient elution profile, injection volume, column temperature and flow rate.

4.1.1 Overview of tested parameters

Table 5 *An overview of all parameters tested; RP column, organic MP, organic additive, gradient elution profile, injection volume, column temperature and flow rate, and final parameters chosen.*

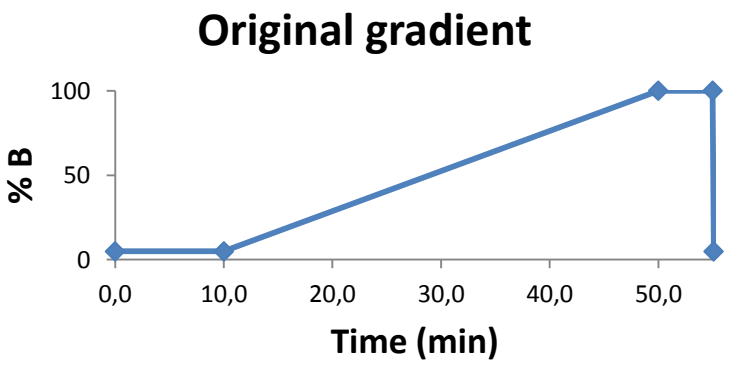
Tested parameters	RP Column	Organic MP	Organic additive	Gradient elution profile	Injection volum (µL)	Column temperature (˚C)	Flow rate (µL/min)	Final parameters
RP Column	See **Table 1** (in Experimental, section 3.2)	MeOH	0.1% FA	See **Table 6** (section 4.1.1)	2	Room	150	C18-Diphenyl
Organic MP	C18-Diphenyl	MeOH and ACN	0.1% FA	See **Table 6** (in section 4.1.1)	2	Room	150	MeOH
Organic additive	C18-Diphenyl	MeOH and ACN	0.1% FA and 10 mM NH_4COOCH_3	See **Table 6** (in section 4.1.1)	2	Room	150	FA
Gradient elution profile	C18-Diphenyl	MeOH	0.1 % FA	See **Figure 19** (section 4.4)	2	Room	150	Gradient 6
Injection volume (µL)	C18-Diphenyl	MeOH	0.1% FA	Gradient 6	2, 10 and 20	Room	150	2
Column temperature (˚C)	C18-Diphenyl	MeOH	0.1% FA	Gradient 6	2	30, 40, 50 and 55	150	30
Flow rate (µL/min)	C18-Diphenyl	MeOH	0.1% FA	Gradient 6	2	30	50, 150 and 300	300

4.2 Choice of reversed phase analytical column

To achieve separation of as many compounds as possible for untargeted metabolomics in DBS, five selected RP analytical columns with different SP were examined. The RP analytical columns tested are shown in **Table 1** in **Experimental** (section 3.2). Spiked DBS were used to compare column performance. For all analyses performed in this experiment the parameters given for "RP column" shown in **Table 5** were used. The gradient elution program used is shown in **Table 6**.

Table 6 *Gradient elution program (left): time and percentage solvent B (MeOH/FA (99.9/0.1, v/v)), and a schematic profile (right) illustrating the percentage solvent B during* t_G.

Time (min)	Solvent B (%)
0.0	5
10.0	5
50.0	100
55.0	100
55.1	5



The separation of spiked DBS was examined for the five RP analytical columns shown in **Table 1**, (in **Experimental**, section 3.2). The separation of spiked DBS for the following columns: C18-Ether, C18-PFP, XB-C18 and Biphenyl is shown as EICs in the **Appendix** (Figure 10-13 in section 6.2) together with associated tables (Table 7-14, section 6.2) showing t_R for all EICs with three replicates together with t_R averages, standard deviations (SD) and coefficients of variation (CV%) for both ionization modes. Results for the C18-Diphenyl column are shown as EICs in **Figure 14** and respectively tables are shown in **Appendix** (Table 15-16, section 6.2.5).

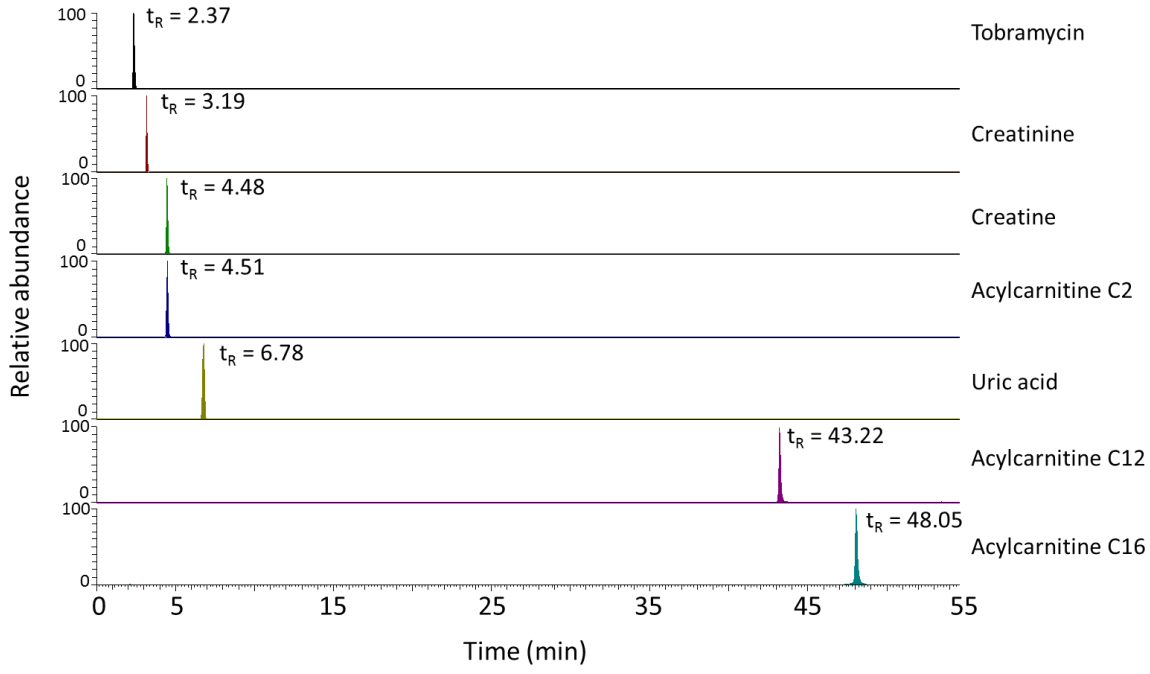


Figure 14 Chromatographic profile of spiked DBS separated on a C18-Diphenyl column. *EICs of tobramycin, creatinine, creatine, acylcarnitine C2, uric acid, acylcarnitine C12 and acylcarnitine C16 are shown.*

As shown in **Figure 14**, all standard components gave nice signals, except for glucose which is not ionized in negative mode, and bilirubin which was not detected in either ionization mode. The lack of signal for bilirubin was most likely due to this compound not being extracted from the DBS. Bilirubin is highly insoluble in water and has to be converted into a soluble conjugate before it can be extracted in an aqueous solution [48, 49].

4.2.1 Retention time (t_R)

For each of the five RP analytical columns tested, data from three replicates (n = 3) in positive and negative ionization mode were collected. The average t_R for all extracted compounds in spiked DBS have been calculated from the three replicates, shown in **Figure 15** and **Figure 16** in positive and negative ionization mode respectively.

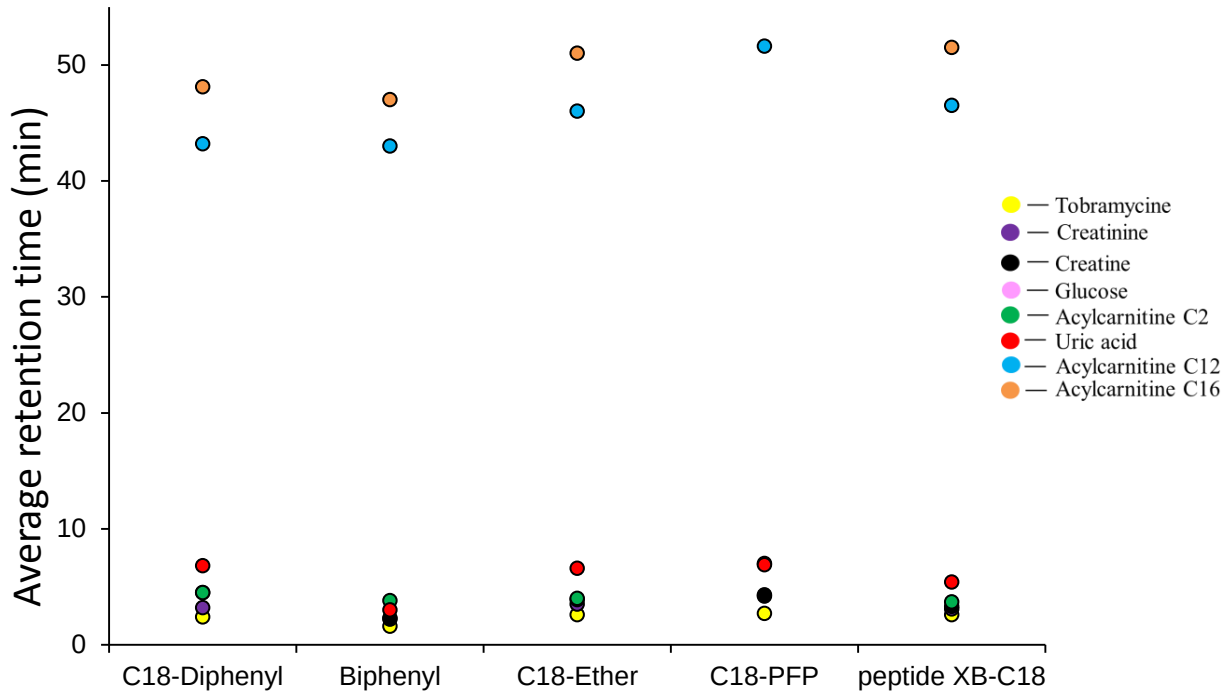


Figure 15 *Average* t_R *(n = 3) of spiked DBS separated on different analytical columns: Data shown for C18-Ether, C18-Diphenyl, C18-PFP, peptide XB-C18 and Biphenyl columns in positive ionization mode.*

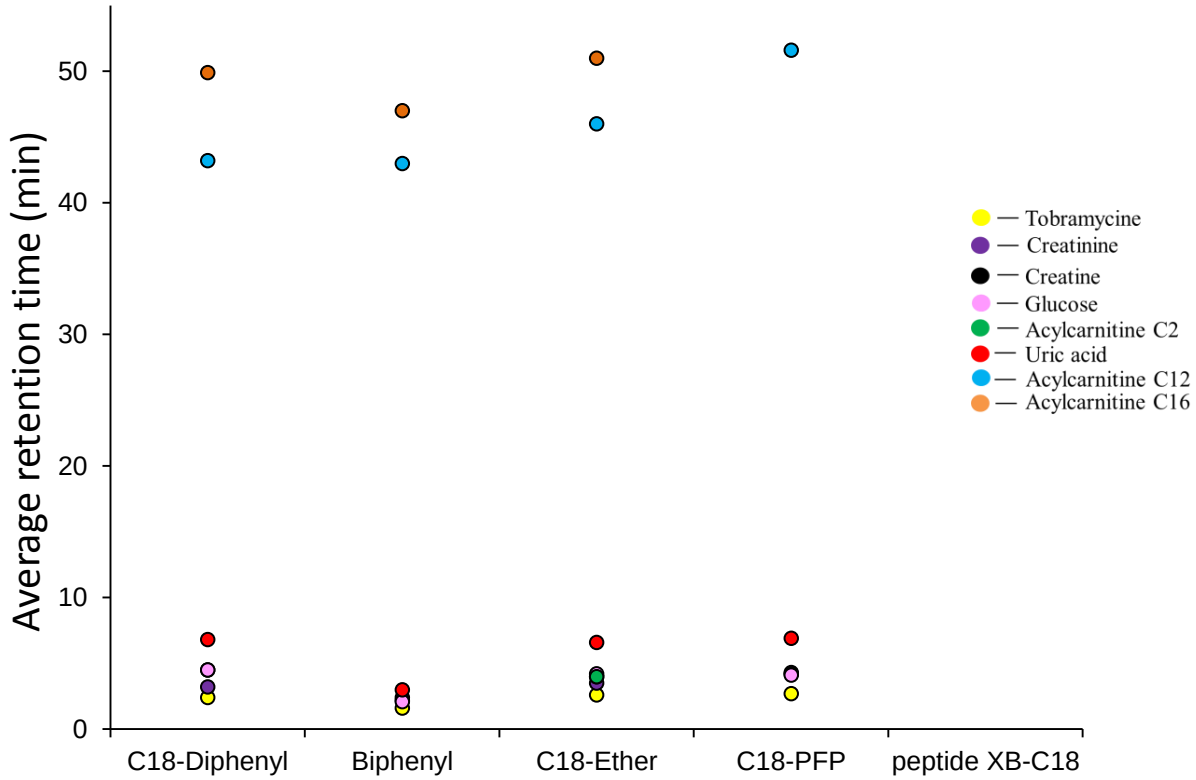


Figure 16 *Average* t_R *(n = 3) of spiked DBS separated on different analytical columns: Data shown for C18-Ether, C18-Diphenyl, C18-PFP, peptide XB-C18 and Biphenyl columns in negative ionization mode.*

The C18-Diphenyl column was chosen for further metabolomics studies in DBS in this master thesis because of the high degree of separation compared to the other columns tested, in addition to the presence of lower background noise than the C18-Ether column, and a better distribution of the hydrophilic compounds than the Biphenyl column. The C18-Diphenyl column manages to separate all compounds in spiked DBS in contrast to the PFP column where acylcarnitine C16 was stuck on the SP and did not elute within a reasonable time (about 55 minutes). The peptide XB-C18 column shows similar results to the Ether column when analyzing in positive ionization mode, but unexpectedly, it does not give results in negative ionization mode. It is conceivable that contamination from the filter paper card can have a negative impact on these analyses. Analysis of extracts from a blank filter paper could have provided more information on this.

4.3 Choice of mobile phase composition

4.3.1 Effect of organic solvent

For all analyses performed in this experiment the parameters given for "MP" shown in **Table 5** were used. To investigate the effect of organic solvent on chromatographic separation, the effects of MeOH/FA (*99/0.1, v/v*) and ACN/FA (*99/0.1, v/v*) as MP solvent B were tested. Data from three replicates (n = 3), with SD and CV% for all extracted compounds in spiked DBS in both ionization mode was collected, shown in the **Appendix** (Table 17-18, section 6.3.1 and Table 19-20, section 6.3.2 respectively). The average t_R for all compounds extracted from spiked DBS for solvent B consisting of ACN/FA (*99/0.1, v/v*) compared to MeOH/FA (*99/0.1, v/v*) have been calculated and are shown in **Figure 17**, with positive ionization mode.

