Laboratory diagnostics: Maximizing sensitivity of a Q-Exactive Orbitrap mass spectrometer for untargeted metabolomics of dried blood spots

Hanne Bendiksen Skogvold

Thesis for the Master's Degree in chemistry

60 credits

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Abstract

Biological samples consist of an unknown, but large, number of regulated metabolites. It is of great interest scientifically and clinically to determine the composition and dynamics of this "metabolome". Metabolomics is the systematic study of the metabolome and requires the development of reliable and sensitive methods. High resolution mass spectrometry is widely used, often in combination with liquid chromatography. It is essential to optimize instrumental parameters for each specific application. In this project, mass spectrometric parameters were optimized in order to detect as many compounds as possible and maximize signal intensity in dried blood spots, a sample material used in newborn screening and increasingly used in laboratory diagnostics. The following parameters were optimized on a Q Exactive Orbitrap mass spectrometer: voltage applied to the spray liquid in the ionization source (electrospray voltage), distance between the electrospray needle and the MS inlet (electrospray needle position), mass resolving power of the mass spectrometer (resolution), automatic gain control target value (which controls the number of ions to be injected into the Orbitrap), and mobile phase flow rate. The following values were chosen for each parameter: electrospray voltage 3.5 kV, electrospray needle position C, resolution 70 000, and automatic gain control target value 1 000 000 ion counts. Mobile phase flow rate was increased from 150 to 300 µL/min, leading to a 50 % reduction of the initial analysis time, without compromising sensitivity.

In certain cases, the optimal values derived for parameters differed unexpectedly from theoretical expectations. This emphasizes the importance of thorough parameter optimization during method development. Using a suboptimal value will decrease the number of detected compounds. This may have serious consequences, especially if compounds which are not detected are clinically relevant. The present work indicates that if extensive optimization of parameters is done during method development, diagnostic opportunities will improve.

Preface

The present work has been performed at the National Unit for Screening and Diagnosis of Congenital Pediatric Metabolic Disorders at Oslo University Hospital, Rikshospitalet, from August 2015 to May 2017.
My supervisors have been Katja B. P. Elgstøen and Anja Østeby at Rikshospitalet, and my supervisor at the Department of Chemistry at the University of Oslo has been Steven R. H. Wilson.

First of all, I would like to thank Katja for giving me the opportunity to work on such an interesting project. Katja is a big role model for me, and I am extremely grateful for her positive attitude and her ability to always think on the spot and provide smart solutions to any challenge.
Anja has been a fantastic mentor, and her positive attitude and wise comments have helped me solve many of the problems we faced during these years. I really appreciate all our long talks in the office.
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Thank you to Alexander Rowe for taking the time to proofread my thesis and helping me with the graphs. Henning Cederkvist also deserves a thank you for all the valuable input he provided. Ingjerd Sæves has been very helpful answering both big and small questions – thank you for that.
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I would like to thank Norsk Kjemisk Selskap - Faggruppe for Analytisk Kjemi for giving me the opportunity to attend «Det 16. norske seminar i massespektrometri» at Hafjell. I presented my work with a poster, which is shown in the Appendix.

Thank you to Per Ola Rønning at Høgskolen i Oslo og Akershus for all your help throughout these years.
I would also like to thank my fellow student Camilla Elene Arnesen. I greatly value our friendship, and your ability to always make my mood shift when I was having a bad day.

Thank you to all my friends for your support and love.

Last, but not least, I want to thank my family: my father for the valuable input he provided and support, my mother for always believing in me, and my amazing sister for being my biggest supporter. An extra thank you to my family for proofreading my thesis! Even B. B. deserves a huge thank you for all his support.

Oslo, Norway, May 2017

Hanne Bendiksen Skogvold

Table of Content

1 Abbreviations and Definitions

1.1 Abbreviations

Abbreviation	Term
AGC	Automatic gain control
C18	Octadecyl
DBS	Dried blood spot
d	Distance between the tip of the electrospray capillary and the counter electrode
Da	Dalton
DC	Direct current
DNA	Deoxyribonucleic acid
E_c	Electric field
EDTA	Ethylenediaminetetraacetic acid
EIC	Extracted ion chromatogram
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ESI-MS	Electrospray ionization-mass spectrometry
FT-ICR	Fourier transform ion cyclotron resonance
FWHM	Full width at half maximum
GC-MS	Gas chromatography-mass spectrometry
HCD	Higher energy collision-induced dissociation
HESI	Heated electrospray ionization
hGH	Human growth hormone
HMDB	Human metabolome database
HPLC	High-performance liquid chromatography
ID	Inner diameter
IEM	Inborn error of metabolism
K	Conductivity of solution
LC	Liquid chromatography

LC-MS	Liquid chromatography-mass spectrometry
LIT	Linear ion trap
LIT/FT-ICR	Linear ion trap-Fourier transform ion cyclotron resonance
m/z	Mass-to-charge ratio
Max IT	Maximum injection time
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NBS	Newborn screening
NMR	Nuclear magnetic resonance
NL	Normalized intensity level
PKU	Phenylketonuria
ppm	Parts per million
Q-TOF	Quadrupole-Time-of-Flight
QqQ	Triple quadrupole
R	Electrospray droplet radius
R_s	Resolution
r_c	Outer radius of electrospray capillary
RF	Radio frequency
RSD	Relative standard deviation
RT	Retention time
TIC	Total ion chromatogram
TOF	Time-of-Flight
UHPLC	Ultra high-performance liquid chromatography
UV	Ultraviolet
V_c	Applied potential/electrospray voltage
V_f	Flow rate (volume/time)
VUS	Variants of uncertain significance
$w_{0.5}$	Peak width at half height
ε	Permittivity of solvent

1.2 Definitions

Term	Definition
Automatic gain control	Controls the number of ions to be stored together in the C-Trap, before being injected into the Orbitrap mass analyzer for detection
Biomarker	A compound that indicates if a disease is present or not
Electrospray ionization	An ionization technique in which a liquid sample is turned into a spray of small droplets which evaporate, releasing gas-phase ions into the MS
Electrospray needle position	The electrospray needle position sets the distance between the electrospray needle and the MS inlet
Electrospray voltage	The voltage applied to the spray liquid in the ionization source as it emerges from the electrospray needle
HPLC	A technique used to separate compounds in a solution
Inborn error of metabolism	An inherited disease characterized by an absent or markedly reduced ability to break down or synthesize one or several specific compounds
Log p	The log p value for a compound is used to describe the compounds' hydrophobicity. It is defined as the ratio of distribution of the compound between an organic phase and an aqueous phase at equilibrium
Mass accuracy	Closeness of accurate *m/z* and exact *m/z*
Mass spectrometry	A technique for identifying compounds, and mass and structure determination
Metabolites	Compounds that participate in or are the end products in the metabolism
Metabolome	The composition of metabolites in a sample/organism
Metabolomics	The systematic study of the metabolome
MS/MS (or "Tandem MS")	Mass spectrometry analysis consisting of mass filtering *and* fragmentation
Resolving power	The resolving power of a mass spectrometer describes how well the mass spectrometer separates peaks with similar masses

Sensitivity	This term can be used to describe the ability of a mass spectrometer to detect low-concentration analytes in a sample
Untargeted metabolomics	Analyzing a large set of metabolites (known or unknown), with the purpose of detecting (and sometimes quantifying) as many metabolites as possible

2 Introduction

2.1 Laboratory Diagnostics

Laboratory diagnostics involves measuring concentrations, or detecting the presence, of biomarkers. Most medical biochemistry analyses are quantitative measurements, i.e. measuring concentrations of compounds [1]. Some analyses are qualitative, meaning that the purpose of the analysis is to see whether the biomarker is present with or without an estimation of approximate or relative amounts. Qualitative analyses are commonly used when the biomarker is pathognomonic. Presence of a pathognomonic compound means that a particular disease is present beyond any doubt. An example of a pathognomonic biomarker is succinylacetone [2]. If succinylacetone is detected in a patient sample, the person is extremely likely to have the disease Tyrosinemia type 1. Many biomarkers are present in clinically healthy people as well as in people with diseases, but in lower or sometimes higher concentrations [1].

The concept of "chemical individuality" was introduced by Sir Archibald Garrod in the early 20th century [3]. Many factors can affect levels of compounds present in the body, including genetic factors, age, gender, diet, physical activity, and weight [1]. Laboratory analysts need to be able to identify pathology, despite this individual variability. To identify pathology, the best comparison may be with the patient him/herself, from a time when he/she was considered clinically healthy, if these samples are available. Since this information is often not available, laboratory results generally have to be compared to what is considered normal in a healthy population. The concept of a "reference range" is often used in this context. The reference range is the range of analysis results of samples from a healthy population, usually excluding the upper and lower 2.5 %. Results from patient samples are then compared to reference ranges. However, it is important that the people these samples are taken from are comparable [1]. A generic reference range is illustrated in **Figure 1**, showing that there is usually an overlap between the "healthy (reference) range" and the "disease range". This can complicate the diagnostics.

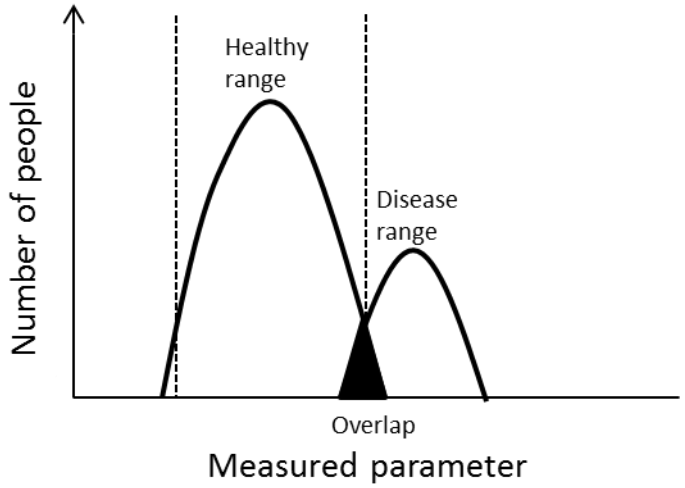


Figure 1 Illustration of reference range (dotted lines). There is usually an overlap between the healthy (reference) range and the disease range distributions.

2.1.1 Newborn Screening

The purpose of newborn screening (NBS) is to detect treatable diseases as early as possible, minimizing mortality and disabilities [4, 5]. Many newborns appear healthy, although they can have a serious disorder. A dried blood spot (DBS) sample from the newborn is screened for abnormally high or low concentrations of specific metabolites using mass spectrometry (see below), immunoassays and measurement of enzyme activity. Small quantities of blood are needed, and the analyses are automated and very fast. Additionally, a large number of compounds can be analyzed simultaneously. There are two main groups of analytes that are measured using mass spectrometry in NBS - amino acids and acylcarnitines [4].