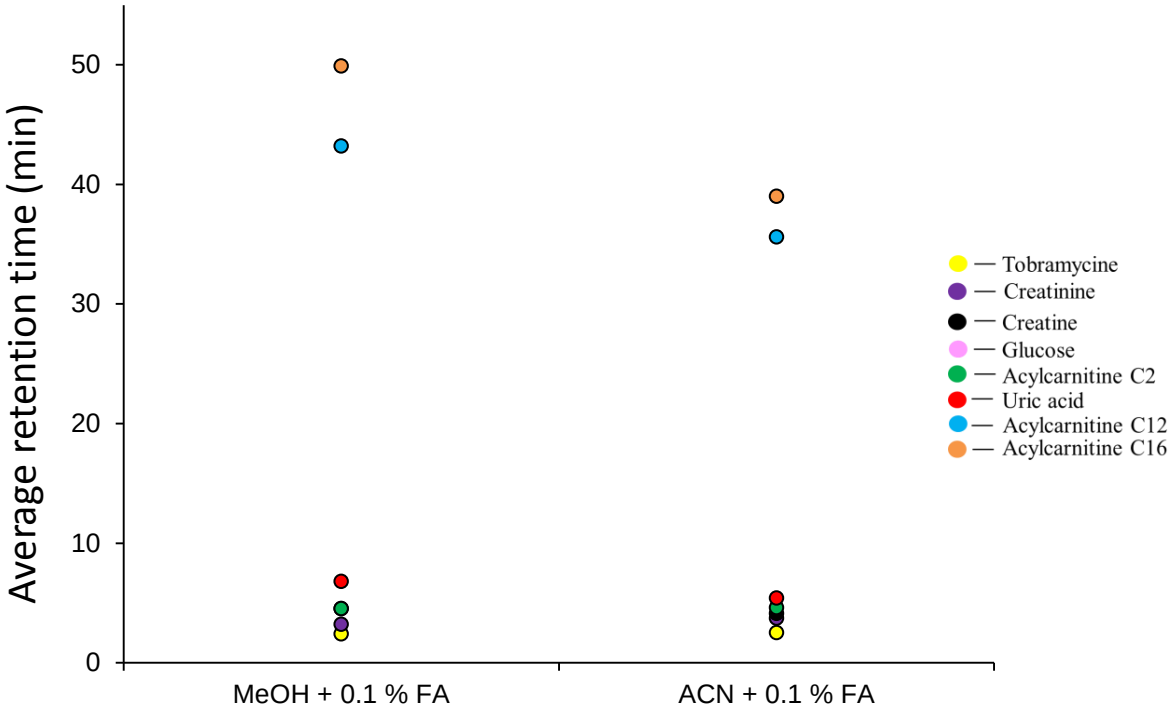


Figure 17 *Average* t_R *(n = 3) for the compounds in spiked DBS separated on a C18-Diphenyl column using two different organic MPs, MeOH/FA (99/0.1, v/v) and ACN/FA (99/0.1, v/v) in positive ionization mode.*

Figure 17 shows that there is no marked difference between the two solvents tested, but provides an illustration that the gap between the hydrophilic and lipophilic compounds is considerably smaller when using ACN/FA (*99/0.1, v/v*). The majority of compounds have a minimal difference in t_R, but uric acid, acylcarnitine C12 and acylcarnitine C16 have a shorter t_R when using ACN/FA (*99/0.1, v/v*).

4.3.2 Effect of organic additives

For all analyses performed in this experiment the parameters given for "organic additive" shown in **Table 5** were used. Two different organic additives were tested, 0.1% FA and 10 mM ammonium acetate (NH_4COOCH_3), to investigate the effect on separation of the compounds in spiked DBS. The composition of solvent A was changed from water/FA (*99.9/0.1, v/v*) to water/NH_4COOCH_3 (*99.9/10 mM, v/v*). Solvent B was changed from MeOH/NH_4COOCH_3 (*99.9/10 mM, v/v*) to ACN/NH_4COOCH_3 (*99.9/10 mM, v/v*). The re-extraction solution was changed accordingly to contain water/MeOH/NH_4COOCH_3 (*95/5/10 mM, v/v/v*) or water/ACN/NH_4COOCH_3 (*95/5/10 mM, v/v/v*).

Two alternatives for solvent B were tested (MeOH and ACN containing NH_4COOCH_3), and data from three replicates (n = 3), with SD and CV% for all extracted compounds in spiked DBS in both ionization modes was collected and is shown in the **Appendix** (Table 21-22, section 6.3.3 and Table 23-24, section 6.3.4 respectively). The average t_R for all compounds extracted from spiked DBS with solvent B consisting of MeOH/NH_4COOCH_3 (*99.9/10 mM, v/v*) and the alternative ACN/NH_4COOCH_3 (*99.9/10 mM, v/v*), have been calculated and are shown in **Figure 18**.

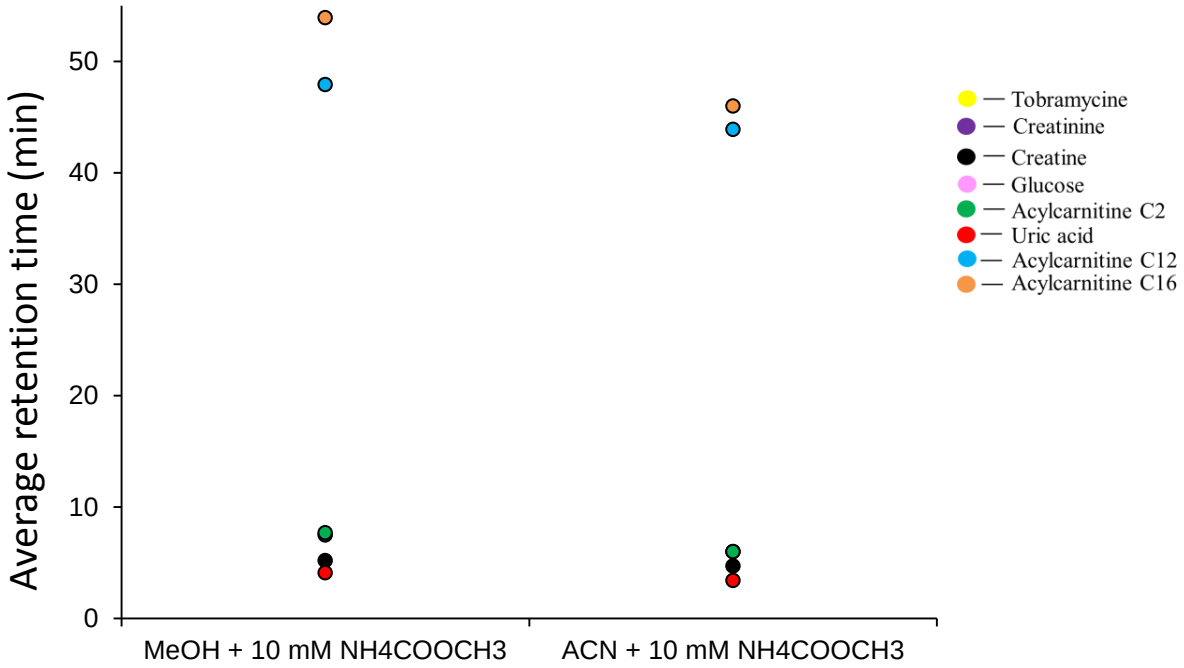


Figure 18 *Average t_R (n = 3) for the compounds in spiked DBS separated on a C18-Diphenyl column using two different MPs, MeOH/NH_4COOCH_3 (99.9/10 mM, v/v) and ACN/NH_4COOCH_3 (99.9/10 mM, v/v) in positive ionization mode.*

As can be seen in **Figure 18**, similar differences between MeOH and ACN were observed to those seen when FA was tested (**Figure 17**), but the average t_R for each compound was somewhat higher when using NH_4COOCH_3 as an additive. Creatine, acylcarnitine C12 and acylcarnitine C16 have a shorter t_R when using ACN/NH_4COOCH_3 (*99.9/10 mM, v/v*), but have a higher average t_R when comparing with ACN/FA (*99/0.1, v/v*). Overall, FA gave the best distribution between the hydrophilic and lipophilic compounds. Furthermore NH_4COOCH_3 contaminated the sample cone to such a large extent that it needed to be cleaned regularly. This is time-consuming and not ideal for routine applications, therefore NH_4COOCH_3 was excluded from further studies.

The organic solvents MeOH and ACN containing the organic additive FA were chosen for further testing with several different gradient elution profiles (since they had only been tested with a fixed gradient, shown in **Table 6**, section 4.2), to investigate which of the organic solvents provides the best distribution for the compounds in spiked DBS with an optimized gradient elution profile.

4.4 Choice of gradient elution profile

For all analyses performed in this experiment the parameters given for "gradient elution profile" shown in **Table 5** were used. Six different solvent gradients were tested using a 55 minute run time program. The aim of this testing was to improve the separation and decrease the gap between the hydrophilic and lipophilic compounds. The six gradient elution profiles are tested with MeOH/FA (*99/0.1, v/v*) and ACN/FA (*99/0.1, v/v*), respectively shown in **Figure 19**.

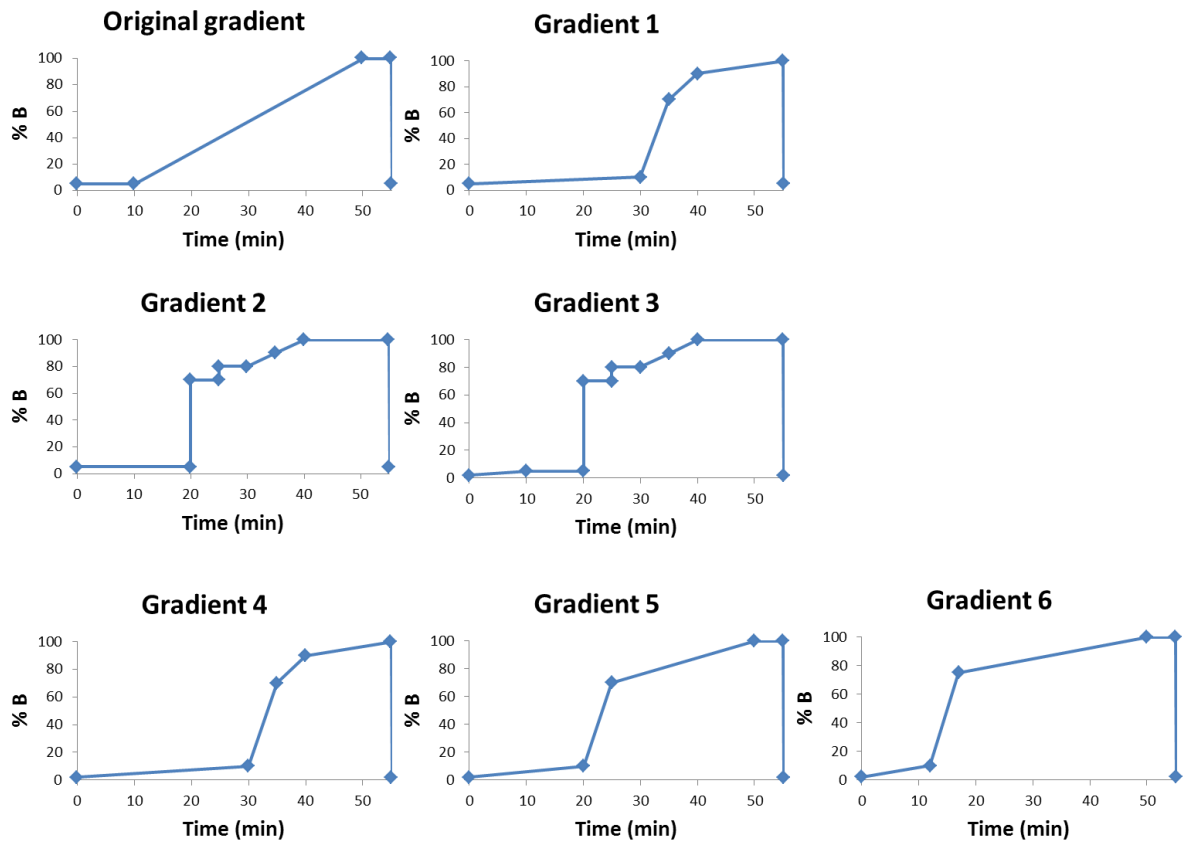


Figure 19 *A schematic overview of the different gradient profiles tested, x-axis: time elution, y-axis: percentage solvent B. The original gradient is the fixed gradient used, while gradients 1-6 were defined based on when the compounds in the spiked DBS elute.*

Additional information about each individual gradient is shown in **Appendix** (Table 25-31, section 6.4). The original gradient was used when working with LC-QTOF on the Bachelor thesis [47] and the method was transferred to the LC-Q-Exactive mass spectrometer when starting on this master thesis. Gradient elution profiles 1-6 were developed based on results from gradients studied; the profile of one gradient was evaluated and the gradient was adjusted to improve separation. The original gradient, and gradients 1 and 2 begin at 5% solvent B, whilst gradients 3-6 begin at 2% solvent B. The percentage was changed with the intention of improving the separation of the hydrophilic compounds.

First, all solvent gradients (original gradient – gradient 6) were tested with solvent B containing MeOH/FA (*99/0.1, v/v*). Data from three replicates (n = 3), with SD and CV% for all compounds extracted from spiked DBS in both ionization modes was collected and is shown in **Appendix** (Table 32-45, section 6.5). Comparisons of all gradient elution profiles

tested with MeOH/FA (*99/0.1, v/v*) are illustrated by the average t_R (n = 3) showing the separation distribution of the compounds in spiked DBS, in **Figure 20**.

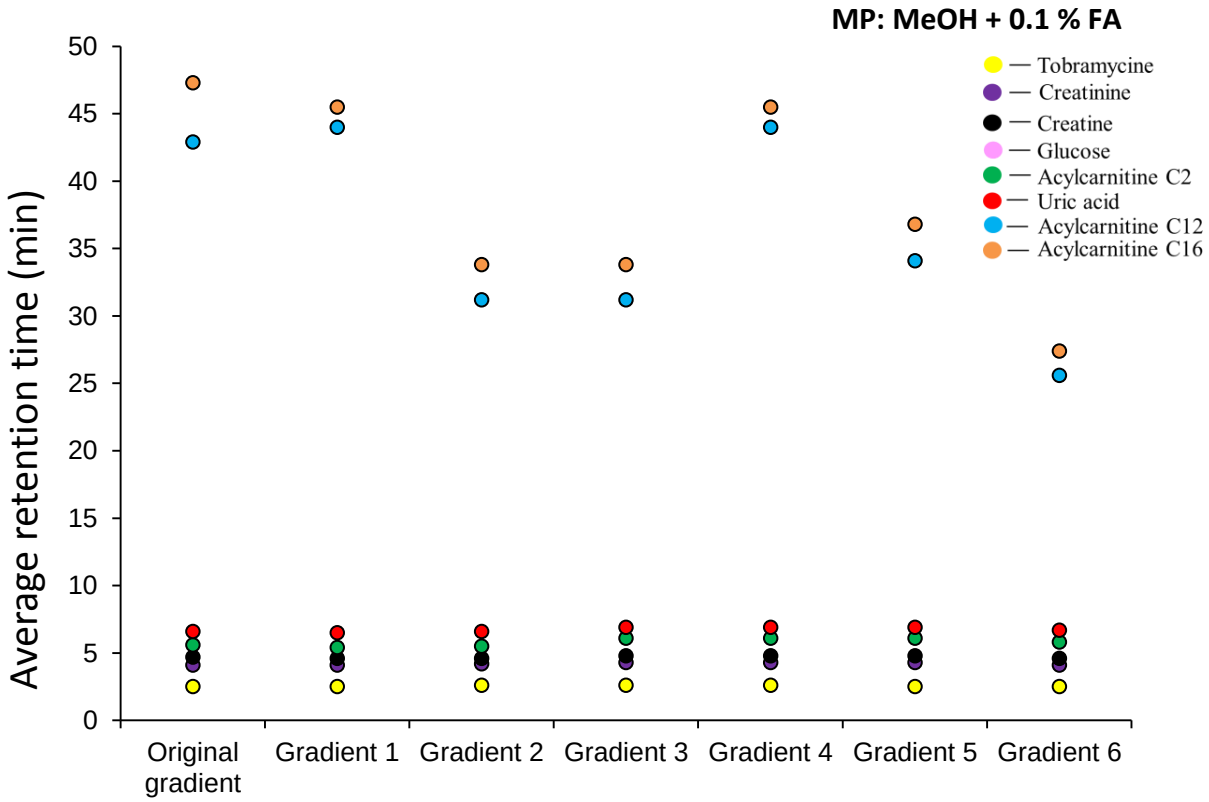


Figure 20 *Average* t_R *(n = 3) for the compounds in spiked DBS separated on a C18-Diphenyl column using the different gradient profiles (original gradient, gradient 1, gradient 2, gradient 3, gradient 4, gradient 5 and gradient 6) when using MeOH/FA (99/0.1, v/v).*

The difference between the original gradient and gradient 6, shown in **Figure 20**, is clearly illustrated in the plot. The average t_R of late eluting compounds acylcarnitine C12 and C16 was significantly reduced from 42.9 and 47.3 minutes respectively when using the original gradient, to 25.6 and 27.4 minutes respectively when gradient 6 was used. The time interval between hydrophilic and lipophilic compounds is significantly reduced when using gradient 6, shown in **Figure 20**, which improves the overall efficiency of the method.

Subsequently, all gradients (original gradient – gradient 6) were tested with solvent B containing ACN/FA (*99/0.1, v/v*). Data from three replicates (n = 3), with SD and CV% for all compounds extracted from spiked DBS in both ionization modes was collected, is shown in the **Appendix** (Table 46-59, section 6.6). Comparison of all gradient elution profiles tested

with ACN/FA (*99/0.1, v/v*) are illustrated by the average t_R (n = 3) showing the separation distribution of the compounds in spiked DBS, shown in **Figure 21**.

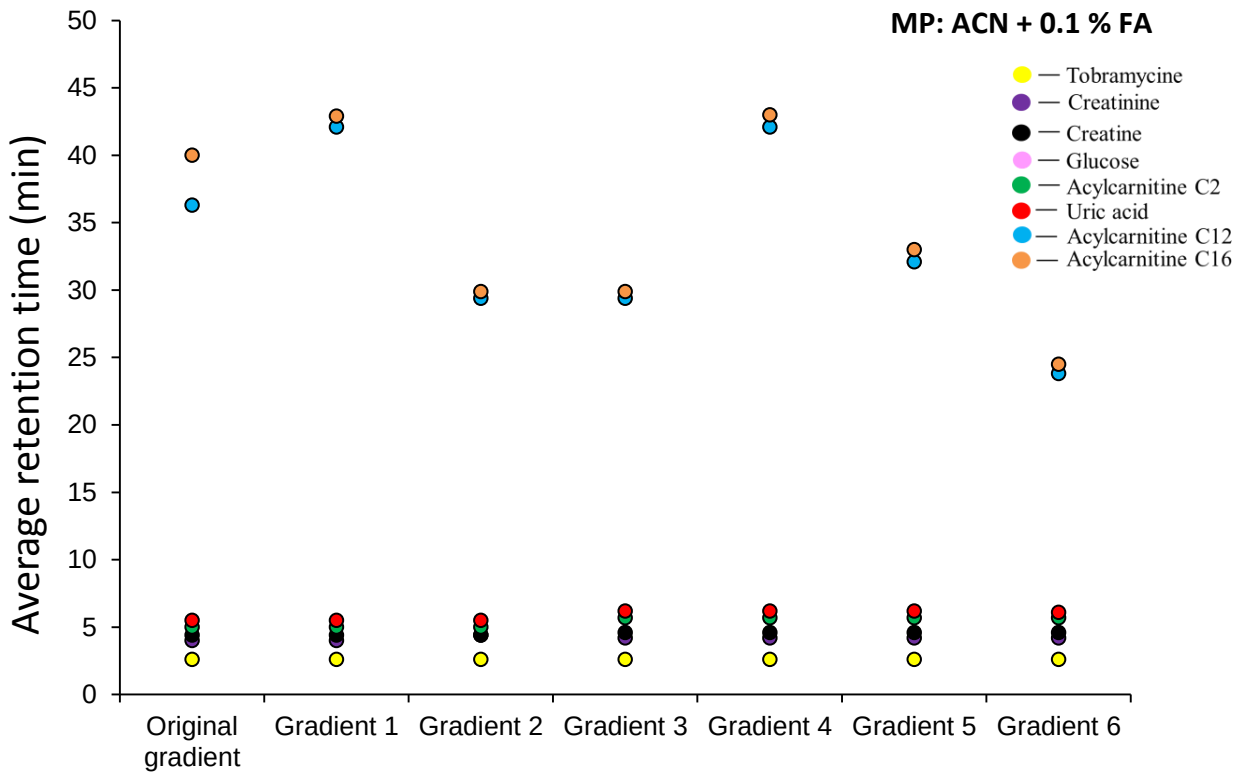


Figure 21 *Average* t_R *(n = 3) for the compounds in spiked DBS separated on a C18-Diphenyl column using the different gradient profiles (original gradient, gradient 1, gradient 2, gradient 3, gradient 4, gradient 5 and gradient 6) when using ACN/FA (99/0.1, v/v).*

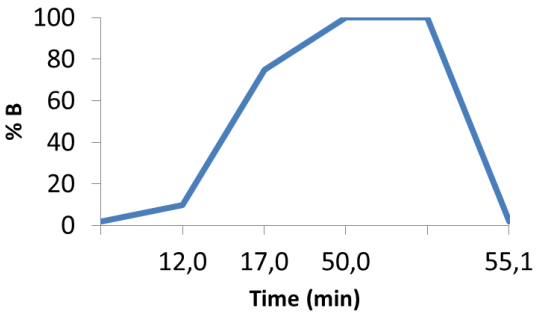
As illustrated in **Figure 21**, a clear difference between the original gradient and gradient 6 was observed. Acylcarnitines C12 and C16 have an average t_R of 36.3 and 40.0 respectively, when using the original gradient, while having a t_R of 23.8 and 24.5 minutes respectively when gradient 6 was used. By comparing **Figure 20 and 21** one can observe that the compounds separated using ACN/FA (*99/0.1, v/v*) have shorter retention times and that the gap between hydrophilic and lipophilic compounds are slightly smaller when using gradient 6.

Gradient 6 was chosen because it gave the most even separation distribution. It decreased the gap between the hydrophilic and lipophilic compounds from 36 to 19 minutes with organic solvent containing MeOH/FA (*99/0.1, v/v*) and from 31 to 18 minutes with the organic solvent containing (ACN/FA (*99/0.1, v/v*). Separation distributions of the hydrophilic compounds have been slightly improved and the gap between the hydrophilic and lipophilic compounds

has been reduced considerably. The gradient elution program for gradient 6 is shown in **Table 60**.

Table 60 *Gradient elution program, gradient 6, (left): time and percentage solvent B (MeOH/FA (99.9/0.1, v/v)), and a schematic profile (right) illustrating the percentage solvent B during* t_G.

Time (min)	Solvent B (%)
0.0	2
12.0	10
17.0	75
50.0	100
55.0	100
55.1	2



No marked difference between MeOH/FA (*99/0.1, v/v*) and ACN/FA (*99/0.1, v/v*) as organic MP was observed when using gradient 6. 2D-plots were used to compare the two organic MP. A 2D-plot represents eluted compounds with bright spots as function of the gradient elution time (min) and mass (*m/z*). 2D-plots were created using the software Xcalibur™. 2D-plots of control DBS analyzed with solvent B containing MeOH/FA (*99/0.1, v/v*) are shown in **Figure 22**, and with ACN/FA (*99/0.1, v/v*) in **Figure 23**.

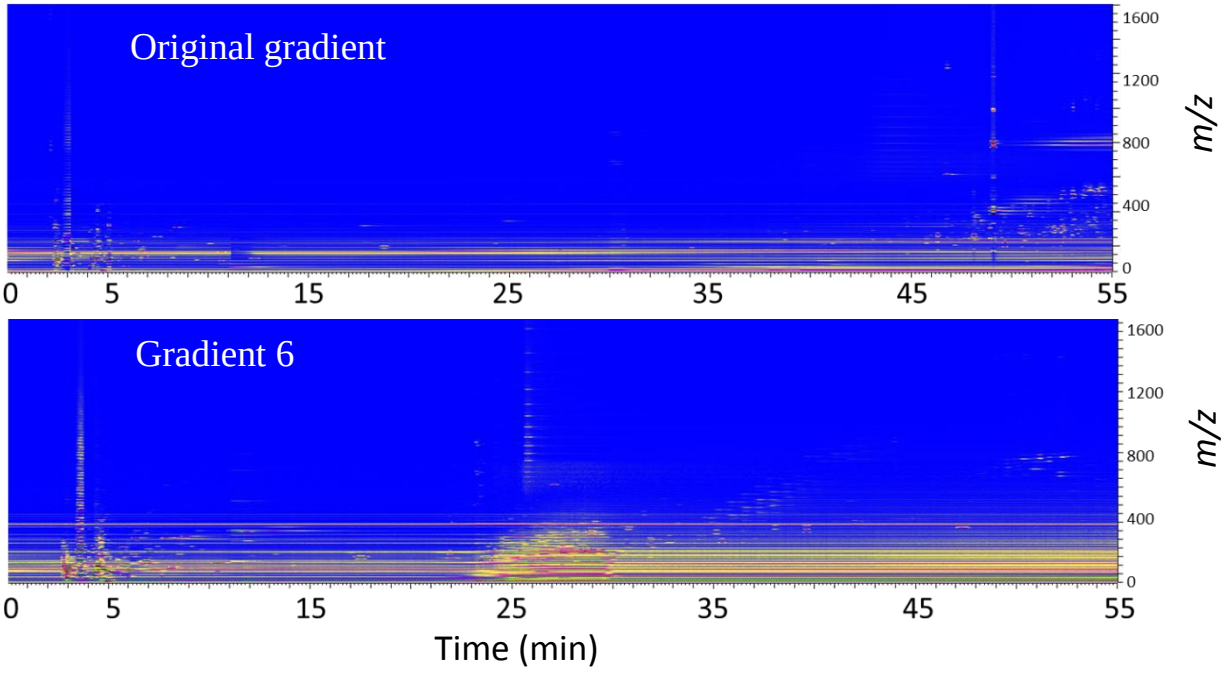


Figure 22 *2D-plots of DBS control when using solvent B containing MeOH/FA (99/0.1, v/v). The gradient elution time is shown on the x-axis, the m/z is shown on the y-axis, top: the original gradient, bottom: gradient 6.*

Comparison of the 2D-plots (original gradient and gradient 6) in **Figure 22** shows marginal differences of the hydrophilic compounds (early eluting) in control DBS, but the lipophilic compounds (late eluting) have moved as much as the acylcarnitines – C12 and C16 shown in **Figure 20**.

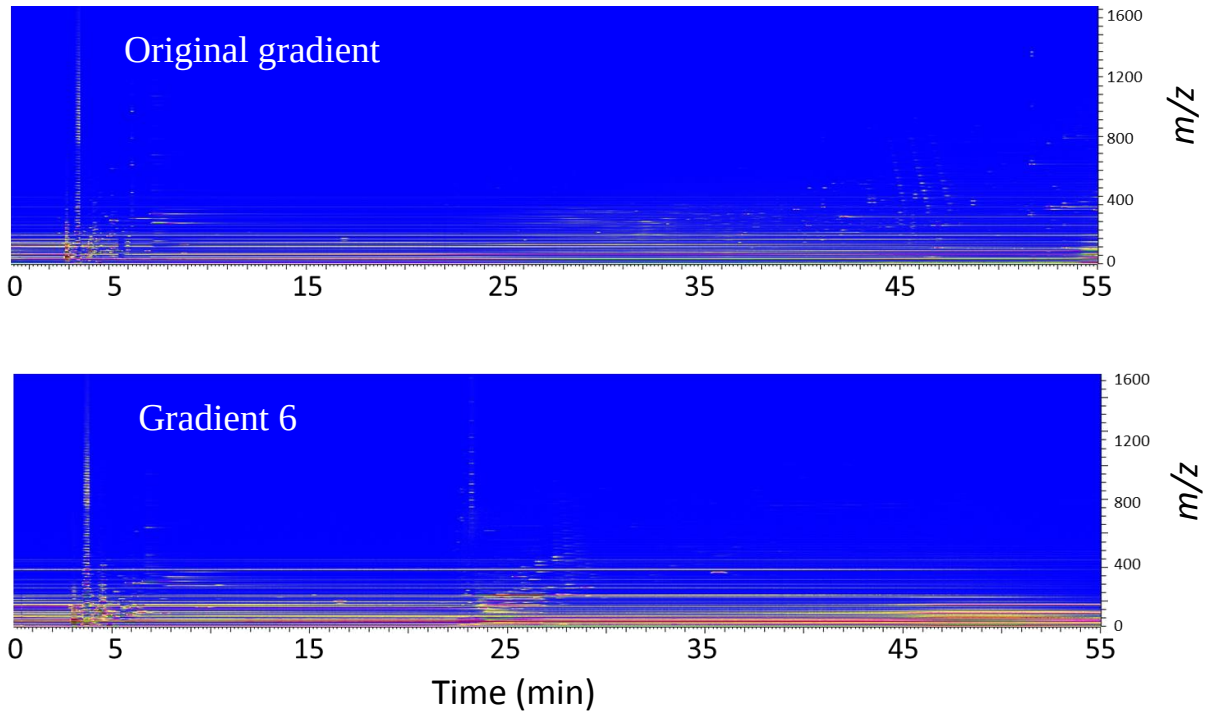


Figure 23 *2D-plots of DBS control when using solvent B containing ACN/FA (99/0.1, v/v), top: original gradient, bottom: gradient 6.*

Comparison of the 2D-plots (original gradient and gradient 6) in **Figure 23** show the exact same trend as **Figure 22**, but there seems to be less background noise when using ACN/FA. The decision was therefore taken to compare the background for MeOH/FA (*99/0.1, v/v*) and ACN/FA (*99/0.1, v/v*) since there was no noticeable difference between them when comparing the 2D-plots. A blank (spot punched from an unused filter paper with similar sample preparation) was analyzed with gradient 6 for both solvents. Backgrounds for the solvents MeOH/FA (*99/0.1, v/v*) and ACN/FA (*99/0.1, v/v*) are shown in **Figures 24 and 25** respectively.

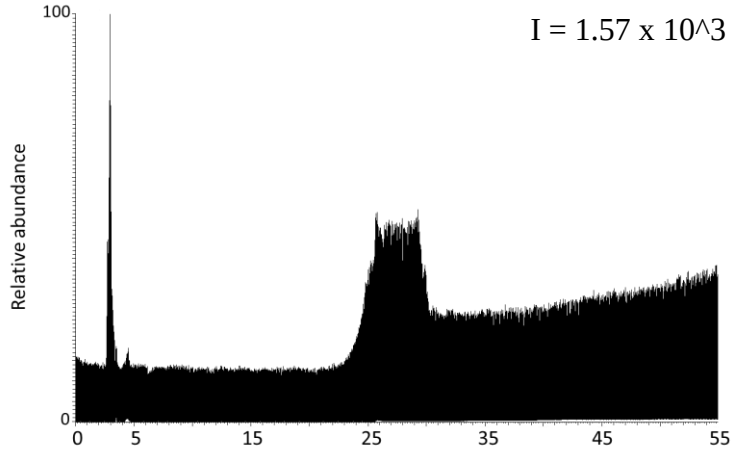


Figure 24 *The background for MeOH/FA (99/0.1, v/v) when analyzing a blank with gradient 6.*

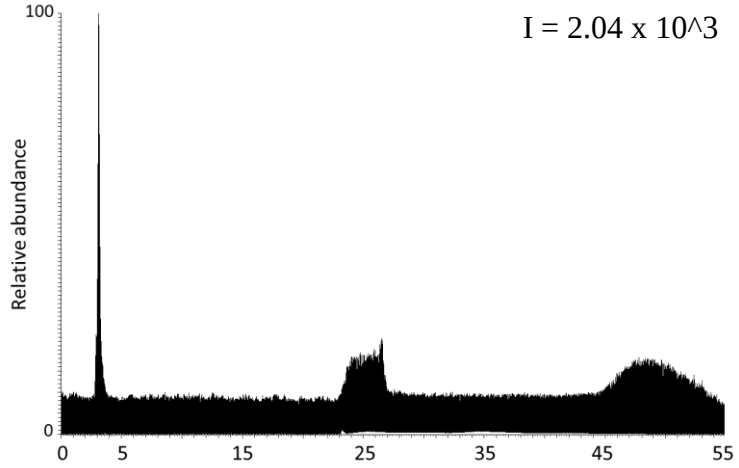


Figure 25 *The background for ACN/FA (99/0.1, v/v) when analyzing a blank with gradient 6.*

Comparing the background in **Figures 24 and 25** shows no major difference between the two solvents. **Figure 24** shows intensity (I) of 1.57 x 10^3 and **Figure 25** shows I of 2.04 x 10^3.
Both organic MP's showed quite similar backgrounds, but MeOH/FA (*99/0.1, v/v*) showed slightly better separation of the hydrophilic compounds (**Figure 20**). MeOH also has the extra advantage of halving reagent costs and often has shorter delivery time, both important considerations for routine applications. Therefore MeOH was determined to be best suited MP component, so the organic MP containing MeOH/FA (*99/0.1, v/v*) was chosen for further studies in this master thesis.

4.5 Calculation of peak capacity

The parameters tested for studying the effect on P_C, were injection volume, column temperature and flow rate. All tests were performed in triplicate (n = 3), and the average P_C

for each parameter tested was used to optimize the final parameters. EICs of the compounds added to spiked DBS were used to calculate the w at 13.4% peak height. P_C for all results below was calculated with Equation 7 (see **Introduction**, section 2.7.1).