NBS was developed in the 1960s in the USA by Dr. Robert Guthrie, with screening for phenylketonuria (PKU) [4-6]. The incidence of certain diseases can vary in different parts of the world, which affects the selection of diseases to be included in the NBS programs. In Norway, the DBS sample is taken 48-72 hours after the baby is born, and the samples are sent to the Department of Newborn Screening at Rikshospitalet [7]. The DBS samples are screened for 23 diseases, of which 21 are inborn errors of metabolism (IEMs).

2.1.2 Inborn Errors of Metabolism

The aforementioned Sir Archibald Garrod linked metabolism to disease, thereby becoming the first person to recognize the disease group of inborn errors of metabolism [3, 8]. An IEM is an inherited disease characterized by the inability to break down or synthesize one or several specific compounds [9, 10]. Thousands of vital reactions occur continuously in living cells. Many of these are dependent on the catalytic effect of a specific enzyme to be able to occur fast enough under physiological conditions. IEMs are commonly caused by mutations in the relevant gene, or in regions controlling the expression of the relevant gene. This typically leads to a failure to produce an important protein (usually an enzyme) or the production of dysfunctional or unstable protein (enzyme). The affected protein can also be a transport protein. Protein malfunction can lead to abnormalities in the metabolism, thereby causing diseases. This can happen because toxic compounds accumulate, or because a vital compound is not produced [9].

Today, about 1500 different IEMs have been described [9]. Although each individual disease is rare, IEMs as a group are more common and the patients are relatively numerous [8]. IEMs can cause cancer, seizures, mental retardation, blindness, many other disabilities, and in the severest cases, death within hours of birth. Many symptoms can be avoided by dietary restriction. An example is the treatment of PKU with a low-phenylalanine formula. Maintaining a strict diet prevents an otherwise inevitable progression of mental retardation [4].

2.1.2.1 Diagnostics of IEMs

Due to the broad spectrum of clinical symptoms, diagnostics of IEMs can be challenging. Clinical observations and information about the patient are important when narrowing down the list of possible diseases [10]. Diagnostics can happen on four different levels: clinical, metabolite, gene product and gene [9]. Diagnostics on a clinical level depend on the fact that many diseases have clinical presentations representing whole organs which have been affected – as well as more unique features such as a characteristic smell that points directly to a specific diagnosis. However, most IEMs do not have symptoms and signs pathognomonic for a specific disease. The clinical examination needs to be supplemented by other analyses. Imaging techniques like magnetic resonance imaging (MRI) may be used to determine whether the brain displays neurological abnormalities. An example of diagnostics on the

metabolite level is the analysis of acid/base status. Metabolic acidosis is either caused by accumulation of an acid, or by lack of a base. Diagnostics at the gene product level can be used when a specific disease is suspected. The activity of the enzyme that causes the disease can be measured. At the gene level deoxyribonucleic acid (DNA) sequencing can be scaled from single gene variants up to the entire genome [9].

There is always a risk of missing out on important information when specific and targeted techniques are used. ELISA (Enzyme-linked immunosorbent assay) is a widely used technique in immunology, but does not provide any information about enzyme activity. Also, ELISA and western blotting (used in proteomics) cannot provide information about thousands of compounds at the same time [11, 12].

Genetic testing is important for diagnostics of many diseases, providing information about the genotype of a patient. The genotype describes the genetic information from both alleles for a specific gene, although the term can also be used about the genetic information in a single nucleotide position or the entire genome. However, the genotype alone does not necessarily provide any information about the phenotype or the gene expression [13]. If a mutation known to cause disease is found (pathogenic mutation), this can be used to diagnose a patient if it is in accordance with clinical and biochemical findings [14]. However, in many cases the DNA variants identified can only be classified as variants of uncertain significance (VUS) [15, 16].

Analysis of the metabolome – the composition of metabolites in a biological sample such as urine or blood [17, 18], is a very important and rapidly growing diagnostic tool. Many metabolites may be closely linked to the phenotype of an organism. This means that metabolomics can be used for many applications, such as phenotyping of genetically modified plants. In this case metabolomics can “close the gap” between genotype and phenotype [19, 20]. Genetic examinations and metabolomics complement each other and together they can provide a definite diagnosis of a suspected disease.

2.1.3 Metabolomics

Metabolomics consists of identifying and quantifying low-molecular weight metabolites (typically <1,000 Da [21, 22]), and provides information about the physiological state of an

organism at the time of sampling. Chromatographic techniques coupled with mass spectrometry are typical tools to use for metabolomics. Targeted metabolomics is used to describe methods where one, or a few, known metabolites are identified or quantified. In untargeted metabolomics, a larger set of metabolites is analyzed without being pre-defined. The metabolites may be known or unknown. The goal is to identify and quantify as many metabolites as possible [19, 22, 23]. The metabolome of the investigated individual can then be compared to the metabolomes of individuals considered to be clinically healthy to look for deviations. This is illustrated in **Figure 2:**

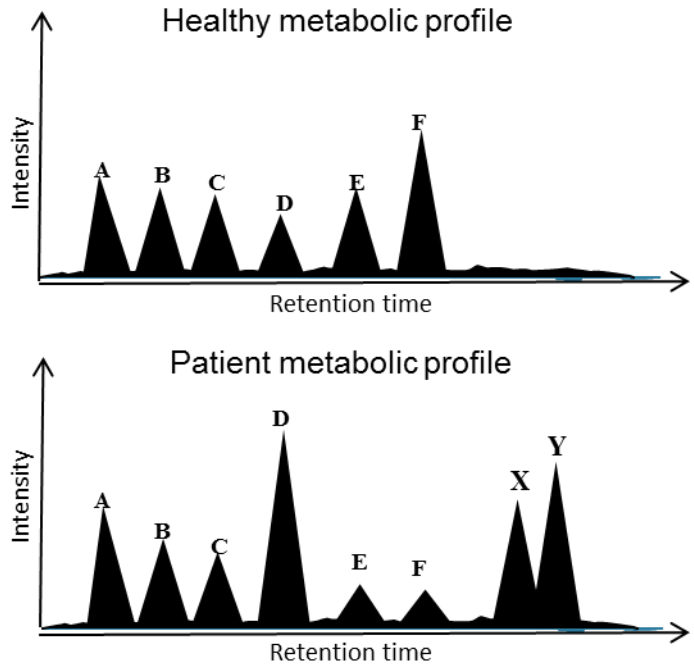


Figure 2 Illustration of healthy vs. patient metabolic profile. The theoretical chromatogram in the upper panel represents a small part of the metabolome of a clinically healthy person. The theoretical chromatogram on the lower panel represents the equivalent small part of the metabolome of a suspected patient. The metabolomes can be compared to look for deviations.

As shown in **Figure 2**, the patient metabolic profile has more of metabolite ”D” than the healthy person, and much less of metabolites ”E” and ”F”, as well as two new metabolites, ”X” and ”Y”. This may indicate (after controlling for external causative factors) that the patient has a disease linked to the metabolism of metabolite ”D”, which leads to compound ”D” accumulating while compounds ”E” and ”F” are not produced in the right amount. The metabolites ”X” and ”Y” could be alternative metabolites produced as a side-effect of the

excessive amount of metabolite ”D”. If compounds D, X and/or Y are toxic, or if E and F are vital compounds, a deviation like this could cause serious diseases.

Many biomarkers are present in the body in very low concentrations [24, 25]. A high sensitivity analysis instrument is necessary to be able to detect these low concentration compounds. When analysis methods are to be used for diagnostics, one needs to know the lowest concentrations of a compound which the method can detect with sufficient accuracy. This is important to avoid both false negative results and false positive results if the precision is low at the level of the detection or decision limits. An example of a low-abundant biomarker is human growth hormone (hGH), which is typically present in the concentration range 1-10 ng/mL in serum, but with approximately a thousand times lower concentration in urine [24].

Targeted metabolomics [26] is a suitable approach when one knows which compounds to look for and in what concentration range they are considered to be pathological, when a certain disease is suspected. As of today, only a small fraction of the thousands of metabolites that exist are used in diagnostics. Efforts are made to maximize the coverage of the metabolome in analytical methods and to identify which are the best biomarkers of disease and treatment response [17, 20].

2.2 High-Performance Liquid Chromatography-Mass Spectrometry

2.2.1 High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is a technique used to separate solutes in time, and is the most common separation technique used for metabolomics because of its versatility [23]. The HPLC system consists of a pump, an injection valve, a column, detector, and a computer [27]. High pressure is used to force the mobile phase, which transports the sample, through the column. The column is filled with stationary phase particles, which the sample compounds will have different affinity to. The most common particles are spherical microporous particles of silica. Typically, a bonded stationary phase is covalently attached to the silica surface. Some of the most common phases to use are amino, cyano, octadecyl (C18) and octyl. Using less polar stationary phases, such as C18, is referred to as “reverse phase”

chromatography. Reverse phase chromatography is a versatile technique, known for giving a high degree of reproducibility [28], and was used in this project.

Sample compounds with different affinity to the stationary phase will elute at different times [27]. Thus compounds tend to have a characteristic retention time. Common detectors are ultraviolet (UV) detectors, spectroscopic detectors, and mass spectrometers. Mass spectrometers are important for many applications. The coupling of liquid chromatography with mass spectrometry (LC-MS) is widely used in the analysis of small molecules in biological samples [21]. Capillary electrophoresis and NMR (nuclear magnetic resonance) are common techniques, but require highly concentrated samples, making LC-MS a better option for many applications because of the superior sensitivity [29, 30].

The endogenous metabolites in the human metabolome consist of several different kinds of compounds, such as amino acids, amines, sugars, and organic acids. The chemical diversity requires a comprehensive analysis [21]. The diversity of the metabolites and their varying concentration range in samples, as well as the presence of many different analytes and isotopes of analytes, necessitates a good chromatographic separation. However, if a mass spectrometer with high resolving power and mass accuracy is used for the analysis, the degree of chromatographic separation required is reduced [23, 31, 32]. LC-MS is more popular for blood spot analysis than gas chromatography-mass spectrometry (GC-MS), as it provides better specificity and sensitivity as well as being more cost effective [33].

2.2.2 Mass Spectrometry

Mass spectrometry (MS) is an extremely powerful analytical technique that can be used for both quantitative and qualitative analyses [27]. As well as identifying compounds, mass spectrometers can be used for mass and structure determination. MS analysis results in a mass spectrum, which displays detector response (signal intensity) versus mass-to-charge ratio (*m/z*). Mass is the mass of the compound, while charge is the charge applied. Only ionic compounds are detected by the mass spectrometer. Different ionization sources can be used, such as electron ionization, chemical ionization, and electrospray ionization (ESI) [27]. ESI was used in this project, and will be discussed below.

MS analysis allows the relevant mass range to be filtered, meaning that only ions with a mass within a certain range reach the detector. The terms MS or MS/MS (”tandem MS”) are used to describe whether the MS analysis consists of mass filtering only before detection, or if mass filtering *and* fragmentation occurs. The fragmentation pattern is unique for each compound; meaning that the pattern can be used for identification. Use of MS/MS is increasing in bioanalytical chemistry. MS/MS can increase the selectivity of the method [34]. The principle of MS/MS is illustrated in **Figure 3**.

Figure 3 Illustration of tandem MS. Tandem MS is when the MS analysis includes mass filtering *and* fragmentation before detection.

There are many different mass spectrometers, each with its own advantages and limitations. Quadrupole instruments are relatively inexpensive and small enough to be bench top instruments, and are known for high reproducibility. However, the resolution can be limited. Time-of-Flight (TOF) instruments are the fastest mass analyzers, and have a high practical mass range, but the precursor ion selectivity for most tandem MS analyses is limited. Trap-based instruments are known for their high resolution, but their dynamic range is limited. Quadrupole ion traps have high sensitivity, but the quantitation ability is poor [35].

Three-dimensional Paul trap (quadrupole ion trap) and the Fourier transform ion cyclotron resonance (FT-ICR) are widely used, but the Paul trap has a low mass resolution and accuracy, and the FT-ICR is a very expensive and complex instrument. Quadrupole-Time of Flight (Q-TOF), which consists of a mass resolving quadrupole coupled with a Time of Flight mass spectrometer, is widely used for both proteomics and metabolomics, and is an alternative to the Paul trap and the FT-ICR. However, the sensitivity of Q-TOF instruments is lower than e.g. the triple quadrupole (QqQ) instruments [31].

The coupling of a linear ion trap (LIT) and FT-ICR (LIT/FT-ICR) has the advantage of high speed, large trapping capacity, versatility, and high mass accuracy, resolving power, sensitivity and dynamic range. But, it is expensive and large, making these instruments less suitable for many laboratories. Orbitrap instruments are good alternatives. These are bench top instruments with a relatively low cost, while still having high resolving power and mass

accuracy [23, 31, 36], making them a suitable tool for increasingly complex biological analyses.

2.2.2.1 Q Exactive Orbitrap

The Q Exactive Orbitrap mass spectrometer has a quadrupole mass filter, a C-Trap, a higher energy collision-induced dissociation (HCD) cell and an Orbitrap analyzer, giving the opportunity to use both MS and MS/MS mode [37]. A schematic view of the Q Exactive is shown in **Figure 4.**

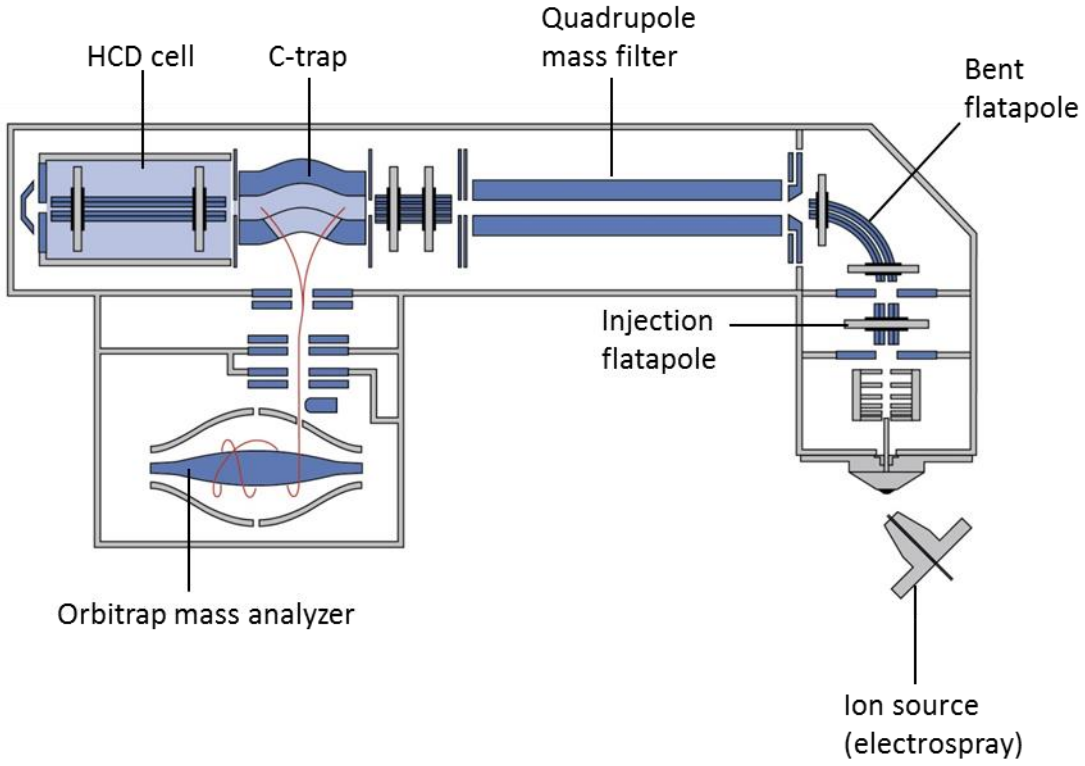


Figure 4 A schematic view of the Q Exactive Orbitrap analyzer. Figure reproduced from Planet Orbitrap [38]. Black lines and names of parts are added by the author of the thesis.

The electrospray ion source transforms the sample molecules, which are part of a liquid solution, into gas phase ions. The resulting ions are focused by the injection flatapole [39]. In the bent flatapole, the ions flow through a 90º arc from the injection flatapole to the quadrupole mass filter. Neutral compounds are removed because they are unable to follow the curved shape. The ions enter the quadrupole mass filter, which consists of four rods. The rods opposite each other are connected electrically, meaning that the four rods practically work as two pairs of two rods. During the scan, the RF (radio frequency) and DC (direct current)

voltages that are applied to the rod pairs are ramped. The voltages applied to each rod pair is of the same amplitude, but opposite in charge. The ratio of RF voltage and DC voltage can be set to fixed values for each injection. This determines which ions are sent to the C-Trap, because for each fixed value, only ions of a certain range of *m/z* ratios will achieve movements with stable oscillations. The ions with unstable oscillations will hit one of the rod surfaces and be neutralized. These compounds are then pumped away or ejected [39].

Ions move from the quadrupole through the RF transfer multipole into the C-Trap, which is a curved linear trap. Here, the ions lose their kinetic energy by colliding with the nitrogen bath gas. When the ions have reached the C-trap, they are either sent directly to the Orbitrap analyzer for detection or to the HCD collision cell for fragmentation [36, 39]. A spindle-like inner electrode and a pair of outer electrodes constitute the Orbitrap mass analyzer. Packets of ions with high energy are sent to the Orbitrap where they start moving in an oscillating motion around the inner electrode. The frequency of these movements is related to the *m/z* of the ions. A Fourier transformation algorithm is used to process the image current picked up by the detector into frequency, and then into *m/z* [37, 39].

The Q Exactive is known for being a suitable instrument for proteomics, but has not been used for metabolomics to the same degree [40]. However, the Q Exactive has been demonstrated to be suitable for metabolomics in cell lines. The results from this study show that both profiling and quantitation of the human metabolome are possible simultaneously when using the Q Exactive, which indicates that the Q Exactive is suitable for metabolomics [40]. Orbitrap MS coupled with HPLC instrumentation has the advantage of high sensitivity, excellent mass accuracy (<5 parts per million (ppm)), and high resolution [31]. Mass accuracy can be expressed as the mass error in ppm, which is defined in **Equation 1**.

$$\frac{\text{Exact mass} - \text{accurate mass}}{\text{Exact mass}} \cdot 10^6 \qquad \text{Eq. 1}$$

Exact mass is calculated by adding up the masses of each atom in a compound (isotope distribution is taken into account), and accurate mass is the experimentally determined mass [41]. Resolution (R_s) is commonly defined as shown in **Equation 2**.

$$R_s = \frac{m/z}{w_{0.5}} \quad \text{Eq. 2}$$

m/z is the mass-to-charge ratio, while $w_{0.5}$ is the peak width at half height (full width at half maximum, FWHM) [41].

2.2.2.2 Parameters Influencing Sensitivity

The term "sensitivity" can be used to describe the ability of a mass spectrometer to detect low-concentration analytes in a sample [42]. High sensitivity is a desired quality, but high sensitivity leads to lower resolution and vice versa. This is because the ions do not reach the detector in a focused beam unless the slits and lenses in the mass spectrometer are narrow and cuts out ions from the center of the beam. This means that many ions are lost, thus leading to decreased sensitivity [43]. The resolving power of an MS tells us how well the instrument separates peaks with similar masses. This is illustrated in **Figure 5**, which shows a mass spectrum obtained from an Orbitrap MS analysis.

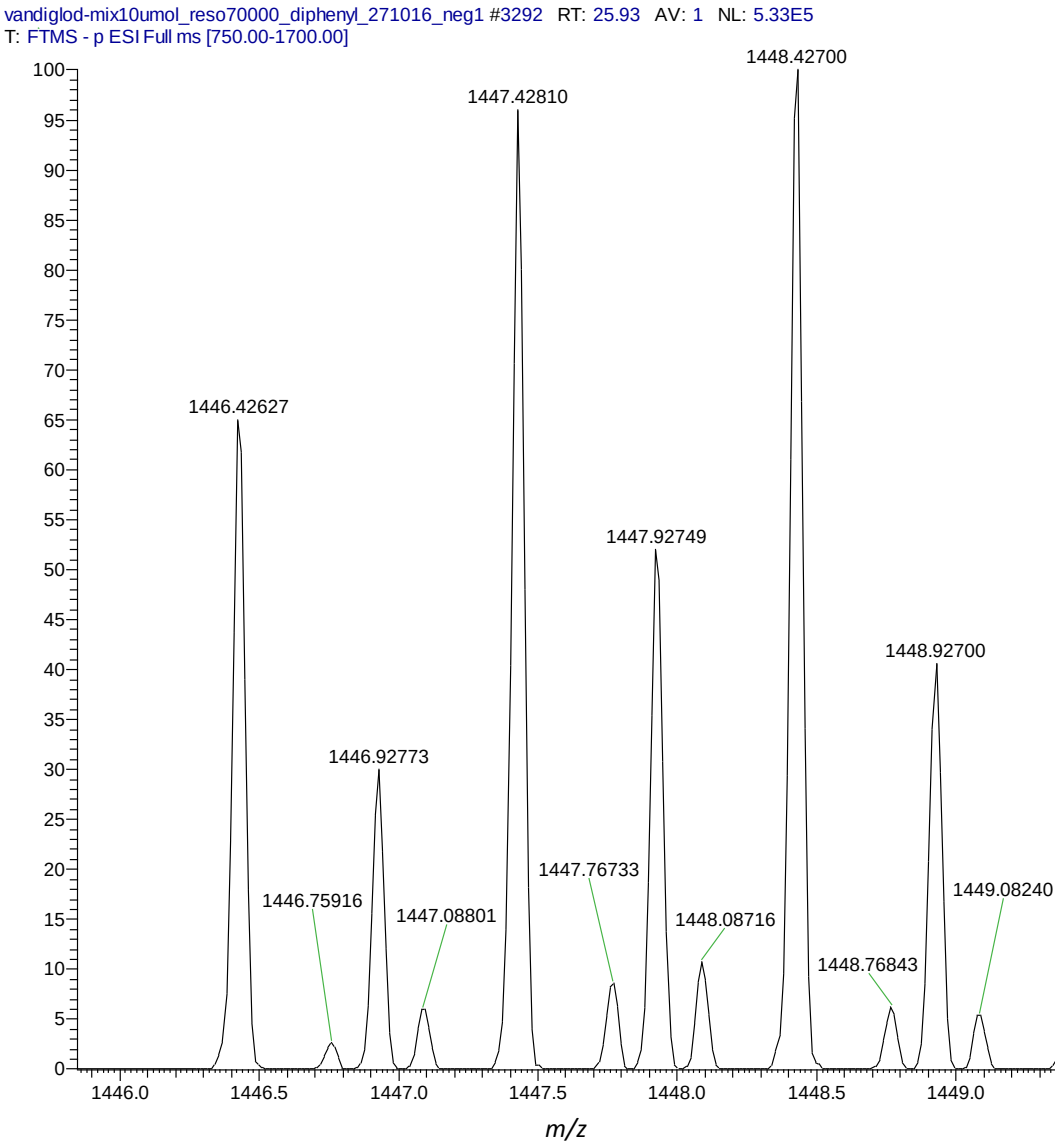


Figure 5 Example of a mass spectrum obtained from an Orbitrap analysis, showing excellent separation of peaks with similar masses.