4.6 Effect of injection volume on peak capacity

For all analyses performed in this experiment the parameters given for "injection volume" shown in **Table 5** were used. Injection volumes of 2, 10 and 20 µL were tested to optimize the chromatographic system and to determine which yielded the highest P_C. P_C was calculated for three replicates (n = 3) and the average, SD and CV% of P_C were calculated for each of the injection volumes. Data (w) from three replicates (n = 3), with average w, P_C, and average P_C for all compounds extracted from spiked DBS in both ionization modes was obtained, and is shown in the **Appendix** (Table 61-66, section 6.7.1). The average P_C of three replicates for each of the injection volumes 2, 10 and 20 µL, analyzed in positive (A) and negative (B) ionization mode are respectively shown in **Figure 26**, and data are presented in the **Appendix** (Table 67 and 68, section 6.7.2).

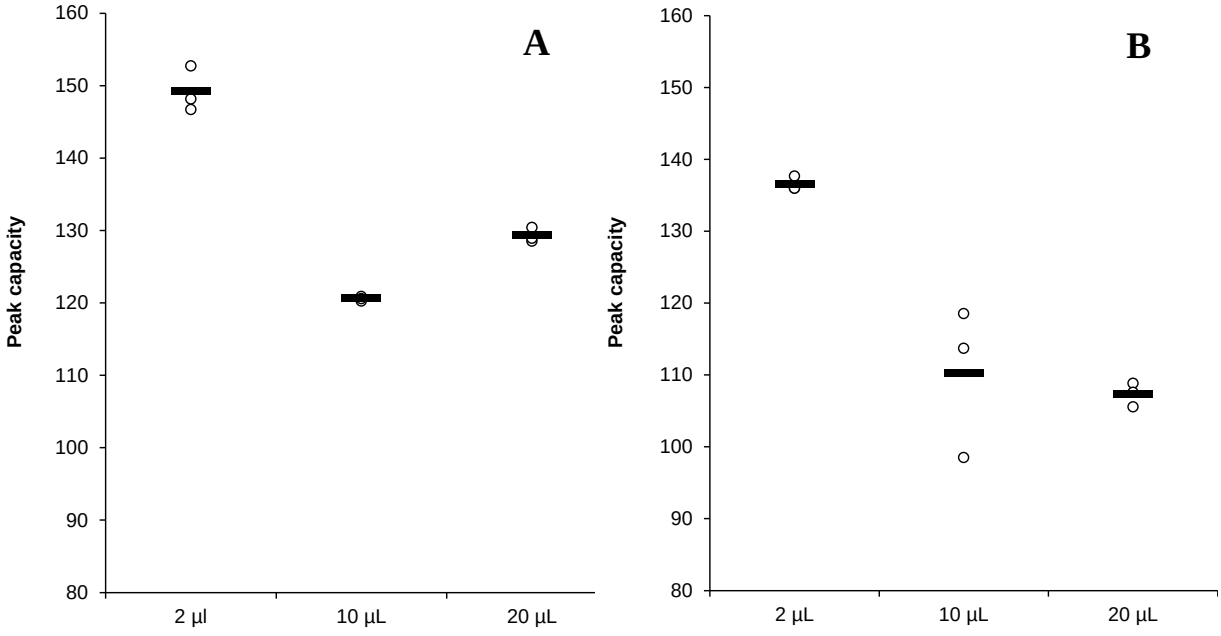


Figure 26 *Average P_C (n = 3)(thick line) for each of the injection volumes 2, 10 and 20 µL analyzed with spiked DBS in positive ionization mode (A) and negative ionization mode (B).*

As is clear from **Figure 26**, an injection volume of 2 µL provides the highest P_C. As shown in **Appendix** (Table 61, 63 and 65, section 6.7.1) these results illustrates that all compounds in spiked DBS are detected when using positive ionization mode for all tested injection volumes.

All compounds, except tobramycin and the acylcarnitines, are also detected in negative ionization mode. However, acylcarnitine C12 was detected in negative mode when injecting a large volume, 10 or 20 µL. Increasing the injection volume has the disadvantage that the peaks become broader and tailing occurs. For some reason there is a greater problem with tailing when using injection volumes of 10 µL compared to 20 µL (the SD and CV% is significantly higher), shown in **Appendix** (Table 68, section 6.7.1). The reason for this increase is due to variation in the tail and the measurement of w is taken in the region where the tail occurs (at 13.4% peak height).

An injection volume of 2 µL was found to be best since this provided the highest P_C, with no particular tailing. An extra advantage of a small injection volume is that it makes the analysis method suitable for use on samples from newborns where only small amounts of sample material are available.

4.7 Effect of column temperature on peak capacity

For all analyses performed in this experiment the parameters given for "column temperature" shown in **Table 5** were used. Temperatures of 30, 40, 50 and 55 ˚C were tested to optimize the chromatographic system and to determine which one yielded the highest P_C. P_C was calculated for three replicates (n = 3) and the average, SD and CV% of P_C were calculated for each of the temperatures.

The temperature experiment was performed twice to investigate how reproducible the results were. This was done by comparing P_C, SD and CV% between the two datasets. Data (w) from three replicates (n = 3), with average w, P_C and average P_C for all compounds extracted from spiked DBS in both ionization modes was gathered for all tested temperatures. Experiment 1 is shown in **Appendix** (Table 69-76, section 6.8.1) and experiment 2 is shown in **Appendix** (Table 77-84, section 6.8.2). The average P_C of three replicates (n = 3) for each of the temperatures 30, 40, 50 and 55 ˚C, **experiment 1 (A)** and **experiment 2 (B)** analyzed in positive ionization mode are shown in **Figure 27** and data are shown in **Appendix** (Table 85 and 86, section 6.8.3).

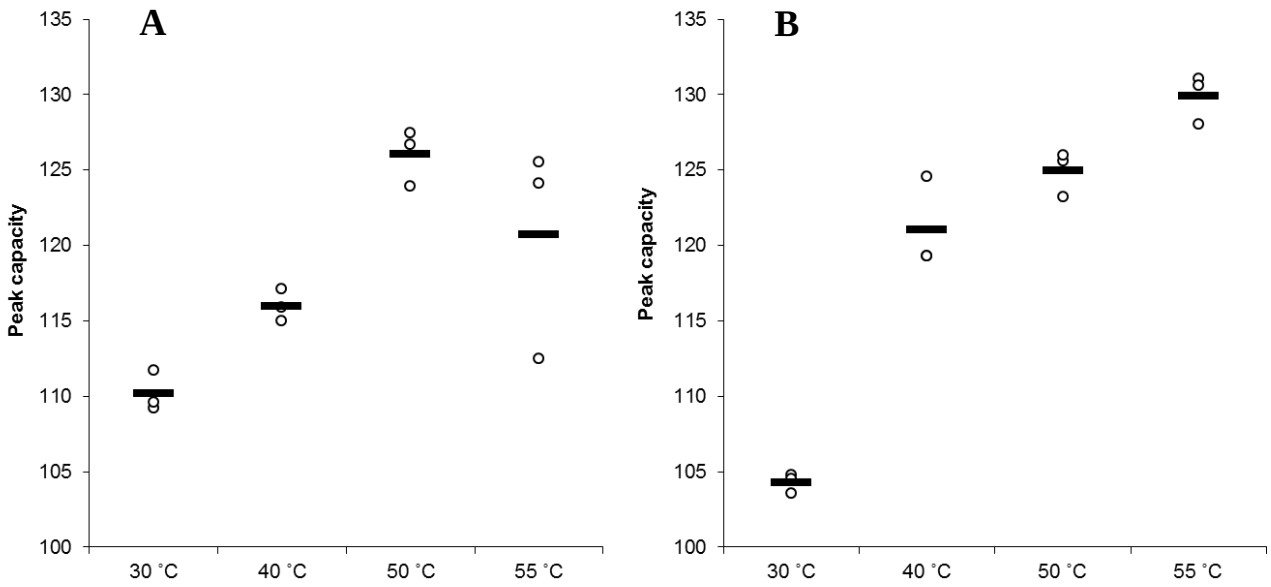


Figure 27 *Average P_C (n = 3)(thick line) for each of the column temperatures 30, 40, 50 and 55˚C, experiment 1 (A) and experiment 2 (B) analyzed with spiked DBS in positive ionization mode.*

In experiment 1, shown **in Figure 27 (A)**, 50 ˚C is the temperature that gives the highest P_C and in experiment 2, shown in **Figure 27 (B)**, 55 ˚C provides the highest P_C. Comparison of A and B shows that there are only small differences in P_C between 30 - 55 ˚C. By increasing the temperature the peaks get somewhat narrower (shown in **Appendix**, Table 69-76, section 6.8.1 and Table 77-84, section 6.8.2) but the chromatographic system seems to be more unstable since tobramycin and acylcarnitine C16 not are detected when using 55 ˚C (**A**), and acylcarnitine C16 was never detected when using 50 or 55 ˚C (**B**). For test temperatures of 30 and 40 ˚C analyzed in positive ionization mode, all compounds in spiked DBS are detected in all runs for both experiments and the results seem to be more stable at these temperatures (**Appendix**, Table 69 and 71, section 6.8.1 and Table 77 and 79, section 6.8.2).

Since this LC-method is intended to be used in future diagnostics it is very important that the method is reliable. The room temperature is in the range of 26-29 ˚C in the laboratory throughout the year, and since there were no major differences between the P_C at 30 and 40 ˚C, it would be prudent to choose 30 ˚C since the temperature is slightly above room temperature and gives the best reproducibility. According to Petterson et al. [41], P_C is dependent upon the coherence between flow rate and temperature; a high temperature provides the highest P_C. In this study the highest temperatures tested (50 and 55 ˚C) provided

the highest P_C. However, a temperature of 30 ˚C was chosen to the prioritization of reproducibility.

4.8 Effect of flow rate on peak capacity

For all analyses performed in this experiment the parameters given for "flow rate" shown in **Table 5** were used. Flow rates of 50, 150 and 300 µL/min were evaluated to optimize the chromatographic system and to see which yielded the highest P_C. P_C was calculated for three replicates (n = 3) and the average, SD and CV% of P_C were calculated for each of the flow rates. When testing 50 and 300 µL/min, the gradient elution time was changed according to the gradient elution program used for a flow rate of 150 µL/min. A flow rate of 50 µL/min is one-third of 150 µL/min so the t_G was changed to 91.8 minutes, and the t_G was halved to 27.6 minutes when using a flow rate of 300 µL/min.

Data (w) from three replicates (n = 3), with average w, P_C and average P_C for all compounds extracted from spiked DBS in both ionization modes was obtained, and is shown in **Appendix** (Table 87-92, section 6.9.1). The average P_C of three replicates (n = 3) for each of the flow rates 50, 150 and 300 µL/min analyzed in positive (**A**) and negative (**B**) ionization mode are shown in **Figure 28** and data are shown in **Appendix** (Table 93 and 94, section 6.9.2)

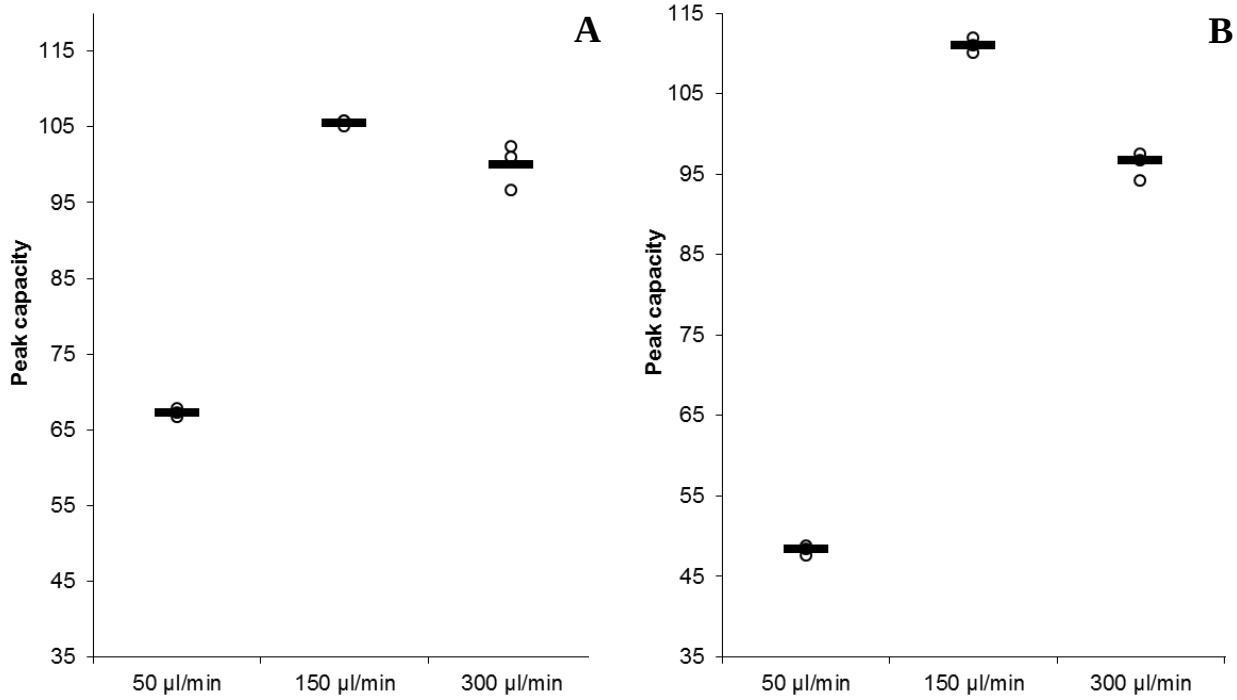


Figure 28 *Average P_C (n = 3) (thick line) at three replicates (n = 3) for each of the flow rates 50, 150 and 300 µL/min analyzed with spiked DBS in positive ionization mode (A) and negative ionization mode (B).*

The results of flow rate testing shows that all compounds in spiked DBS are detected when analyzing in positive ionization mode at all flow rates, and that all except the acylcarnitines are detected when analyzing in negative ionization mode (**Appendix**, Table 88, 90 and 92, section 6.9.1). Tobramycin was not detected in negative ionization mode when testing the flow rates 50 and 300 µL/min (**Appendix**, Table 88 and 92, section 6.9.1). The trend shows that by increasing the flow rate from 50 to 150 µL/min the P_C was almost doubled, but when doubling the flow rate from 150 to 300 µL/min and halving the t_G the P_C remains almost unchanged.

When increasing the flow rate, w become narrower and the t_R reduces (**Appendix**, Table 87, 89, 91, section 6.9.1). The flow rate of 50 µL/min has the lowest P_C and gave broad peaks (e.g. 2.59 minutes for creatine) that were not symmetrical. The w for the compounds analyzed with the flow rate 300 µL/min are almost halved compared to their w with a flow rate of 150 µL/min, but this has little impact on P_C since the t_G is halved. P_C was also calculated with another formula (Eq. 8, see **Introduction**, section 2.7.1) to illustrate the difference in P_C when calculating with and without the gradient elution time. The new P_C calculated is almost halved, but both of the equations show the same trend. Results when calculating P_C with Equation 8 are shown in **Appendix** (Table 95 and Figure 29, section 6.9.3), where Table 95 shows data from three replicates (n = 3) with the average, SD and CV% of P_C for each flow rate. Figure 29 shows the average P_C for each flow rate (50, 150 and 300 µL/min).
The flow rate that gave the highest P_C was 150 µL/min, but 300 µL/min yielded almost the same P_C, as shown in **Figure 28**.

In accordance with the observations of Wang et. al [39], an intermediate flow rate was found to give the highest P_C in this study. However, 300 µL/min was chosen since P_C was approximately the same and the flow rate of 300 µL/min allowed a halving of the t_G; 27.55 vs. 55.1 minutes. In accordance with Wren and Wang et. al [33, 39], a long t_G generally provides high P_C, which was found in this study; 150 µL/min (55.1 minutes) provided the highest P_C. However it is a great advantage to choose a flow rate with half the t_G, since an analysis can be performed in only half the time. When using flow rates of 150 µL/min and 300 µL/min, the maximum pressures were approximately 200 and 400 bar, respectively. A packed column with UHPLC instrumentation can withstand a maximum pressure of 600 bar [21], so a maximum pressure of 400 bar is within a reasonable parameters. Based on these results, the

flow rate of 300 µL/min was evaluated to be the best flow rate given the markedly shorter t_G gained without significant loss of P_C. The final gradient elution program customized to the flow rate 300 µL/min is shown in **Table 4** (see **Experimental**, section 3.4.5).

4.9 More advanced methods for untargeted metabolomics

The challenges of developing fast, robust, and accurate LC-MS methods for separation of closely related compounds is discussed by Guillarme et. al [50]. Employment of UHPLC provides improved metabolite separation and higher resolution compared to conventional HPLC columns, as narrower chromatographic peaks can be achieved resulting in increased P_C. Gradient conditions were selected to elute analytes over a wide range of polarity for multi-component screening, with FA added to the MP. Guillarme et. al conclude that the analysis time can be reduced by a factor of 3-5, and higher chromatographic resolution achieved attributed to increased peak capacity, when using UHPLC rather than HPLC.

In a publication by Yin et. al [51], state-of-the-art non-targeted metabolomics based on LC-MS with special emphasis in clinical applications is discussed. They describe the advantage of using two dimensional (2D) LC systems to achieve better separation rather than one dimensional LC for analysis of complex biological samples. Comprehensive 2D systems can achieve higher peak capacity for metabolic profiling since two analytical columns with different separation mechanisms are applied A disadvantage of 2D-LC system is the extended separation time and extra equipment, not available in most routine labs, needed

Contrepois et. al [52] optimized analytical procedures for the untargeted metabolomic profiling of plasma with a combined HILIC-RP LC-MS method. They found that by combining the optimized HILIC an RPLC-MS method using a column temperature of 60 ˚C, the coverage of the metabolome was greatly expanded compared with RPLC alone.

Although highly sophisticated methods including 2D LC and/or high temperatures might give the best performance characteristic, they are seldom well suited in a high throughput clinical laboratory due to more complex instrumentation, lack of the robustness needed, higher costs, and need for specially trained personnel.

5 Conclusions and summary

The aim of this study was to optimize LC parameters of a LC-MS method to achieve separation of as many compounds as possible for untargeted metabolomics of DBS. This was done by studying how each parameter affects P_C. A DBS spiked with nine selected compounds representing the metabolome was used for method development. The parameters tested were RP analytical column, MP composition, gradient elution profile, injection volume, column temperature and flow rate. The LC method developed was merged with the MS method developed in parallel (master thesis by Hanne B. Skogvold [53]). The goal of the LC-MS method was to create a robust and reliable tool for untargeted metabolomics suitable for clinically relevant samples.

RP analytical column

The C18-Diphenyl column was chosen because of the high degree of separation compared to the other columns tested, lower background noise when compared to the Ether column, and superior retention of the hydrophilic compounds compared to the Biphenyl column. The C18-Diphenyl column manages to separate all compounds in spiked DBS unlike the PFP column where acylcarnitine C16 was stuck on the SP and did not elute within a reasonable time. The peptide XB-C18 column showed quite similar results to the Ether column when analyzing in positive ionization mode but, unexpectedly, provided no results in negative ionization mode.

MP composition

Organic solvent

ACN and MeOH as organic MP were tested with no marked difference observed. However, MeOH gave slightly better separation of the polar compounds and has the extra advantage of halving the reagent cost.

Organic additive

Two different organic additives were tested; 0.1% FA and 10 mM NH_4COOCH_3. 0.1% FA was found to be the best on account of the contamination of the sample cone and significantly higher t_R of creatine, acylcarnitine C12 and acylcarnitine C16 experienced when using 10 mm NH_4COOCH_3.

Gradient elution program

Six different solvent gradients were tested using a 55 minute run time program. The gradient chosen was "gradient 6"; a step gradient that gave the most even separation time distribution between the hydrophilic and the lipophilic compounds. The gap between these compound classes decreased by 17.4 minutes when using MeOH + 0.1 % FA, and by 13.2 minutes when using ACN + 0.1% FA.

Injection volume

Injection volumes of 2, 10 and 20 µL were tested, and 2 µL was found to give the highest P_C, with no particular tailing. When increasing injection volume (10 and 20 µL) tailing and w occurred.

Temperature

Temperatures of 30, 40, 50 and 55 ˚C were tested, and 30 ˚C was chosen due to best reproducibility although increased temperature gave increased P_C.

Flow rate

Flow rates of 50, 150 and 300 µL/min were evaluated, and 300 µL/min was chosen due to shortest gradient elution time without significant loss of P_C.

This Master thesis describes the development of the LC part of a LC-MS method for untargeted metabolomics of DBS that is now ready for validation and subsequent testing in clinically relevant samples. P_C obtained by the final optimized LC-parameters was 100.

5.1 Future work

A full factorial design could be performed to estimate robustness of the optimized LC-method and to investigate which combination of all the parameters tested gives the highest P_C. Before the merged LC-MS method can be used in clinical research or diagnostics a full method validation should be performed to ensure the acceptability of the performance and the reliability of analytical results. It will be critical to determine the stability of the analytes (metabolites) in the biological matrix, matrix effects, selectivity, the lower limit of quantification, accuracy, precision and carry-over. After validation, a clinical validation including analysis of samples from patients with known diseases should be performed to evaluate the methods ability to detect metabolic aberrations.

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6 Appendix

6.1 Additional information about MS

MS is an analytical technique with high sensitivity that is well suited to the detection of substances over a large range of molecular weights and polarity. A limitation of MS is that non-volatile substances or non-ionizable substances not will be detected. Several different mass analyzers are used in MS, of which quadrupole, ion trap and time-of-flight (TOF) are most commonly used today.[22]

Quadrupole time of flight (Q-TOF)-MS is one of the most commonly used methods for metabolomics studies, often coupled to a LC system. Even though it offers only moderate mass resolution and mass accuracy, the Q-TOF compensates for this with very fast scanning rates, which enable a better circumscription of chromatographic peaks. [20] Coupling either GC or LC to Q-TOF mass analyzers is generally more suitable than coupling these chromatographic columns to high-resolution mass analyzers such as orbitrap due to the fact that the latter have slow scanning rates which compromise chromatographic resolution. [20]

Orbitrap technology combines orbital trapping with image current detection. There are three instrument families: LTQ Orbitrap, Orbitrap Fusion and (Q) Exactive mass spectrometers [54]. The samples can be introduced into the API source at the front by a variety of methods including direct infusion or via a LC system. The ions are transferred into the C-Trap through four stages of differential pumping. In the C-Trap, the ions are accumulated and their energy is dampened using a bath gas (nitrogen). The ions are then injected through three further stages of differential pumping using a lens system (Z-lens) into the Orbitrap analyzer where mass spectra are acquired by image current detection. The vacuum inside the Orbitrap analyzer is maintained below 1E-9 mbar.

Ions are passed through the C-Trap into the HCD cell, if the instrument is equipped with it. The HCD cell adds a Higher Energy Collision Induced Dissociation capability to the instrument. This allows all-ion fragmentation (AIF) experiments to be performed. After the ions have been fragmented in the HCD cell, the HCD cell voltages are ramped up and the ions are transferred back into the C-Trap, from where they are injected into the Orbitrap analyzer for detection. [55]

An Orbitrap mass analyzer is composed of a spindle-like central electrode and a barrel-like outer electrode.

When ions are injected into the orbitrap at a z-position offset from z = 0, the ion packet will begin coherent axial oscillation without the need for any additional excitation. Ions of different m/z values are injected at different times, with larger m/z ions arriving later. After ions of all m/z values have entered the orbitrap, the voltage on the central electrode and deflector is held constant to prevent mass shifts during detection. [56] After stabilizing the central electrode voltage, stable ion trajectories within the orbitrap involve axial oscillations along and rotation around the central electrode. At a given electric field strength, ion trajectories are determined by their initial kinetic energy. [56] Axial frequencies are completely independent of initial ion parameters. Therefore, ions of the same m/z ratio continue to oscillate along the z-axis together, remaining in-phase for hundreds of thousands of oscillations [56].

6.2 Supplementary results of the other reversed phase columns tested

6.2.1 The C18-Ether column

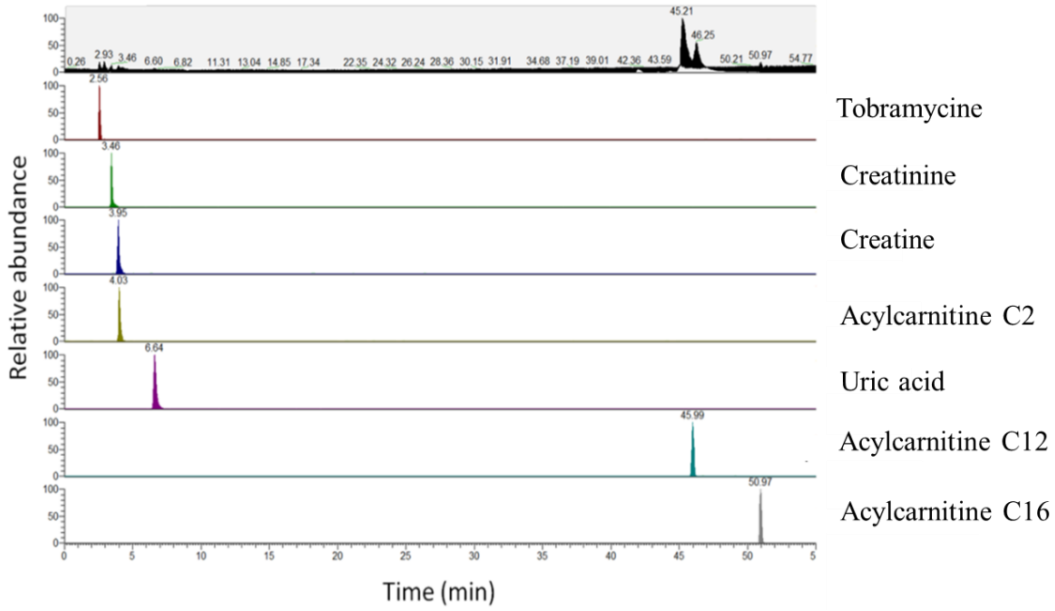

Figure 10 Chromatographic profile of spiked DBS separated on a C18-Ether column. *Top.panel: TIC, bottom panel: EICs of tobramycine, creatinine, creatine, acylcarnitine C2, uric acid, acylcarnitine C12 and acylcarnitine C16 are shown.*

Table 7 *The t_R on the C18-Ether column for three replicates (n = 3) of the compounds in spiked DBS analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,006	0,002
Creatinine	3,5	3,5	3,5	3,5	0,010	0,003
Creatine	4,0	4,0	3,9	3,9	0,015	0,004
Acylcarnitine C2	4,0	4,0	4,0	4,0	0,006	0,001
Uric acid	6,7	6,6	6,6	6,6	0,010	0,002
Acylcarnitine C12	46,0	46,0	46,0	46,0	0,006	0,000
Acylcarnitine C16	51,0	51,0	50,9	51,0	0,021	0,000

Table 8 *The t_R on the C18-Ether column for three replicates (n = 3) of the compounds in spiked DBS analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,026	1,018
Creatinine	3,5	3,5	3,5	3,5	0,025	0,725
Creatine	4,0	4,0	4,0	4,0	0,015	0,385
Glucose	4,2	4,2	4,3	4,2	0,015	0,360
Acylcarnitine C2	4,0	-	-	4,0	-	-
Uric acid	6,7	6,6	6,7	6,6	0,035	0,528
Acylcarnitine C12	46,0	46,0	46,0	46,0	0,035	0,075
Acylcarnitine C16	51,0	51,0	50,9	51,0	0,026	0,052

6.2.2 The C18-PFP column

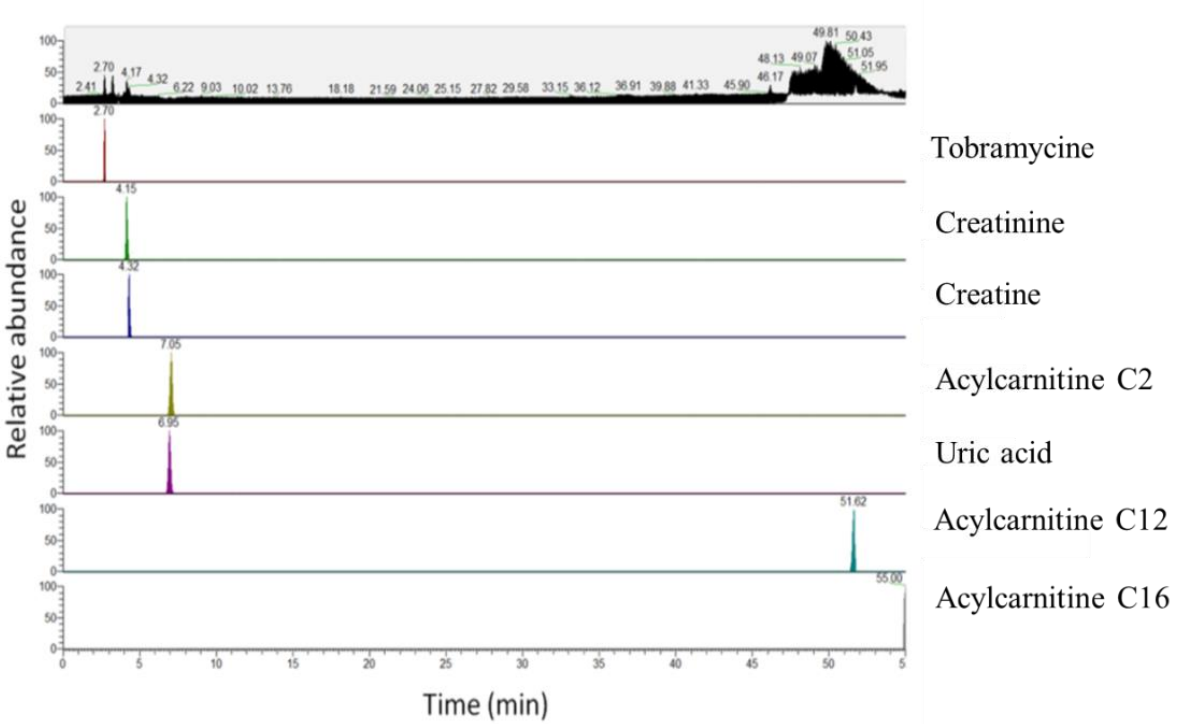

Figure 11 Chromatographic profile of spiked DBS separated on a C18-PFP column. *Top.panel: TIC, bottom panel: EICs of tobramycine, creatinine, creatine, acylcarnitine C2, uric acid, acylcarnitine C12 and acylcarnitine C16 are shown.*

Table 9 *The t_R on the C18-PFP column for three replicates (n = 3) of the compounds in spiked DBS analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,7	2,7	2,7	2,7	0,006	0,002
Creatinine	4,2	4,2	4,2	4,2	0,012	0,003
Creatine	4,3	4,3	4,3	4,3	0,012	0,003
Acylcarnitine C2	7,0	7,1	7,1	7,0	0,012	0,002
Uric acid	7,0	7,0	6,9	6,9	0,015	0,002
Acylcarnitine C12	51,7	51,6	51,6	51,6	0,021	0,000
Acylcarnitine C16	-	-	-	-	-	-

Table 10 *The t_R on the C18-PFP column for three replicates (n = 3) of the compounds in spiked DBS analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,7	2,7	2,7	2,7	0,015	0,562
Creatinine	4,2	4,2	4,2	4,2	0,015	0,366
Creatine	4,3	4,3	4,3	4,3	0,006	0,134
Glucose	4,1	4,1	4,1	4,1	0,006	0,142
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	7,0	6,9	6,9	6,9	0,021	0,300
Acylcarnitine C12	51,6	51,5	51,6	51,6	0,049	0,096
Acylcarnitine C16	-	-	-	-	-	-