Two peaks can appear to be a single peak if the peaks are too close; therefore, resolving power is an important parameter. High resolution makes identification of analytes possible without the use of MS/MS analysis. The Q Exactive is a high resolution mass spectrometer, meaning that its identification abilities are good (a compound database or a reference compound is required if the analyte is unknown). One study reported that 12 563 unique peptides on average were identified in each run when a Q Exactive was used for proteomics [37]. The mass accuracy of an instrument depends on the resolving power. Increasing resolving power means increased mass accuracy [31]. Although high resolving power in general is considered an advantage, it can negatively affect the analysis results as a longer scan time is needed. This means that the number of data points across the peak decreases,

negatively affecting the sensitivity (and making quantification more challenging) [32]. This can especially affect low-concentration compounds, as there is a risk of compounds eluting when the mass spectrometer is slowly scanning other parts of the mass range. When analyzing complex samples the right balance between the rate of acquisition and resolution is critical in order to get sufficient resolution and number of ion counts [32, 44]. The scan rate needs to be fast enough for the mass spectrometer to be able to scan the whole range of interest faster than the time it takes the peak to elute from the column [45]. When analyzing complex mixtures, a fast scan rate is important as this means the MS acquires more data throughout the separation [46], resulting in higher sensitivity.

During analysis, a prescan in the linear trap to find the ion current within the chosen mass range is performed by the instrument. Then, a defined number (automatic gain control (AGC) target value) of ions with the relevant mass is stored in the C-Trap, before being injected into the Orbitrap mass analyzer [47]. The AGC is connected to the maximum ion injection time (max IT; the maximum amount of time that the ions are accumulating in the C-trap). When the max IT is reached, the ions will be injected into the Orbitrap regardless of whether the AGC target is reached. High AGC target values will result in a slower scan rate and possibly space charge effects, negatively affecting the sensitivity and mass accuracy [48].

2.2.2.3 Electrospray Ionization

ESI is a widely used ionization technique for LC-MS analysis [49], in which ions in a solution are transformed into gas phase ions. ESI is a popular technique because it can be used for both large (e.g. proteins) and small (e.g. metabolites) molecules [50]. The liquid sample passes through a capillary. There is a coaxial flow of warm nitrogen gas surrounding the capillary, which turns the sample into a spray of small droplets as it leaves the capillary ("Taylor cone"). Evaporation of the solvent, together with an electric field, leads to formation of smaller and electrically unstable droplets with repellent electrical forces inside. The repellent forces increase, and when the repellent forces overcome the surface tension of the droplet, the droplet explodes, releasing ions into gas phase. The ions are led to the mass spectrometer by electric fields in the chamber [51, 52]. **Figure 6** shows the ESI process (the formation of ions in gas phase). The simplified sketch in **Figure 6** shows a linear geometry design of the electrospray capillary relative to the MS inlet. An orthogonal placement of the capillary relative to the MS inlet may be an advantage as this reduces the amount of neutrals entering

the MS. This, in turn, reduces contamination and the potential signal decrease which can be caused by neutrals [53, 54].

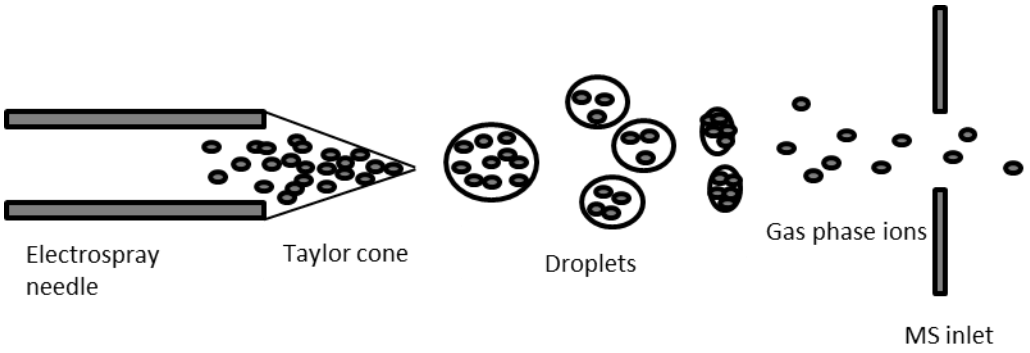


Figure 6 Simplified sketch of the electrospray ionization process. The droplets evaporate, releasing gas phase ions, which then enter the MS interface.

ESI is strongly dependent on the generation of an electric field. **Equation 3** shows the parameters that influence the electric field (E_c) in the vicinity of the electrospray capillary tip.

$$E_c = \frac{2V_c}{r_c \ln\frac{4d}{r_c}} \qquad \text{Eq. 3}$$

V_c is the applied potential to the spray liquid in the electrospray ionization source as it emerges from the electrospray needle (electrospray voltage), r_c is the outer radius of the electrospray capillary (and thereby a constant value for a given capillary), and d is the distance between the tip of the electrospray capillary and the counter electrode (the MS inlet) [51]. The generation of an electric field is what makes ionic compounds move from the electrospray needle and into the MS. As **Equation 3** shows, the strength of the electric field in the vicinity of the capillary tip increases with increasing electrospray voltage, and decreases with increasing distance between the electrospray needle and the MS inlet.

The distance between the ESI needle and the MS inlet can affect the ionization efficiency. A shorter distance means that more of the spray liquid will enter the MS inlet; however, a shorter distance also means that the time for the droplets to evaporate and gas phase ions to be released is decreased. For higher mobile phase flow rates, a longer distance is generally used, while a shorter distance is generally used for lower flow rates [55].

The optimal voltage at the beginning of a mobile phase gradient is not necessarily optimal for the end of the gradient with regards to droplet composition. Still, a constant spray voltage is used for most LC-MS analyses. Previous experience with similar analyses can help to decide the appropriate voltage [56]. ESI is a soft technique [51], meaning that it does not lead to a significant fragmentation of the ions, and is therefore suitable for LC-MS analysis. The electrospray needle position and spray voltage should be set to ensure a stable spray. The position affects the potential gradient between the capillary and the counter electrode, which can affect the stability. Using a high electrospray voltage increases the risk of discharges, especially when using negative ionization mode, which adversely affects the stability and reproducibility. Therefore, it is recommended to use lower spray voltages [57, 58].

The efficiency of ESI is also negatively affected by the presence of salts, making samples with a high content of salts, such as urine, difficult to analyze due to matrix effects [59]. Matrix effects can complicate an analysis, especially in the case of complex matrices, like biological fluids. Compounds such as proteins, phospholipids and salts can cause ionic suppression or enhancement if they co-elute with the analyte. ESI is described as a competitive process, because the number of charged sites at the droplet surface is limited, and the analyte can be outcompeted by other compounds for these sites. Efficient sample preparation and good chromatographic separation is important to avoid suppression or enhancement [34, 57]. Some compounds are ionized more easily when positive ionization mode is used, while other compounds are ionized more easily with negative ionization. Some compounds ionize in positive or negative mode only [22].

2.2.2.3.1 Electrospray ionization, column ID and flow rate

Using smaller-diameter columns as opposed to conventional columns can increase the sensitivity. A smaller inner diameter (ID) reduces the radial dilution, which is proportional to the square of the column radius [60]. As the sample is less diluted, the sample compounds enter the MS in more concentrated bands, leading to better sensitivity for concentration dependent detectors, such as ESI-MS (electrospray ionization-mass spectrometry). This is especially advantageous for samples only available in limited volumes, and for low-concentration samples [60]. Decreasing the column diameter increases peak response. Using smaller diameter columns means that the flow rate is decreased, and less solvent is needed [61]. The electrospray response depends on the analyte concentration and on the flow rate.

The signal increases when the flow rate decreases. A smaller solvent volume means that the concentration of the sample molecules increases (given that the equal sample amount is injected), which in turn increases the electrospray response [62]. Using a low flow rate decreases the droplet size, which is an advantage for concentration dependent detectors. Higher flow rates decreases signal intensity and can decrease stability [63].

Equation 4 shows the parameters that influence the droplet radius, R.

$$R \approx \left(\frac{V_f \varepsilon}{K}\right)^{\frac{1}{3}} \quad \text{Eq. 4}$$

V_f is the flow rate (volume/time), ε is the permittivity of the solvent, and K is the conductivity of the solution [51]. As **Equation 4** shows, increasing flow rate means increasing droplet radius.

2.3 Dried Blood Spots

Dried blood spots were used in this project. DBS are drops of whole blood dripped on filter paper card, usually obtained by finger- or heel pricking. The blood is adsorbed onto the cellulose-based paper, and the paper is left to dry after the sample is taken [64]. Blood is used for many types of biochemical analyses. Whole blood, plasma, or serum can be analyzed, depending on the purpose of the analysis. Whole blood is the blood circulating in the blood vessels, containing plasma and blood cells. Whole blood will coagulate over time, and this makes it necessary to have an anti-coagulant, such as ethylenediaminetetraacetic acid (EDTA) in the sample tubes when these samples are taken [1]. With DBS, coagulation will not occur as the blood is dried on the filter paper. Sample collection of DBS is easy, and the cards can be transported at ambient temperature [65]. When storing for a long period of time, the cards should be kept in a freezer, preferably at -80 ºC. A big advantage with DBS is that the biohazard risks of handling blood samples are greatly reduced compared to whole blood samples [64]. This is because compounds are stabilized and inactivated when dried on filter paper. Additionally, the use of DBS prevents bacterial growth [65].

The fact that the blood compounds are adsorbed and dried makes them more stable than whole blood [64]. To make sure they stay dry, they should be kept in a sealed bag with

desiccant, such as silica. Filter papers can be chemically treated or untreated. The untreated papers are pure cellulose. These are the most commonly used filter papers, especially in NBS [64]. The volume collected in DBS samples is low (about a few dozen µL). Therefore, when a larger volume of blood is needed for an analysis, blood samples should be collected in tubes. In cases where sample material is limited, DBS is a good option. This makes DBS a suitable sample type when samples are taken from newborns. The low blood volume requires high sensitivity analysis instruments [33, 64, 65].

A challenge with DBS samples is hematocrit levels [64]. This varies between people, and affects the viscosity of the blood, thereby affecting the spreadability on the filter card. This means that concentration of analytes can vary on different parts of the DBS, which is important to consider if quantification of the analyte is wanted. For quantitative applications, determination of hematocrit may be an advantage [33, 65]. In this project, no quantification was done.

Analytes can be extracted from the filter paper in a solution [64]. This makes it possible to analyze the samples using LC-MS. Use of MS is the most common technique for DBS analysis [33]. The development of "global" metabolite profiles can be used to potentially find new biomarkers. Metabolomics studies using DBS could be a suitable tool for this purpose [66]. Use of DBS for untargeted metabolomics studies has been successfully reported [67, 68], and DBS seems to be a promising sample type for future metabolomics studies.

2.4 Aim of study

The aim of this study was to optimize parameters on a Q Exactive Orbitrap for untargeted metabolomics of dried blood spots, with the purpose of maximizing both the number of compounds detected and peak intensity. Parameters that were optimized were: electrospray voltage, electrospray needle position, resolution, AGC target value, and mobile phase flow rate. **Figure 7** shows which part of the Q Exactive mass spectrometer each tested parameter (green writing) affects (except for flow rate, which flows to the ion source from the LC instrumentation).

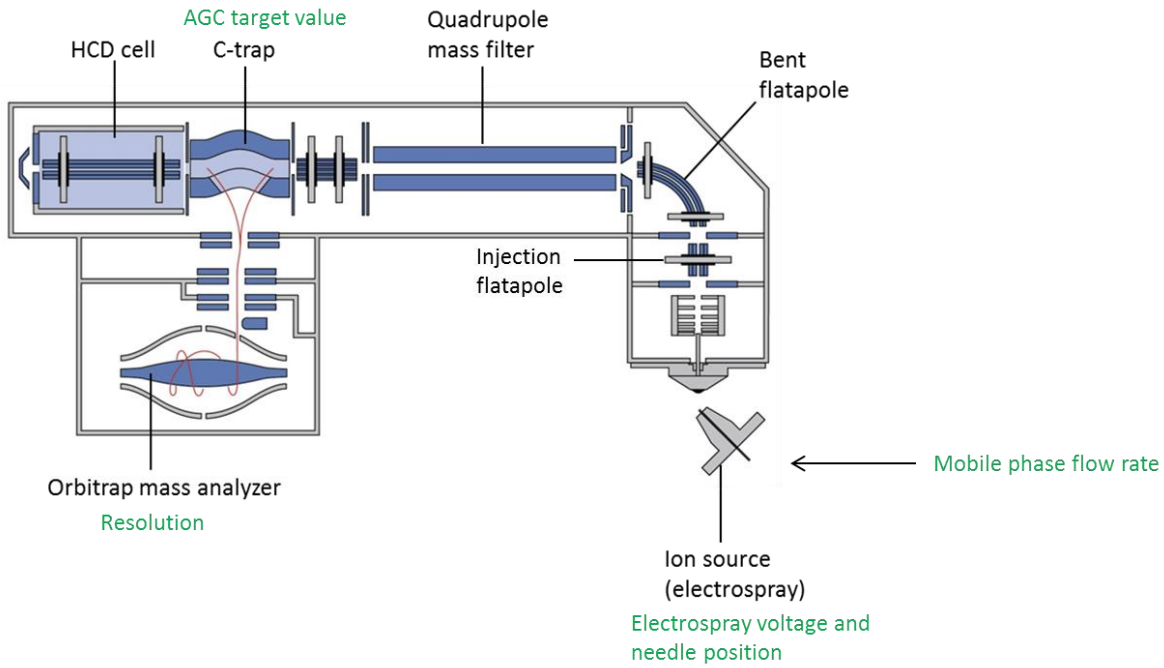


Figure 7 A schematic view of the Q Exactive Orbitrap analyzer, with names of important parts and tested parameters. Figure reproduced from Planet Orbitrap [38]. Black lines and names of parts and parameters are added by the author of the thesis.

3 Experimental

3.1 Chemicals

3.1.1 Analytes and solvents

D2 glycolic acid, D6 glucose and acylcarnitines D3 C2, D3 C12 and D3 C16 were obtained from Larodan (Malmö, Sweden). D4 succinic acid was purchased from Sigma Aldrich (Darmstadt, Germany). ^{13}C creatine was purchased from Isotec (Sigma Aldrich). Vancomycin (1000 mg powder) was obtained from MIP Pharma GmbH (Blieskastel, Germany). $^{13}C_2$ guanidinoacetate was obtained from Dr. H Ten Brink (VU University Medical Center, Amsterdam, The Netherlands).

All water used was of type 1, obtained from MilliQ ultrapure water purification system (Merck Millipore, Darmstadt, Germany). Methanol (purity (GC) ≥ 99.9 %) was purchased from Merck (Darmstadt, Germany). Formic acid (98 %) was obtained from Sigma (Fluka, Sigma Aldrich).

3.1.2 Solutions

A mix of nine compounds was made and used in the optimization experiments (named "standard mix"). Aqueous stock solutions of each compound were made, before making an aqueous mix of all standard mix compounds. Stock solutions were made as follows: the following were weighed in (using an analytical balance (model AG 245) from Mettler-Toledo (Columbus, OH, USA)) and mixed with water to a final volume of 100 mL each: 0.0062 g of D4 succinic acid, 0.0068 g of ^{13}C creatine, and 0.0040 g of D2 glycolic acid. The following were mixed with water to a final volume of 10.0 mL each: 36.0 µL vancomycin (200 mg/mL), 5.00 µL D6 glucose (1 000 mM), 1.20 mL D3 acylcarnitine C2 (4.17 mM), 5.00 mL D3 acylcarnitine C12 (1.00 mM), 8.50 mL D3 acylcarnitine C16 (0.59 mM), and 1.00 mL $^{13}C_2$ guanidinoacetate (5 mM). Concentration of each compound in each stock solution, and pipetted volume of each stock solution to a final volume of 100 mL water (standard mix), is shown in **Table 1**. All pipettes used were PIPETMAN from Gilson (Gilson, Inc., Middleton, WI, USA).

Table 1 Concentration and pipetted volume of each stock solution to a total of 100 mL water.

Compound	Concentration in stock solution (µmol/L)	Pipetted volume (mL)
D3 acylcarnitine C2	500	2.00
D3 acylcarnitine C12	500	2.00
D3 acylcarnitine C16	502	1.99
^{13}C creatine	515	1.94
$^{13}C_2$ guanidinoacetate	500	2.00
D2 glycolic acid	513	1.95
D4 succinic acid	508	1.97
D6 glucose	500	2.00
Vancomycin	497	2.01

After about 10 months, a new aqueous standard mix was made by thawing 2.00 mL of each stock solution (which were kept in a freezer at -80 °C) and mixing them, before adding water to a final volume of 100 mL. The concentrations of the compounds in the two standard mixes are shown in **Table 2**.

Table 2 Concentrations of each compound in the two standard mixes.

Compound	Concentration in the first standard mix (µmol/L)	Concentration in the second standard mix (µmol/L)
D3 acylcarnitine C2	10.0	10.0
D3 acylcarnitine C12	10.0	10.0
D3 acylcarnitine C16	9.98	10.0
^{13}C creatine	9.98	10.3
$^{13}C_2$ guanidinoacetate	10.0	10.0
D2 glycolic acid	9.99	10.3
D4 succinic acid	10.0	10.2
D6 glucose	10.0	10.0
Vancomycin	9.99	9.94

For the sake of simplicity, the concentration of each individual compound has been rounded to the nearest µmol/L. Since the concentration was approximately 10 µmol/L in all cases, it will be referred to as 10 µmol/L. The first standard mix was used in the dilution, electrospray voltage, and electrospray needle position experiments. The second was used in the resolution, AGC target value, and flow rate experiments. The standard mix was stored in a refrigerator at 4 ºC.

Each of the nine compounds was injected in separate aqueous solutions to establish retention times. To find the exact mass of each compound, the Mass Calculator in the Tune software was used. The masses are listed in **Table 3**.

Table 3 Calculated masses of the standard mix compounds, obtained from the Mass Calculator in Tune software.

Compound	Mass in negative ionization mode	Mass in positive ionization mode
D3 acylcarnitine C2	205.12731	207.14186
D3 acylcarnitine C12	345.28381	347.29837
D3 acylcarnitine C16	401.34641	403.36097
^{13}C creatine	131.06555	133.08011
$^{13}C_2$ guanidinoacetate	118.05326	120.06781
D2 glycolic acid	77.02132	79.03587
D4 succinic acid	121.04444	123.05899
D6 glucose *	185.09377	187.10833
Vancomycin	1446.42292	1448.43748

**: Mass extraction by the software (in the extracted ion chromatograms) of the calculated mass of D6 glucose provided no results. The detected mass from the analysis of the D6 glucose aqueous standard in negative ionization mode was used instead (185.07593). D6 glucose was not detected in positive mode in any of the standard mix samples analyzed. Endogenous glucose was detected in a dried blood spot sample (without addition of standard mix) with negative ionization mode, indicating that the mass extraction problem was caused by the D6 glucose standard, although the exact reason for this is unclear.*

Mobile phase A consisted of water with 0.1 % formic acid. Mobile phase B consisted of methanol with 0.1 % formic acid. Each mobile phase was prepared with 1.00 mL formic acid diluted to 1000 mL of water and methanol, respectively.

3.2 Materials and equipment

3.2.1 LC-MS instrumentation and settings

All LC-MS analyses were performed using LC instrumentation (Dionex Ultimate 3000 UHPLC system pump, column department and autosampler, Thermo Scientific, Waltham, MA, USA) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). Electrospray was used as ionization source, and all samples were analyzed in both positive and negative mode. The analytical column was a Pursuit XRs C18 Diphenyl column (250 x 2.0 mm, particle size 3 µm), from Agilent Technologies (Santa Clara, CA, USA).

A mobile phase flow rate of 150 µL/min was used in all experiments unless otherwise written. Injection volume was 2 µL. Mobile phase gradients were performed according to **Table 4** and **Figure 8**.

Table 4 Mobile phase gradient used in all experiments described, except parts of the flow rate experiments. Mobile phase A consisted of type 1 water with 0.1 % formic acid. Mobile phase B consisted of methanol with 0.1 % formic acid. Equilibration was performed for ten minutes after each analysis.