6.2.3 The peptide XB-C18 column

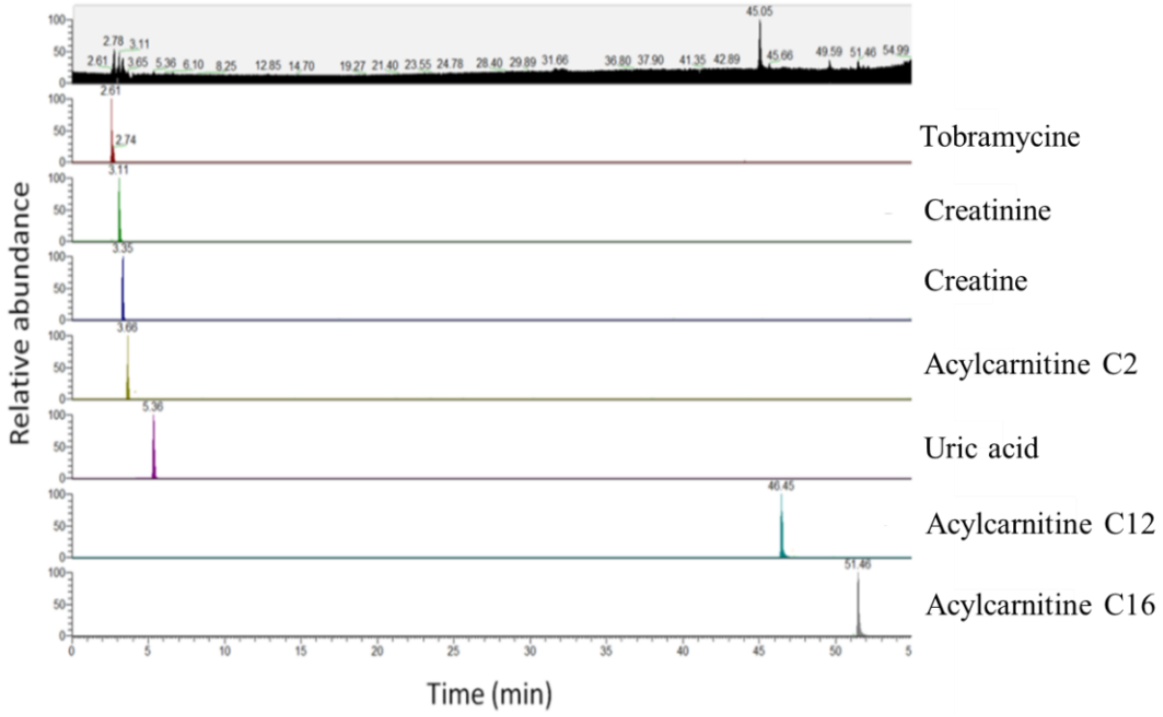

Figure 12 Chromatographic profile of spiked DBS separated on a peptide XB-C18 column.
Top.panel: TIC, bottom panel: EICs of tobramycine, creatinine, creatine, acylcarnitine C2, uric acid, acylcarnitine C12 and acylcarnitine C16 are shown.

Table 11 *The t_R on the peptide XB-C18 column for three replicates (n = 3) of the compounds in spiked DBS analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,017	0,007
Creatinine	3,1	3,1	3,1	3,1	0,017	0,006
Creatine	3,3	3,4	3,3	3,3	0,017	0,005
Acylcarnitine C2	3,7	3,7	3,7	3,7	0,010	0,003
Uric acid	5,4	5,4	5,3	5,4	0,021	0,004
Acylcarnitine C12	46,5	46,5	46,4	46,5	0,032	0,001
Acylcarnitine C16	51,5	51,5	51,4	51,5	0,025	0,000

Table 12 *The* t_R *on the peptide XB-C18 column for three replicates (n = 3) of the compounds in spiked DBS analyzed in negative ionization mode with their average* t_R*, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	-	-	-	-	-	-
Creatinine	-	-	-	-	-	-
Creatine	-	-	-	-	-	-
Glucose	-	-	-	-	-	-
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	-	-	-	-	-	-
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

6.2.4 The Biphenyl column

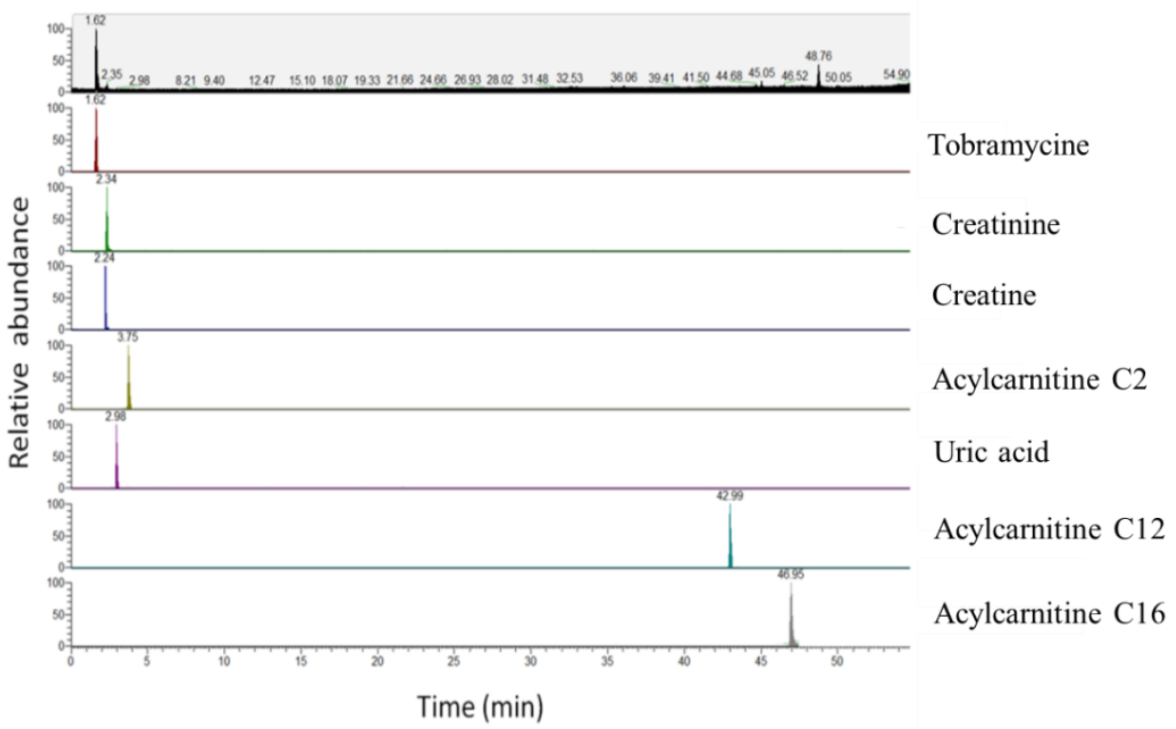

Figure 13 Chromatographic profile of spiked DBS separated on a C18-Biphenyl column. *Top.panel: TIC, bottom panel: EICs of tobramycine, creatinine, creatine, acylcarnitine C2, uric acid, acylcarnitine C12 and acylcarnitine C16 are shown.*

Table 13 *The t_R on the Biphenyl column for three replicates (n = 3) of the compounds in spiked DBS analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	1,7	1,6	1,7	1,6	0,017	0,011
Creatinine	2,3	2,3	2,4	2,3	0,012	0,005
Creatine	2,2	2,2	2,3	2,2	0,006	0,003
Acylcarnitine C2	3,7	3,8	3,8	3,8	0,010	0,003
Uric acid	3,0	3,0	3,0	3,0	0,010	0,003
Acylcarnitine C12	43,0	43,0	43,0	43,0	0,006	0,000
Acylcarnitine C16	47,0	47,0	47,0	47,0	0,015	0,000

Table 14 *The t_R on the Biphenyl column for three replicates (n = 3) of the compounds in spiked DBS analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	1,6	1,6	1,6	1,6	0,012	0,707
Creatinine	2,3	2,4	2,4	2,4	0,010	0,426
Creatine	2,2	2,3	2,3	2,2	0,012	0,515
Glucose	2,1	2,1	2,1	2,1	0,006	0,281
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	3,0	3,0	3,0	3,0	0,012	0,388
Acylcarnitine C12	43,0	43,0	43,0	43,0	0,025	0,059
Acylcarnitine C16	47,0	47,0	47,0	47,0	0,010	0,021

6.2.5 The C18-Diphenyl column

Table 15 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,4	2,4	2,4	2,4	0,030	0,013
Creatinine	3,2	3,2	3,2	3,2	0,029	0,009
Creatine	4,6	4,5	4,5	4,5	0,036	0,008
Acylcarnitine C2	4,6	4,5	4,5	4,5	0,036	0,008
Uric acid	6,8	6,8	6,8	6,8	0,035	0,005
Acylcarnitine C12	43,2	43,2	43,2	43,2	0,015	0,000
Acylcarnitine C16	48,1	48,1	48,1	48,1	0,015	0,000

Table 16 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,4	2,4	2,4	2,4	0,017	0,728
Creatinine	3,2	3,2	3,2	3,2	0,006	0,183
Creatine	4,5	4,5	4,5	4,5	0,006	0,129
Glucose	4,5	4,5	4,5	4,5	0,006	0,128
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,8	6,8	6,8	6,8	0,000	0,000
Acylcarnitine C12	43,2	43,2	43,2	43,2	0,015	0,035
Acylcarnitine C16	48,0	48,1	53,7	49,9	3,288	6,585

6.3 Supplementary results of the MP compositions tested

6.3.1 MeOH and FA

Table 17 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,4	2,4	2,4	2,4	0,030	1,250
Creatinine	3,2	3,2	3,2	3,2	0,029	0,900
Creatine	4,6	4,5	4,5	4,5	0,036	0,799
Acylcarnitine C2	4,6	4,5	4,5	4,5	0,036	0,799
Uric acid	6,8	6,8	6,8	6,8	0,035	0,509
Acylcarnitine C12	43,2	43,2	43,2	43,2	0,015	0,035
Acylcarnitine C16	48,1	48,1	48,1	48,1	0,015	0,032

Table 18 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,4	2,4	2,4	2,4	0,017	0,728
Creatinine	3,2	3,2	3,2	3,2	0,006	0,183
Creatine	4,5	4,5	4,5	4,5	0,006	0,129
Glucose	4,5	4,5	4,5	4,5	0,006	0,128
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,8	6,8	6,8	6,8	0,000	0,000
Acylcarnitine C12	43,2	43,2	43,2	43,2	0,015	0,035
Acylcarnitine C16	48,0	48,1	48,0	48,0	0,035	0,073

6.3.2 ACN and FA

Table 19 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,5	2,5	2,5	0,006	0,230
Creatinine	3,7	3,7	3,7	3,7	0,012	0,309
Creatine	4,1	4,1	4,1	4,1	0,000	0,000
Acylcarnitine C2	4,6	4,6	4,6	4,6	0,012	0,253
Uric acid	5,4	5,4	5,4	5,4	0,010	0,186
Acylcarnitine C12	35,6	35,6	35,6	35,6	0,012	0,032
Acylcarnitine C16	39,0	39,0	39,0	39,0	0,006	0,015

Table 20 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,5	2,5	2,5	0,040	1,594
Creatinine	3,7	3,7	3,7	3,7	0,000	0,000
Creatine	4,1	4,1	4,1	4,1	0,010	0,245
Glucose	4,3	4,3	4,3	4,3	0,006	0,134
Acylcarnitine C2	4,6	4,6	4,6	4,6	0,021	0,453
Uric acid	5,4	5,4	5,4	5,4	0,006	0,107
Acylcarnitine C12	35,6	35,6	35,6	35,6	0,025	0,071
Acylcarnitine C16	39,0	39,0	39,0	39,0	0,006	0,015

6.3.3 MeOH and NH_4COOCH_3

Table 21 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 10 mM NH_4COOCH_3 , analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	-	-	-	-	-	-
Creatinine	7,6	7,5	7,5	7,5	0,044	0,580
Creatine	5,3	5,2	5,2	5,2	0,029	0,553
Acylcarnitine C2	7,8	7,7	7,7	7,7	0,032	0,417
Uric acid	4,2	4,1	4,1	4,1	0,032	0,781
Acylcarnitine C12	47,9	47,9	47,9	47,9	0,015	0,032
Acylcarnitine C16	53,9	53,9	54,0	53,9	0,032	0,060

Table 22 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 10 mM NH_4COOCH_3 , analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	-	-	-	-	-	-
Creatinine	7,5	7,5	7,5	7,5	0,031	0,407
Creatine	5,2	5,2	5,2	5,2	0,026	0,508
Glucose	4,6	4,6	4,7	4,7	0,032	0,691
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	4,1	4,1	4,2	4,1	0,038	0,920
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

6.3.4 ACN and NH_4COOCH_3

Table 23 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 10 mM NH_4COOCH_3 , analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	-	-	-	-	-	-
Creatinine	6,0	6,1	6,0	6,0	0,026	0,439
Creatine	4,7	4,7	4,7	4,7	0,000	0,000
Acylcarnitine C2	6,0	6,0	6,0	6,0	0,000	0,000
Uric acid	3,4	3,4	3,4	3,4	0,006	0,169
Acylcarnitine C12	43,9	43,9	44,0	43,9	0,023	0,053
Acylcarnitine C16	46,0	46,0	46,0	46,0	0,036	0,078

Table 24 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 10 mM NH_4COOCH_3 , analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	-	-	-	-	-	-
Creatinine	6,0	6,0	6,0	6,0	0,000	0,000
Creatine	4,7	4,7	4,7	4,7	0,006	0,123
Glucose	4,4	4,4	4,4	4,4	0,000	0,000
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	3,5	3,4	3,5	3,4	0,006	0,168
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

6.4 The gradient elution profiles tested

Table 25 *The original gradient elution profile: time (min) and percentage organic solvent B.*

Time (min)	Solvent B (%)
0.00	5
10.00	5
50.00	100
55.00	100
55.10	5

Table 26 *The gradient 1 elution profile: time (min) and percentage organic solvent B.*

Time (min)	Solvent B (%)
0.00	5
30.00	10
35.00	70
40.00	90
55.00	100
55.10	5

Table 27 *The gradient 2 elution profile: time (min) and percentage organic solvent B.*

Time (min)	Solvent B (%)
0.00	5
20.00	5
20.00	70
25.00	70
25.00	80
30.00	80
35.00	90
40.00	100
55.00	100
55.10	5

Table 28 *The gradient 3 elution profile: time (min) and percentage organic solvent B.*

Time (min)	Solvent B (%)
0.00	2
10.00	5
20.00	5
20.00	70
25.00	70
25.00	80
30.00	80
35.00	90
40.00	100
55.00	100
55.10	2

Table 29 *The gradient 4 elution profile: time (min) and percentage organic solvent B.*

Time (min)	Solvent B (%)
0.00	2
30.00	10
35.00	70
40.00	90
55.00	100
55.10	2

Table 30 *The gradient 5 elution profile: time (min) and percentage organic solvent B.*

Time (min)	Solvent B (%)
0.00	2
20.00	10
25.00	70
50.00	100
55.00	100
55.10	2

Table 31 *The gradient 6 elution profile: time (min) and percentage organic solvent B.*

Time (min)	Solvent B (%)
0.00	2
12.00	10
17.00	75
50.00	100
55.00	100
55.10	2

6.5 Supplementary results for the gradient elution profiles tested with MeOH and FA

Table 32 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1 % FA and the original gradient elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,5	2,5	2,5	2,5	0,015	0,607
Creatinine	4,3	4,1	4,1	4,1	0,098	2,367
Creatine	4,8	4,6	4,6	4,7	0,091	1,944
Acylcarnitine C2	6,1	5,4	5,4	5,6	0,384	6,829
Uric acid	6,9	6,5	6,5	6,6	0,237	3,570
Acylcarnitine C12	42,9	43,0	43,0	42,9	0,021	0,048
Acylcarnitine C16	47,4	47,3	47,3	47,3	0,006	0,012

Table 33*The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the original gradient elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,5	2,5	2,5	0,021	0,828
Creatinine	4,1	4,1	4,1	4,1	0,015	0,372
Creatine	4,6	4,6	4,7	4,6	0,015	0,329
Glucose	4,6	4,6	4,7	4,6	0,015	0,329
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,5	6,5	6,5	6,5	0,012	0,177
Acylcarnitine C12	42,9	43,0	42,9	42,9	0,045	0,105
Acylcarnitine C16	47,3	47,3	47,3	47,3	0,021	0,044