Time (min)	% B
0	5
10	5
50	100
55	100
55.1	5

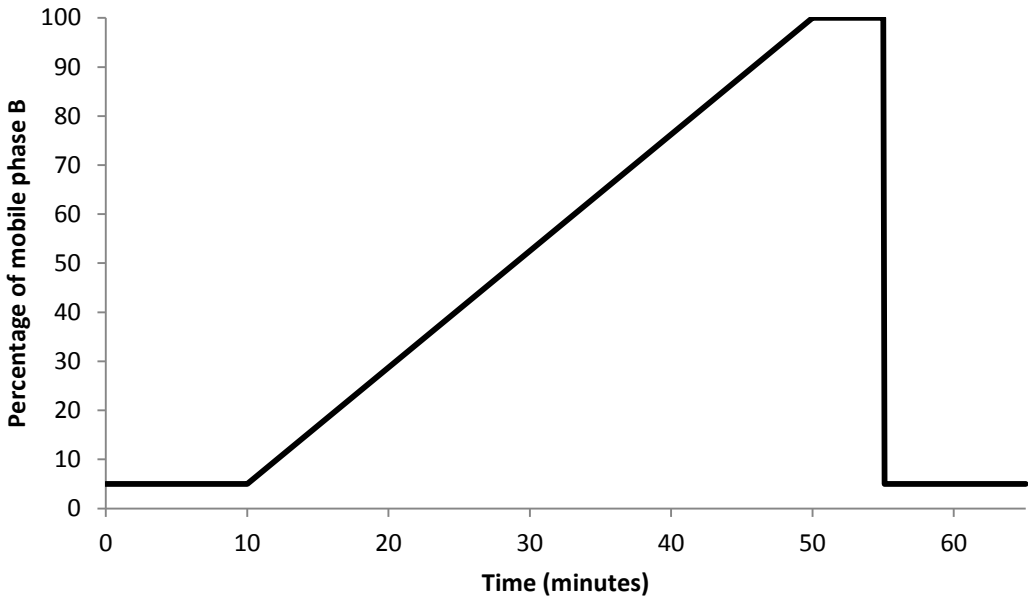


Figure 8 Mobile phase gradient used in all experiments described, except parts of the flow rate experiments. The black line shows the percentage of mobile phase B (methanol with 0.1 % formic acid). Mobile phase A consisted of type 1 water with 0.1 % formic acid.

The initial mass spectrometric settings (shown in **Table 5**) were chosen based on suggestions from Thermo Fisher, settings suggested by the auto default settings of the HESI (Heated electrospray ionization) part in the Tune software, and settings given by Liu [40].

Table 5 Initial mass spectrometric settings. All samples were analyzed in both positive and negative ionization mode.

Parameter	Setting
Scan type	Full MS
Scan ranges	*m/z* 50-750, 750-1700
Fragmentation	None
Resolution	70 000
Microscans	1
Lock masses	Off
Automatic gain control target	3 000 000 ion counts
Maximum injection time	200 ms
Electrospray needle position	C
Sheath gas (N_2) flow rate	40 (a.u.)
Auxiliary gas (N_2) flow rate	10 (a.u.)
Sweep gas (N_2) flow rate	2 (a.u.)
Electrospray voltage	3.5 kV
Capillary temperature	250 °C
S-lens RF level	50
Auxiliary gas heater temperature	300 °C

3.2.2 Computer software

Software used was Xcalibur (Version 3.0 63), Tune (version 2.5 Build 2042), and Chromeleon Xpress (version 6.80), all from Thermo Scientific.

3.3 Sample preparation

Filter cards used in all experiments were Whatman 903 Protein Saver cards, obtained from GE Healthcare Life Sciences (Chicago, IL, USA). The cards are not chemically treated. These filter cards are one of two card types recommended by the Clinical & Laboratory Standards Institute guidelines [33].

Three sample types were used in the optimization experiments: aqueous standard mix (referred to as "standard mix"), standard mix spotted onto filter paper (referred to as "standard mix spot"), and standard mix mixed with whole blood 50:50 (v/v) and spotted onto filter

paper (referred to as “standard mix + blood spot”). The dried spot cards that were made of standard mix and standard mix + whole blood were made using a dropper to transfer one drop to each field. Spot samples were either made prior to the experiment or stored in a freezer at -80 ºC until used. During sample preparation, bits of the DBS were cut out (bits are referred to as ”punches”). One punch has a diameter of 3.2 mm, which is equivalent to about 3 µL whole blood. Whole blood samples were taken from a healthy volunteer and either mixed with the standard mix prior to spotting, or stored in a freezer at -80 ºC and thawed before being mixed with the standard mix, and finally spotting. **Figure 9** shows a DBS sample.

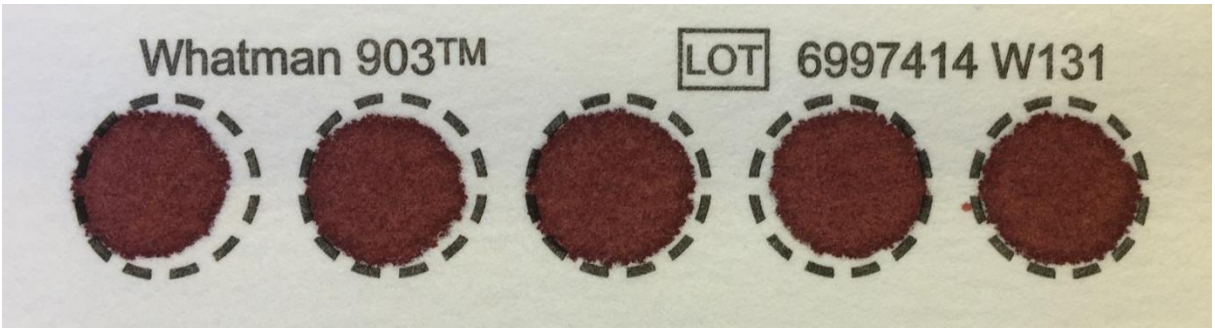


Figure 9 Dried blood spot sample.

The following procedure was used for sample preparation of all dried spot samples:

One punch was cut out (using a puncher from McGill (Jacksonville, FL, USA)) from one of the fields on the filter card and transferred to a micro tube (from Sarstedt, Nümbrecht, Germany). 100 µL 80 % aqueous methanol with 0.1 % formic acid was added to the tube. A thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) was used for extraction for 45 minutes at 45 ºC (700 rpm). The sample solution was reduced to dryness in glass tubes (obtained from VWR (Radnor, PA, USA)) under a stream of dry nitrogen (using TurboVap LV, Caliper Life Sciences, Waltham, MA, USA) at 40 ºC, for about 10 minutes, and then dissolved in 100 µL 5 % aqueous methanol with 0.1 % formic acid. The sample solution was mixed on a table vortex (Genie2, Scientific Industries, Bohemia, NY, USA) and transferred to an HPLC vial (from Matriks AS, Oslo, Norway) for analysis.

Punches were consistently taken from the centre of the spot, not the outer edges. It is recommended to be consistent with the choice of punching location to reduce bias [33].

Choice of sample preparation was based on sample preparation procedures at the Department of Newborn Screening at Oslo University Hospital (Rikshospitalet), and on a bachelor thesis which tested several of the newborn screening sample preparation steps with the goal of maximizing the number of molecular features detected using a Q-TOF mass spectrometer [69].

In all experiments, each sample was injected three times in both positive ionization mode and negative ionization mode.

4 Results and discussion

4.1 Framework of study

The human metabolome consists of compounds with a wide variety of molecular sizes, polarities, and structures. The standard mix compounds (D2 glycolic acid, D6 glucose, acylcarnitines D3 C2, D3 C12 and D3 C16, D4 succinic acid, ^{13}C creatine, vancomycin, and $^{13}C_2$ guanidinoacetate) were carefully chosen as a representative subset of the human metabolome. Structures, molecular formula, average molecular weight, and log P of all compounds in the standard mix are shown in **Table 6**. Log p values are used to describe a compounds' hydrophobicity, and is defined as the ratio of distribution of the compound between an organic phase (with octanol as the most commonly used model of the organic phase) and an aqueous phase at equilibrium [70].

Compounds in the standard mix ranged from the polar, such as ^{13}C creatine, to less polar acylcarnitines with long hydrophobic tails, including D3 C16. The standard mix compounds are regularly analyzed in routine analyses at Oslo University Hospital, meaning that it is possible to compare the quantitative and qualitative achievements of the developed method against routine analyses. All compounds in the standard mix, except vancomycin (which is a drug), were isotopically labelled. This was to ensure that the measured signal intensity was a result of parameter optimization, not the endogenous value of compounds in blood samples.

Three sample types were used in the optimization experiments: aqueous standard mix ("standard mix"), standard mix spotted onto filter paper ("standard mix spot"), and standard mix mixed with whole blood 50:50 (v/v) and spotted onto filter paper ("standard mix + blood spot"). Standard mix spots and standard mix + blood spots were prepared as described in section **3.3**. Three different sample types were used in order to investigate the effect of spotting, i.e. if any of the compounds were not extractable from the filter paper. Additionally, using a sample that also included whole blood made it possible to investigate matrix effects. As some compounds ionize only in positive *or* negative mode, all samples were analyzed in both modes in order for the method to cover as many compounds of the DBS metabolome as possible. In general, the results from the analyses of the standard mix + blood spot sample were considered the most important when results of the different sample types did not point to

the same choice of value. This is because standard mix + blood spot was deemed to be the most representative of a patient sample.

For practical reasons, whole blood used in the samples was sometimes taken right before spotting onto filter paper, or stored in a freezer at -80 °C and thawed before spotting. One could argue that the blood should have been taken consistently throughout the project – either frozen and thawed, or always taken right before making of the spots. However, the components of blood circulating in the vessels are not consistent from day to day – they can be affected by diet or other factors, meaning that this would not have solved the issue of analyzing "different" blood each time. One way to work around this could have been to take a large blood sample and freeze many aliquots, but; then there could be potential effects of prolonged storage. In this project, the potential alteration of blood when it was frozen and thawed and the day-to-day variation of circulating blood was not considered to be of relevance since the analytes of interest were spiked into the sample. The same blood sample was, however, used in all experiments for a chosen parameter. This provided a minimum level of standardization for all experimental conditions for each parameter.

In negative ionization mode, the acylcarnitines were not detected in spot samples (with one exception for D3 acylcarnitine C12, see Table 41 in the **Appendix**, section 6.1), while D2 glycolic acid and D6 glucose were not detected in any positive mode analyses. Additionally, D4 succinic acid and vancomycin were not detected (with one exception for D4 succinic acid, see Table 42 in the **Appendix**, section 6.1) in positive mode spot samples. The reason for these missing components may be found in the compound's structures (shown in **Table 6**). Acylcarnitines are zwitterionic, and in the presence of an acidic mobile phase, it is likely that the acylcarnitines are more easily positively ionized. The routine MS method at Oslo University Hospital for acylcarnitines uses positive mode. For D2 glycolic acid and D6 glucose, it is more likely that they are deprotonated than protonated. In the Human Metabolome Database (HMDB) [71], only negative ionization mode LC-ESI-MS spectra were found (for positive mode, only predicted spectra are available) for glycolic acid, glucose and succinic acid, in agreement with the results presented in this thesis.

Vancomycin is a large compound, and many isotope peaks were detected. Thus, the *m/z* used to search for vancomycin (1446.42292 in negative mode and 1448.43748 in positive mode,

calculated by the mass calculator in Tune software) was not always sufficient for detection. The summarized molecular ion intensity, distributed over 4-6 molecular ion peaks, might have been sufficiently high, but the single molecular ion used for detection appeared to be insufficient. The only two compounds that were detected in both ionization modes in all sample types were ^{13}C creatine and $^{13}C_2$ guanidinoacetate. Based on their structures (see **Table 6**), it seems likely that they are both easily ionized positively as well as negatively. Although it seems more likely that the sample compounds will be positively ionized as the mobile phase is acidic, so called "wrong-way-round" ionization has been reported [57, 72, 73]. This is in correspondence with the results presented in this thesis, in which several of the compounds seemed to be easily negatively ionized. The fact that some compounds only ionize in positive *or* negative mode emphasizes the importance of using both ionization modes for untargeted metabolomics, to ensure detection of as many metabolites as possible.

Overall, there were more compounds detected in the aqueous standard mix than in the standard mix spot and standard mix + blood spot samples, and a higher signal was measured. This was expected as there were several steps during sample preparation where there was a risk of losing a fraction of the sample, e.g. during extraction from the filter paper, or after evaporating to dryness and re-extraction. The combination of sample preparation and weak ionization in positive or negative mode could be the reason that some compounds were not detected in spot samples. All standard mix compounds were detected in either positive or negative mode in all sample types, meaning that all the standard mix compounds are extractable from the filter paper. The LC parameters for the developed method were optimized in another master project [74], and were thus not tested in this project, except for mobile phase flow rate as flow rate should, in theory, affect sensitivity.

In the following pages, arrows in figures point to values of the tested parameter with which a compound was first detected. This highlights that the number of detected standard mix compounds was an important criterion for which value of each parameter to be chosen. For practical reasons, a logarithmic scale was used in the graphs. Using a logarithmic scale can mask differences between numbers to some extent, however; it still conveys the trends in the results. In this project, overarching trends in results and the number of detected standard mix compounds were considered to be the most important aspect, not differences between measured levels of individual markers.

In general, the peak intensity was similar for the standard mix spot and the standard mix + blood spot samples. A matrix effect causing reduction of intensity due to ion suppression from blood compounds was expected. This was not observed, indicating that the matrix effects were limited, probably due to sufficient chromatographic run time resulting in sufficient ionization capability of the source for the compounds eluting. In fact, the analyte concentration was halved in the samples mixed with blood when compared to the standard mix spot. The somewhat surprising intensity trend observed could be a result of either matrix effects causing signal enhancement, or lack of linearity of the detector in the concentration range used. However, assessing linearity and measurement of higher concentrations was beyond the scope of this thesis.

Relative standard deviation (RSD) % for the measured intensities of all compounds in the optimization experiments are shown in **Appendix**, section 6.2, Tables 56-93.

Table 6 Structure, molecular formula, average molecular weight, and log P of all compounds in the standard mix. Colored dots show the color of each compound in all figures. All values were found at HMDB [71]. Isotope labels are not shown.

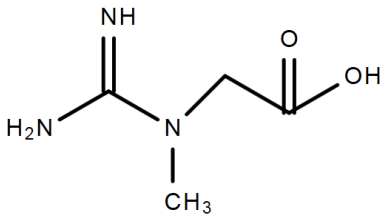
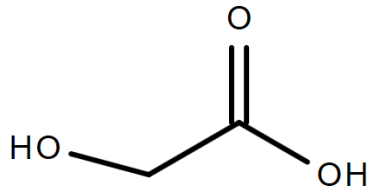
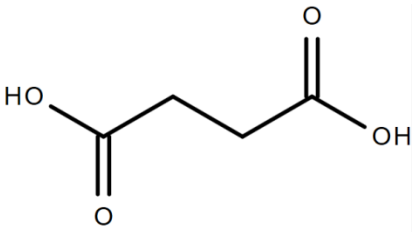
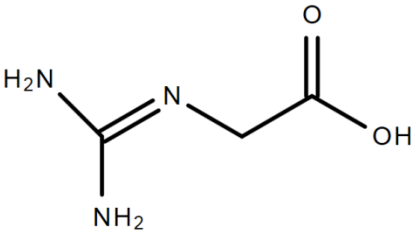
Compound	Structure	Molecular formula	Average molecular weight (g/mol)	Log P	Color in all figures shown
Creatine		$C_4H_9N_3O_2$	131.133	-1.6*	
Glycolic acid		$C_2H_4O_3$	76.051	-1.11	
Succinic acid		$C_4H_6O_4$	118.088	-0.59	
Guanidinoacetate		$C_3H_7N_3O_2$	117.107	-1.8*	

Table 6 continued.

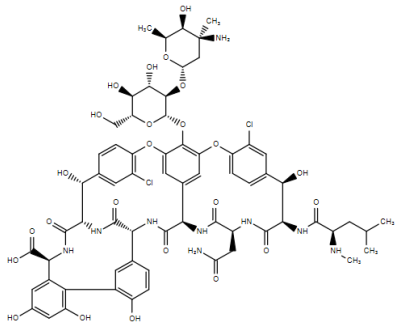
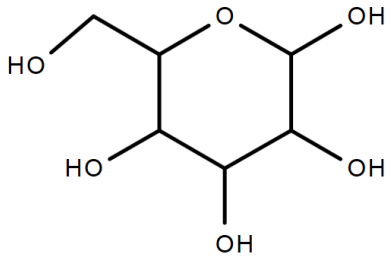
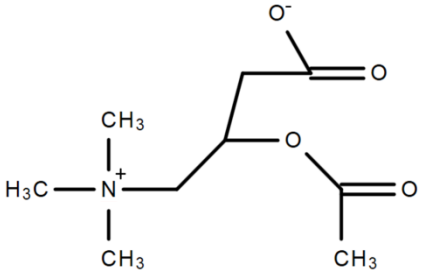
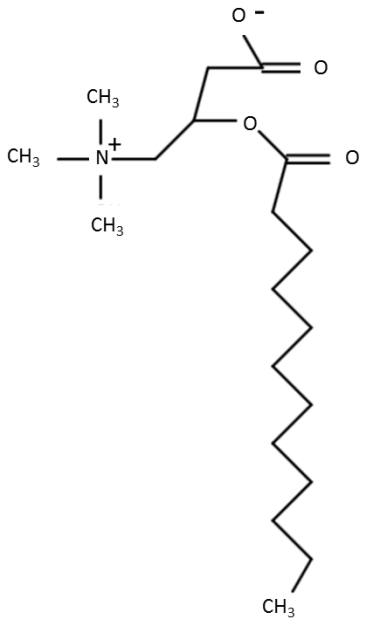
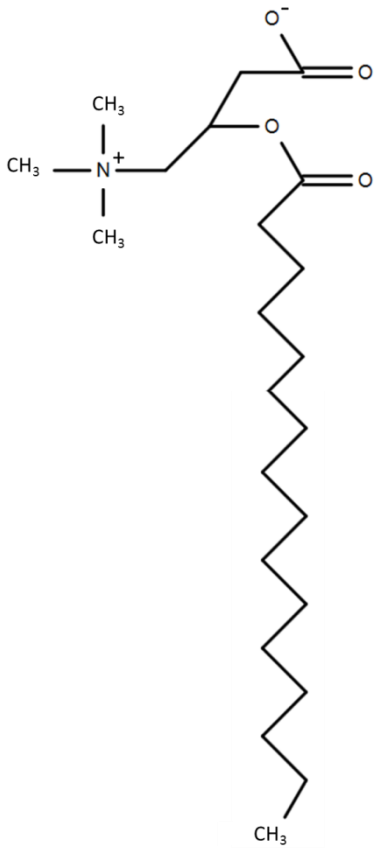
Vancomycin		$C_{66}H_{75}Cl_2N_9O_{24}$	1449.254	-3.1	●
Glucose		$C_6H_{12}O_6$	180.156	-3.24	●
Acylcarnitine C2		$C_9H_{17}NO_4$	203.236	-2.4*	●

Table 6 continued.

Acylcarnitine C12		$C_{19}H_{37}NO_4$	343.501	0.03*	
Acylcarnitine C16		$C_{23}H_{45}NO_4$	399.608	1.77*	

* Predicted log P (experimentally derived log P not available), found at HMDB [71], obtained from Virtual Computational Chemistry Laboratory [75].

4.1.1 Criteria to define compound detection

A compound was considered to be detected if there were 10 points or more across the chromatographic peak, and the intensity of the peak was 10^3 (NL (normalized intensity level) (counts per second)) or higher. Another criterion was that the compound eluted within a retention time (RT) window of three minutes around the RT of the compound measured when the compounds were injected in separate solutions (this criterion was not used in the mobile phase flow rate experiments, as flow rate changes inevitably affect each compound's RT). An RT window of three minutes may be considered too large for diagnostic use in an analytical method; however, as this was a method development project, the decision to use a relatively large RT window was taken. In general, the RT variation of each compound was not large (typically less than one minute). If the mass error in ppm was larger than five, the compound was not considered to be detected. Finally, the compound had to be detected in all three injections of the sample.

4.2 Effect of dilution on sensitivity

A concentration of 10 µmol/L of each compound in the standard mix and standard mix spot samples and 5 µmol/L in the standard mix + blood spot sample was considered to be the best choice for further optimization experiments. All compounds were detected in either positive or negative mode with these concentrations, a crucial step enabling us to see how the compounds are affected by the different values of each parameter in the optimization experiments. See section **4.1.1** for criteria of what was considered a detected compound. Tables showing the average intensity of each compound in each sample for all concentrations analyzed are shown in the **Appendix**, section 6.1 (Tables 8-13).

Total ion chromatograms (TICs) and extracted ion chromatograms (EICs) of all detected compounds in standard mix and standard mix + blood spot for the chosen concentrations are shown in **Figures 10-13**. Only one of the three injections of each sample is shown. For the standard mix spots, TICs and EICs are shown in the **Appendix**, section 6.3 (Figures 46-47).