Table 34 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 1 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,5	2,5	2,5	0,006	0,231
Creatinine	4,1	4,1	4,1	4,1	0,012	0,282
Creatine	4,6	4,6	4,6	4,6	0,021	0,451
Acylcarnitine C2	5,4	5,4	5,4	5,4	0,010	0,185
Uric acid	6,5	6,5	6,5	6,5	0,015	0,235
Acylcarnitine C12	44,0	44,0	44,0	44,0	0,015	0,035
Acylcarnitine C16	45,5	45,5	45,5	45,5	0,006	0,013

Table 35 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 1 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,5	2,5	2,5	0,031	1,220
Creatinine	4,1	4,1	4,1	4,1	0,025	0,616
Creatine	4,6	4,6	4,6	4,6	0,006	0,126
Glucose	4,6	4,6	4,6	4,6	0,006	0,126
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,5	6,5	6,5	6,5	0,035	0,534
Acylcarnitine C12	44,0	44,0	44,0	44,0	0,000	0,000
Acylcarnitine C16	45,5	-	45,7	45,6	0,106	0,233

Table 36 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 2 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,5	2,6	2,6	0,032	1,254
Creatinine	4,2	4,2	4,2	4,2	0,035	0,829
Creatine	4,6	4,6	4,7	4,6	0,032	0,695
Acylcarnitine C2	5,5	5,5	5,5	5,5	0,036	0,658
Uric acid	6,5	6,6	6,6	6,6	0,030	0,458
Acylcarnitine C12	31,2	31,3	31,2	31,2	0,010	0,032
Acylcarnitine C16	33,8	33,8	33,8	33,8	0,006	0,017

Table 37 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 2 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,6	2,6	2,6	0,056	2,158
Creatinine	4,2	4,2	4,2	4,2	0,006	0,139
Creatine	4,6	4,6	4,6	4,6	0,010	0,216
Glucose	4,7	4,6	4,6	4,6	0,012	0,249
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,6	6,5	6,5	6,5	0,025	0,386
Acylcarnitine C12	36,3	36,4	36,4	36,4	0,036	0,099
Acylcarnitine C16	-	-	-	-	-	-

Table 38 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 3 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,021	0,811
Creatinine	4,3	4,3	4,4	4,3	0,026	0,611
Creatine	4,8	4,7	4,8	4,8	0,036	0,756
Acylcarnitine C2	6,1	6,1	6,2	6,1	0,035	0,574
Uric acid	6,9	6,9	7,0	6,9	0,040	0,584
Acylcarnitine C12	31,2	31,2	31,2	31,2	0,031	0,098
Acylcarnitine C16	33,8	33,8	33,8	33,8	0,006	0,017

Table 39 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 3 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,5	2,6	2,6	0,020	0,784
Creatinine	4,3	4,3	4,3	4,3	0,015	0,355
Creatine	4,8	4,8	4,8	4,8	0,012	0,242
Glucose	4,6	4,7	4,7	4,6	0,006	0,124
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,9	6,9	6,9	6,9	0,032	0,465
Acylcarnitine C12	31,2	36,4	36,3	34,7	2,982	8,604
Acylcarnitine C16	33,8	-	-	-	-	-

Table 40 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 4 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,5	2,6	0,015	0,598
Creatinine	4,3	4,3	4,3	4,3	0,006	0,134
Creatine	4,8	4,8	4,7	4,8	0,010	0,211
Acylcarnitine C2	6,1	6,1	6,1	6,1	0,010	0,164
Uric acid	6,9	6,9	6,9	6,9	0,017	0,251
Acylcarnitine C12	44,0	44,0	44,0	44,0	0,015	0,035
Acylcarnitine C16	45,5	45,6	45,5	45,5	0,006	0,013

Table 41 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 4 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds		**n (1-3)**		**Average**	**SD**	**CV (%)**
		(t_R = min)		**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,7	2,7	2,6	2,6	0,078	2,935
Creatinine	4,4	4,3	4,3	4,3	0,032	0,745
Creatine	4,8	4,8	4,8	4,8	0,025	0,527
Glucose	4,7	4,6	4,7	4,7	0,026	0,568
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	7,0	6,9	6,9	6,9	0,050	0,729
Acylcarnitine C12	47,8	47,6	47,7	47,7	0,085	0,179
Acylcarnitine C16	-	-	-	-	-	-

Table 42 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 5 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,5	2,5	2,5	0,017	0,687
Creatinine	4,3	4,3	4,3	4,3	0,010	0,235
Creatine	4,8	4,8	4,8	4,8	0,000	0,000
Acylcarnitine C2	6,1	6,1	6,1	6,1	0,017	0,285
Uric acid	6,9	6,9	6,9	6,9	0,021	0,302
Acylcarnitine C12	34,1	34,2	34,1	34,1	0,021	0,061
Acylcarnitine C16	36,8	36,8	36,8	36,8	0,006	0,016

Table 43 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 5 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,5	2,5	2,5	0,015	0,608
Creatinine	4,2	4,2	4,2	4,2	0,006	0,136
Creatine	4,8	4,8	4,8	4,8	0,020	0,419
Glucose	4,6	4,6	4,6	4,6	0,006	0,125
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,9	6,9	6,9	6,9	0,006	0,084
Acylcarnitine C12	34,2	34,1	34,2	34,2	0,040	0,117
Acylcarnitine C16	-	-	-	-	-	-

Table 44 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,5	2,5	2,5	2,5	0,031	1,229
Creatinine	3,9	4,2	4,2	4,1	0,191	4,681
Creatine	4,4	4,7	4,8	4,6	0,197	4,239
Acylcarnitine C2	5,5	6,0	6,0	5,8	0,307	5,284
Uric acid	6,2	6,9	6,9	6,7	0,387	5,810
Acylcarnitine C12	25,6	25,6	25,6	25,6	0,026	0,103
Acylcarnitine C16	27,4	27,4	27,4	27,4	0,000	0,000

Table 45 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,5	2,5	2,5	2,5	0,032	1,282
Creatinine	4,2	4,2	4,2	4,2	0,017	0,414
Creatine	4,8	4,8	4,8	4,8	0,006	0,121
Glucose	4,6	4,7	4,6	4,6	0,012	0,249
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,9	6,9	6,9	6,9	0,025	0,364
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

6.6 Supplementary results for the gradient elution profiles tested with ACN and FA

Table 46 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the original gradient elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,6	2,6	2,6	2,6	0,012	0,450
Creatinine	4,0	4,1	4,0	4,0	0,006	0,143
Creatine	4,4	4,4	4,4	4,4	0,012	0,263
Acylcarnitine C2	5,0	5,0	5,0	5,0	0,015	0,305
Uric acid	5,5	5,5	5,5	5,5	0,006	0,105
Acylcarnitine C12	36,3	36,4	36,4	36,3	0,006	0,016
Acylcarnitine C16	40,0	40,0	40,0	40,0	0,025	0,063

Table 47 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the original gradient elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,5	2,6	2,5	0,006	0,227
Creatinine	4,0	4,1	4,1	4,1	0,023	0,569
Creatine	4,4	4,4	4,4	4,4	0,026	0,604
Glucose	4,5	4,5	4,5	4,5	0,010	0,222
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	5,5	5,5	5,5	5,5	0,000	0,000
Acylcarnitine C12	-	36,4	36,4	36,4	0,007	0,019
Acylcarnitine C16	-	-	-	-	-	-

Table 48 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 1 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,006	0,225
Creatinine	4,0	4,1	4,1	4,0	0,012	0,286
Creatine	4,4	4,4	4,4	4,4	0,006	0,131
Acylcarnitine C2	5,0	5,0	5,0	5,0	0,012	0,231
Uric acid	5,5	5,5	5,5	5,5	0,012	0,209
Acylcarnitine C12	42,1	42,1	42,1	42,1	0,010	0,024
Acylcarnitine C16	42,9	42,9	42,9	42,9	0,010	0,023

Table 49 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 1 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,5	2,6	0,031	1,196
Creatinine	4,1	4,0	4,1	4,1	0,012	0,285
Creatine	4,4	4,4	4,4	4,4	0,006	0,132
Glucose	4,5	4,5	4,5	4,5	0,006	0,128
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	5,6	5,5	5,5	5,5	0,025	0,455
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

Table 50 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 2 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,006	0,224
Creatinine	4,1	4,1	4,1	4,1	0,006	0,142
Creatine	4,4	4,4	4,4	4,4	0,010	0,227
Acylcarnitine C2	5,1	5,0	5,0	5,0	0,012	0,229
Uric acid	5,5	5,5	5,5	5,5	0,017	0,314
Acylcarnitine C12	29,4	29,4	29,4	29,4	0,015	0,052
Acylcarnitine C16	29,9	29,9	29,9	29,9	0,015	0,051

Table 51 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 2 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,5	2,6	2,6	0,021	0,814
Creatinine	4,1	4,0	4,1	4,1	0,012	0,285
Creatine	4,4	4,4	4,4	4,4	0,017	0,396
Glucose	4,5	4,5	4,5	4,5	0,006	0,128
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	5,5	5,5	5,5	5,5	0,010	0,181
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

Table 52 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 3 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,010	0,389
Creatinine	4,2	4,2	4,2	4,2	0,006	0,136
Creatine	4,6	4,6	4,6	4,6	0,000	0,000
Acylcarnitine C2	5,7	5,7	5,7	5,7	0,012	0,203
Uric acid	6,1	6,1	6,2	6,2	0,017	0,282
Acylcarnitine C12	29,4	29,4	29,4	29,4	0,006	0,020
Acylcarnitine C16	29,9	29,9	29,9	29,9	0,006	0,019

Table 53 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 3 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,020	0,772
Creatinine	4,2	4,2	4,3	4,2	0,015	0,361
Creatine	4,6	4,6	4,6	4,6	0,010	0,218
Glucose	4,6	4,6	4,6	4,6	0,006	0,126
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,2	6,2	6,2	6,2	0,006	0,094
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

Table 54 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 4 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,012	0,449
Creatinine	4,2	4,2	4,2	4,2	0,006	0,136
Creatine	4,6	4,6	4,6	4,6	0,006	0,126
Acylcarnitine C2	5,7	5,7	5,7	5,7	0,010	0,176
Uric acid	6,2	6,2	6,2	6,2	0,012	0,187
Acylcarnitine C12	42,1	42,1	42,1	42,1	0,015	0,036
Acylcarnitine C16	43,0	43,0	43,0	43,0	0,021	0,048

Table 55 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 4 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,025	0,978
Creatinine	4,2	4,2	4,2	4,2	0,010	0,236
Creatine	4,6	4,6	4,6	4,6	0,012	0,251
Glucose	4,5	4,6	4,6	4,6	0,021	0,456
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,2	6,2	6,2	6,2	0,010	0,162
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

Table 56 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 5 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,006	0,224
Creatinine	4,2	4,2	4,2	4,2	0,010	0,236
Creatine	4,6	4,6	4,6	4,6	0,006	0,126
Acylcarnitine C2	5,7	5,7	5,7	5,7	0,023	0,406
Uric acid	6,2	6,2	6,2	6,2	0,006	0,094
Acylcarnitine C12	32,1	32,1	32,1	32,1	0,029	0,090
Acylcarnitine C16	33,0	33,0	33,1	33,0	0,006	0,017

Table 57 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 5 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,025	0,978
Creatinine	4,2	4,3	4,3	4,2	0,006	0,136
Creatine	4,6	4,6	4,6	4,6	0,026	0,575
Glucose	4,5	4,6	4,6	4,5	0,015	0,336
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,2	6,2	6,2	6,2	0,012	0,187
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

Table 58 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average t_R, SD and CV%.*

Compounds	**n (1-3)**			**Average**	**SD**	**CV (%)**
	(t_R = min)			**(t_R = min)**		
	1	**2**	**3**			
Tobramycin	2,6	2,6	2,6	2,6	0,006	0,223
Creatinine	4,2	4,2	4,2	4,2	0,010	0,236
Creatine	4,6	4,6	4,6	4,6	0,025	0,549
Acylcarnitine C2	5,7	5,7	5,7	5,7	0,021	0,364
Uric acid	6,1	6,2	6,1	6,1	0,032	0,525
Acylcarnitine C12	23,8	23,7	23,8	23,8	0,020	0,084
Acylcarnitine C16	24,5	24,5	24,5	24,5	0,006	0,024

Table 59 *The t_R on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a MP composition of ACN + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average t_R, SD and CV%.*

Compounds	n (1-3)			Average	SD	CV (%)
	(t_R = min)			(t_R = min)		
	1	2	3			
Tobramycin	2,6	2,6	2,6	2,6	0,015	0,591
Creatinine	4,2	4,3	4,2	4,2	0,015	0,361
Creatine	4,6	4,6	4,6	4,6	0,010	0,219
Glucose	4,5	4,6	4,6	4,6	0,020	0,439
Acylcarnitine C2	-	-	-	-	-	-
Uric acid	6,2	6,2	6,1	6,1	0,006	0,094
Acylcarnitine C12	-	-	-	-	-	-
Acylcarnitine C16	-	-	-	-	-	-

6.7 Supplementary results for the injection volume testing

6.7.1 Peak width (w)

Table 61 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with an injection volume of 2 µL, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min), P_C and average P_C.*

Compounds	n (1-3)			Average
	w (min)			(t_R = min)
	1	2	3	
Tobramycin	0,15	0,15	0,15	2,54
Creatinine	0,38	0,32	0,34	4,28
Creatine	0,35	0,35	0,34	4,75
Acylcarnitine C2	0,52	0,45	0,49	6,87
Uric acid	0,64	0,63	0,67	17,92
Acylcarnitine C12	0,25	0,27	0,28	25,61
Acylcarnitine C16	0,26	0,29	0,29	27,43
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	148	153	147	
Average P_C	149			

Table 62 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with an injection volume of 2 µL, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	-	-	-	-
Creatinine	0,25	0,25	0,25	4,28
Creatine	0,52	0,55	0,55	4,78
Glucose	0,35	0,35	0,36	4,62
Acylcarnitine C2	-	-	-	-
Uric acid	0,49	0,48	0,47	6,87
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	4	4	4	
Gradient elution time (min)	55	55	55	
P_C	138	136	136	
Average P_C	137			

Table 63 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with an injection volume of 10 µL, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,31	0,3	0,3	2,58
Creatinine	0,42	0,41	0,43	4,44
Creatine	0,45	0,44	0,5	4,84
Acylcarnitine C2	0,62	0,61	0,61	6,87
Uric acid	0,66	0,72	0,65	17,75
Acylcarnitine C12	0,34	0,33	0,32	25,67
Acylcarnitine C16	0,36	0,34	0,35	27,40
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	120	121	121	
Average P_C	121			

Table 64 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with an injection volume of 10 µL, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	-	-	-	
Creatinine	0,29	0,27	0,24	4,40
Creatine	0,67	0,66	0,61	4,87
Glucose	0,58	0,56	0,53	4,68
Acylcarnitine C2	-	-	-	-
Uric acid	0,68	0,67	1,25	6,88
Acylcarnitine C12	0,24	0,18	0,19	25,64
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	113	119	99	
Average P_C	110			

Table 65 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with an injection volume of 20 µL, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,38	0,38	0,38	2,64
Creatinine	0,38	0,38	0,39	4,53
Creatine	0,5	0,47	0,43	4,94
Acylcarnitine C2	0,64	0,63	0,69	6,86
Uric acid	0,64	0,62	0,62	17,71
Acylcarnitine C12	0,24	0,24	0,23	25,63
Acylcarnitine C16	0,25	0,27	0,25	27,36
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	129	130	129	
Average P_C	129			

Table 66 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with an injection volume of 20 µL, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	-	-	-	-
Creatinine	0,37	0,4	0,37	4,49
Creatine	0,57	0,59	0,59	4,95
Glucose	0,6	0,62	0,64	4,71
Acylcarnitine C2	-	-	-	-
Uric acid	0,81	0,8	0,8	6,92
Acylcarnitine C12	0,2	0,22	0,18	25,61
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	109	106	108	
Average P_C	107			

6.7.2 Peak capacity (P_C)

Table 67 P_C *for three replicates (n = 3) of the injection volumes 2, 10 and 20 µL analyzed in positive ionization mode with their average* P_C*, SD and CV%.*

	P_C		
	w (min)		
n (1-3)	**2**	**10**	**20**
1	148	120	129
2	153	121	130
3	147	121	129
Average P_C	149	121	129
SD	3.1	0.3	1.0
CV (%)	2.1	0.3	0.8

Table 68 *P_C for three replicates (n = 3) of the injection volumes 2, 10 and 20 µL analyzed negative ionization mode with their average P_C, SD and CV%.*

	P_C		
	w (min)		
n (1-3)	**2**	**10**	**20**
1	138	114	109
2	149	119	106
3	136	99	108
Average P_C	141	110	107
SD	6.9	10.4	1.7
CV (%)	4.9	9.5	1.5

6.8 Supplementary results for the column temperature testing

6.8.1 Peak width (w) - experiment 1

Table 69 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 30 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min), P_C and average P_C.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,14	0,14	0,15	2,49
Creatinine	0,53	0,5	0,51	4,16
Creatine	0,55	0,53	0,53	4,76
Acylcarnitine C2	0,67	0,67	0,65	6,85
Uric acid	0,64	0,64	0,65	17,18
Acylcarnitine C12	0,44	0,44	0,44	25,56
Acylcarnitine C16	0,45	0,45	0,44	27,51
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	109	112	112	
Average P_C	111			

Table 70 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 30 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,24	0,24	0,17	2,48
Creatinine	0,33	0,33	0,33	4,17
Creatine	0,63	0,66	0,65	4,77
Glucose	0,57	0,57	0,59	4,62
Acylcarnitine C2	-	-	-	-
Uric acid	0,72	0,74	0,73	6,87
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	111	109	112	
Average P_C	111			

Table 71 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 40 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds		**n (1-3)**		**Average**
		w (min)		**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,16	0,16	0,14	2,48
Creatinine	0,5	0,51	0,48	4,04
Creatine	0,55	0,54	0,54	4,65
Acylcarnitine C2	0,65	0,66	0,64	6,36
Uric acid	0,62	0,65	0,61	15,07
Acylcarnitine C12	0,4	0,41	0,38	25,40
Acylcarnitine C16	0,33	0,3	0,36	26,90
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
PC	116	115	117	
Average PC	116			

Table 72 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 40 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,3	0,18	0,18	2,45
Creatinine	0,43	0,42	0,4	4,05
Creatine	0,63	0,64	0,66	4,67
Glucose	0,69	0,68	0,64	4,61
Acylcarnitine C2	-	-	-	-
Uric acid	0,64	0,68	0,66	6,36
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	103	107	109	
Average P_C	106			

Table 73 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 50 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,12	0,12	0,11	2,52
Creatinine	0,46	0,48	0,47	4,09
Creatine	0,56	0,54	0,55	4,57
Acylcarnitine C2	0,59	0,64	0,65	5,97
Uric acid	0,62	0,62	0,63	13,61
Acylcarnitine C12	0,33	0,33	0,32	25,04
Acylcarnitine C16	0,38	0,38	0,37	26,34
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	127	12	124	
Average P_C	126			

Table 74 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 50 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,25	0,26	0,29	2,53
Creatinine	0,28	0,38	0,38	4,10
Creatine	0,54	0,62	0,64	4,54
Glucose	0,59	0,68	0,68	4,62
Acylcarnitine C2	-	-	-	-
Uric acid	0,69	0,66	0,67	5,94
Acylcarnitine C12	-	-	-	
Acylcarnitine C16	-	-	-	
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	118	107	104	
Average P_C	110			

Table 75 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 55 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	-	-	-	-
Creatinine	0,48	0,5	0,51	4,52
Creatine	0,4	0,38	0,42	4,63
Acylcarnitine C2	0,67	0,57	0,55	5,78
Uric acid	0,69	0,61	0,57	13,88
Acylcarnitine C12	0,13	0,14	0,14	25,30
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	112	124	126	
Average P_C	121			

Table 76 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 55 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin				
Creatinine	0,28	0,23	0,18	4,46
Creatine	0,49	0,47	0,48	4,65
Glucose	0,49	0,5	0,53	4,59
Acylcarnitine C2				
Uric acid	0,63	0,61	0,63	5,77
Acylcarnitine C12				
Acylcarnitine C16				
n (number of peaks)	4	4	4	
Gradient elution time (min)	55	55	55	
P_C	117	123	122	
Average P_C	121			

6.8.2 Peak width (w) - experiment 2

Table 77 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 30 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,14	0,15	0,17	2,52
Creatinine	0,48	0,47	0,47	4,28
Creatine	0,55	0,55	0,55	4,74
Acylcarnitine C2	0,61	0,6	0,61	6,76
Uric acid	0,68	0,68	0,69	17,27
Acylcarnitine C12	0,52	0,57	0,57	25,59
Acylcarnitine C16	0,62	0,56	0,54	27,45
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	105	105	104	
Average P_C	104			

Table 78 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 30 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,18	0,21	0,25	2,48
Creatinine	0,25	0,25	0,27	4,26
Creatine	0,66	0,69	0,66	4,75
Glucose	0,59	0,6	0,59	4,60
Acylcarnitine C2	-	-	-	
Uric acid	0,67	0,71	0,68	6,77
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	118	113	113	
Average P_C	115			

Table 79 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 40 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,15	0,16	0,16	2,50
Creatinine	0,49	0,48	0,47	4,14
Creatine	0,55	0,52	0,53	4,63
Acylcarnitine C2	0,62	0,6	0,6	6,28
Uric acid	0,67	0,65	0,63	15,15
Acylcarnitine C12	0,5	0,54	0,5	25,29
Acylcarnitine C16	0,29	0,29	0,25	26,87
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	119	119	125	
Average P_C	121			

Table 80 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 40 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,24	0,26	0,28	2,49
Creatinine	0,32	0,3	0,31	4,15
Creatine	0,62	0,65	0,67	4,63
Glucose	0,64	0,68	0,68	4,57
Acylcarnitine C2	-	-	-	-
Uric acid	0,65	0,63	0,63	6,29
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	112	110	108	
Average P_C	110			

Table 81 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 50 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,12	0,12	0,15	2,51
Creatinine	0,46	0,47	0,44	4,04
Creatine	0,54	0,52	0,52	4,55
Acylcarnitine C2	0,61	0,62	0,61	5,94
Uric acid	0,66	0,6	0,63	13,28
Acylcarnitine C12	0,43	0,43	0,39	25,03
Acylcarnitine C16	-	-	-	-
n (number of peaks)	6	6	6	
Gradient elution time (min)	55	55	55	
P_C	123	126	126	
Average P_C	125			

Table 82 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 50 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,25	0,26	0,28	2,47
Creatinine	0,32	0,32	0,34	4,05
Creatine	0,62	0,65	0,63	4,55
Glucose	0,69	0,71	0,7	4,58
Acylcarnitine C2	-	-	-	-
Uric acid	0,6	0,65	0,64	5,92
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	112	107	107	
Average P_C	109			

Table 83 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 55 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,12	0,13	0,14	2,49
Creatinine	0,46	0,44	0,46	4,00
Creatine	0,55	0,52	0,52	4,51
Acylcarnitine C2	0,53	0,56	0,59	5,76
Uric acid	0,62	0,64	0,66	12,45
Acylcarnitine C12	0,34	0,34	0,36	24,89
Acylcarnitine C16	-	-	-	-
n (number of peaks)	6	6	6	
Gradient elution time (min)	55	55	55	
P_C	131	131	128	
Average P_C	130			

Table 84 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a temperature at 55 °C, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,19	0,18	0,21	2,48
Creatinine	0,35	0,34	0,35	4,00
Creatine	0,64	0,59	0,58	4,49
Glucose	0,65	0,66	0,68	4,58
Acylcarnitine C2	-	-	-	-
Uric acid	0,62	0,62	0,6	5,76
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	113	116	115	
Average P_C	115			

6.8.3 Peak capacity (P_C)

Table 85 P_C *for three replicates (n = 3) of the temperatures 30, 40, 50 and 55 ˚C from experiment 1, analyzed in positive ionization mode with their average* P_C*, SD and CV%.*

	P_C			
	Temperature (C˚)			
n (1-3)	**30**	**40**	**50**	**55**
1	109	116	127	113
2	112	115	127	124
3	110	117	124	126
Average P_C	110	116	126	121
SD	1.3	1.1	1.9	7.2
CV (%)	1.2	0.9	1.5	5.9

Table 86 *P_C for three replicates (n = 3) of the temperatures 30, 40, 50 and 55 ˚C from experiment 2, analyzed in positive ionization mode with their average P_C, SD and CV%.*

	P_C			
	Temperature (C˚)			
n (1-3)	**30**	**40**	**50**	**55**
1	105	120	123	131
2	105	119	126	131
3	104	125	126	128
Average P_C	104	121	125	129
SD	0.6	3.1	1.5	3.6
CV (%)	0.6	2.5	1.2	2.8

6.9 Supplementary results for the flow rate testing

6.9.1 Peak width (w)

Table 87 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a flow rate at 50 µL/min, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min), P_C and average P_C.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,92	0,91	0,92	6,71
Creatinine	1,04	1,05	1,06	9,70
Creatine	2,7	2,59	2,53	10,63
Acylcarnitine C2	2	1,99	2,05	14,27
Uric acid	1,52	1,44	1,5	13,36
Acylcarnitine C12	0,96	0,96	0,96	55,83
Acylcarnitine C16	0,92	0,9	0,87	60,47
n (number of peaks)	7	7	7	
Gradient elution time (min)	91,7	91,7	91,7	
P_C	67	68	67	
Average P_C	67			

Table 88 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a flow rate at 50 µL/min, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n, gradient elution time (min),* P_C *and average* P_C*.*

Compounds	**n (1-3)**			**Average**
	w (min)			**(t_R = min)**
	1	**2**	**3**	
Tobramycin	-	-	-	-
Creatinine	0,85	0,77	0,82	9,79
Creatine	2,88	3,08	2,82	11,29
Glucose	1,96	2,04	2,06	13,20
Acylcarnitine C2	-	-	-	-
Uric acid	2	1,99	2,06	13,98
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	4	4	4	
Gradient elution time (min)	91,7	91,7	91,7	
P_C	49	48	48	
Average P_C	48			

Table 89 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a flow rate at 150 µL/min, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds		**n (1-3)**		**Average**
		w (min)		**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,16	0,17	0,15	2,49
Creatinine	0,47	0,49	0,48	4,21
Creatine	0,57	0,58	0,5	4,75
Acylcarnitine C2	0,62	0,63	0,64	6,75
Uric acid	0,7	0,67	0,7	17,02
Acylcarnitine C12	0,54	0,52	0,5	25,52
Acylcarnitine C16	0,51	0,49	0,55	27,43
n (number of peaks)	7	7	7	
Gradient elution time (min)	55	55	55	
P_C	105	106	106	
Average P_C	106			

Table 90 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a flow rate at 150 µL/min, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds		**n (1-3)**		**Average**
		w (min)		**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,24	0,23	0,26	2,47
Creatinine	0,29	0,29	0,29	4,21
Creatine	0,69	0,68	0,7	4,75
Glucose	0,58	0,58	0,57	4,60
Acylcarnitine C2	-	-	-	-
Uric acid	0,72	0,7	0,68	6,76
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	5	5	5	
Gradient elution time (min)	55	55	55	
P_C	110	112	111	
Average P_C	111			

Table 91 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a flow rate at 300 µL/min, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in positive ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds		**n (1-3)**		**Average**
		w (min)		**(t_R = min)**
	1	**2**	**3**	
Tobramycin	0,07	0,1	0,09	1,24
Creatinine	0,23	0,23	0,23	2,13
Creatine	0,29	0,27	0,32	2,39
Acylcarnitine C2	0,29	0,3	0,3	3,44
Uric acid	0,39	0,38	0,37	8,63
Acylcarnitine C12	0,32	0,36	0,33	12,74
Acylcarnitine C16	0,3	0,26	0,33	13,71
n (number of peaks)	7	7	7	
Gradient elution time (min)	27,5	27,5	27,5	
P_C	102	101	97	
Average P_C	100			

Table 92 *The w (min) on the C18-Diphenyl column for three replicates (n = 3) of the compounds in spiked DBS with a flow rate at 300 µL/min, MP composition of MeOH + 0.1% FA and the gradient 6 elution profile, analyzed in negative ionization mode with their average w (min), n (number of peaks), gradient elution time (min),* P_C *and average* P_C*.*

Compounds		**n (1-3)**		**Average**
		w (min)		**(t_R = min)**
	1	**2**	**3**	
Tobramycin	-	-	-	-
Creatinine	0,13	0,13	0,14	2,14
Creatine	0,4	0,39	0,39	2,41
Glucose	0,26	0,23	0,23	2,31
Acylcarnitine C2	-	-	-	-
Uric acid	0,39	0,39	0,39	3,44
Acylcarnitine C12	-	-	-	-
Acylcarnitine C16	-	-	-	-
n (number of peaks)	4	4	4	
Gradient elution time (min)	27,5	27,5	27,5	
P_C	94	97	97	
Average P_C	96			

6.9.2 Peak capacity (P_C)

Table 93 *P_C for three replicates (n = 3) of the flow rates 50, 150 and 300 µL/min with their average P_C, SD and CV%, analyzed in positive ionization mode.*

	P_C		
	Flow rate (µL/min)		
n (1-3)	**50**	**150**	**300**
1	67	105	102
2	68	106	101
3	67	106	97
Average P_C	67	106	100
SD	0.5	0.4	3.0
CV (%)	0.8	0.4	3.0

Table 94 *PC for three replicates (n = 3) of the flow rates 50, 150 and 300 µL/min with their average PC, SD and CV%, analyzed in negative ionization mode.*

	P_C		
	Flow rate (µL/min)		
n (1-3)	**50**	**150**	**300**
1	49	110	94
2	48	111	98
3	48	111	97
Average P_C	48	111	96
SD	0.6	0.9	1.7
CV (%)	1.2	0.8	1.8

6.9.3 Example of calculating peak capacity using another equation

P_C was calculated using Equation 8 (see **Introduction**, section 2.7.1)

The average P_C at three replicates (n = 3) for each of the flow rates 50, 150 and 300 µL/min calculated with Equation 6, analyzed in positive ionization mode are shown in Figure 38. The P_C for the three replicates (n = 3), average P_C, SD and CV% are given in Table 95.

Table 95 *P_C for three replicates (n = 3) of the flow rates 50, 150 and 300 µL/min with their average P_C, SD and CV%, analyzed in positive ionization mode.*

	P_C		
	Flow rate (µL/min)		
n (1-3)	**50**	**150**	**300**
1	38	47	46
2	39	48	45
3	39	48	43
Average P_C	39	47	45
SD	0.3	0.2	1.3
CV (%)	0.9	0.4	2.9

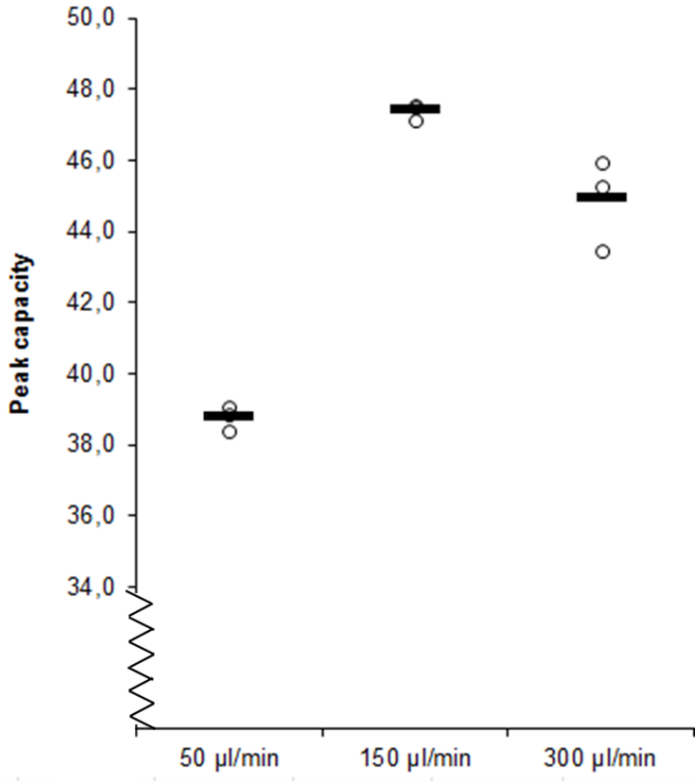

Figure 29 *Average P_C (thick line) at three replicates (n = 3) for each of the flow rates 50, 150 and 300 µL/min analyzed with spiked DBS with positive ionization mode.*