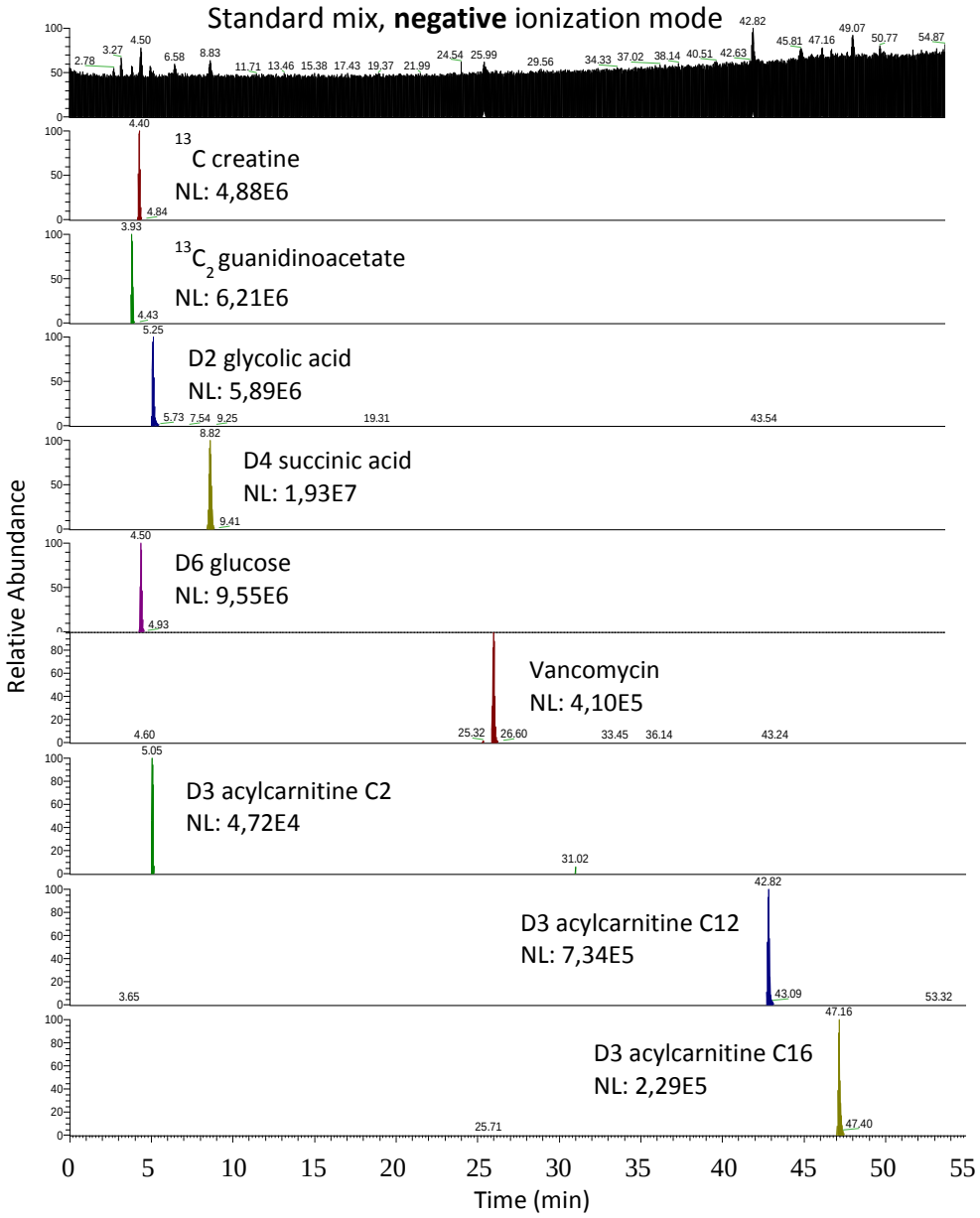


Figure 10 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix, negative ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed.

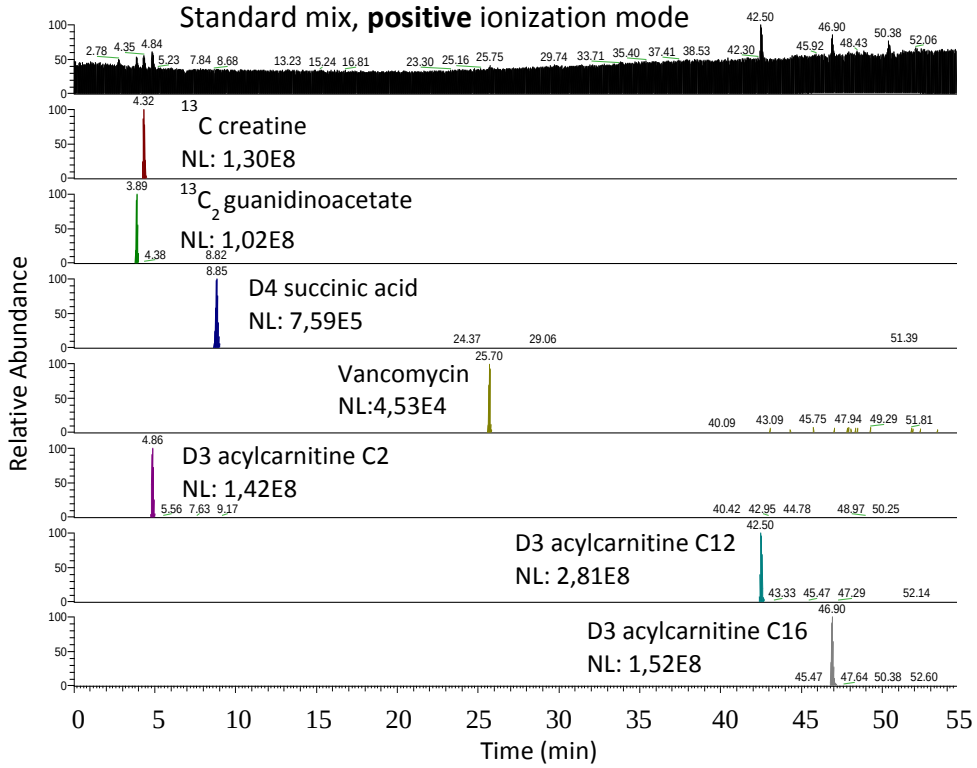


Figure 11 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix, positive ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed.

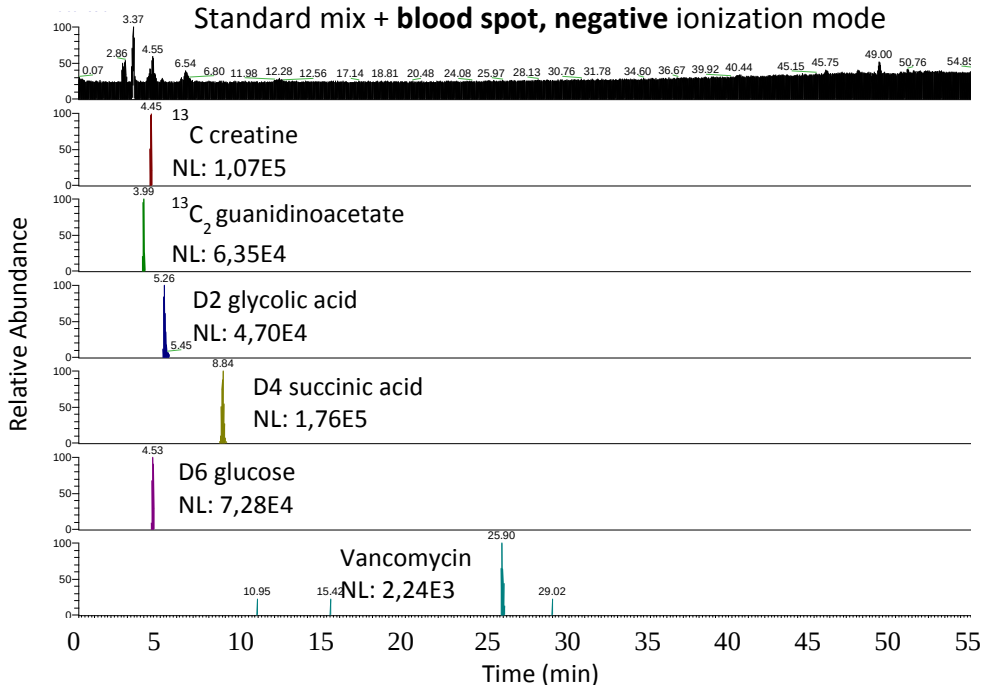


Figure 12 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix + blood spot, negative ionization mode, with a concentration of 5 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed.

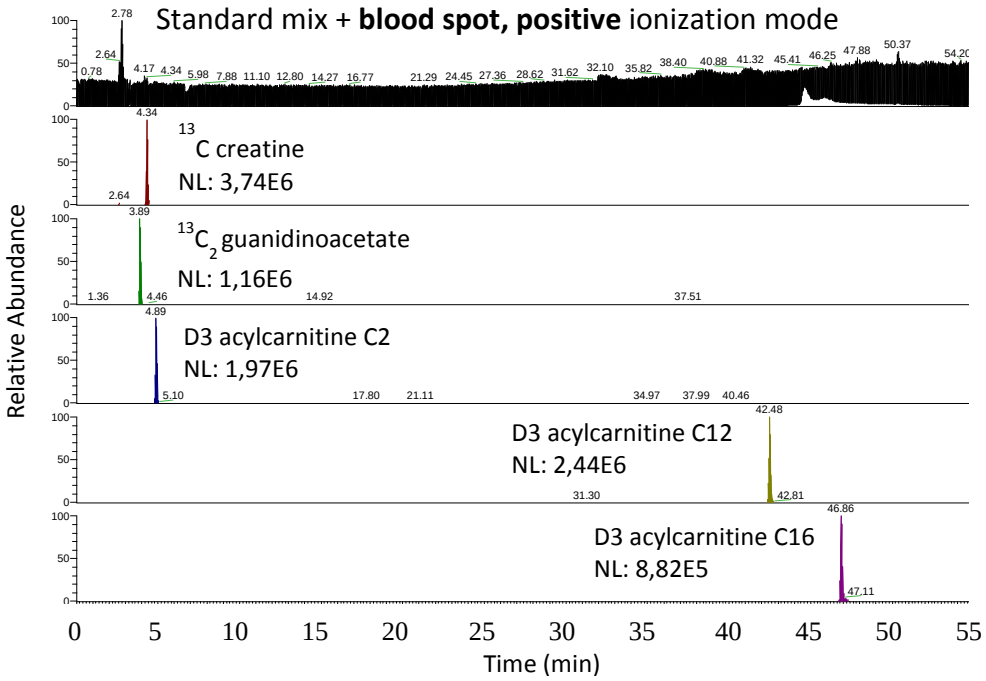


Figure 13 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix + blood spot, positive ionization mode, with a concentration of 5 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed.

In summary, the concentrations chosen for optimization experiments were 10 µmol/L of each compound in the standard mix and standard mix spot samples and 5 µmol/L in the standard mix + blood spot sample.

4.3 Effect of electrospray voltage on sensitivity

As **Equation 3** shows (see section **2.2.2.3** for more details), the strength of the electric field (E_c) in the vicinity of the electrospray capillary tip increases with increasing electrospray voltage (applied potential, V_c) [51]. This should in turn increase sensitivity, as a stronger electric field means that there is a stronger force pulling the spray liquid/ions into the MS. With this in mind, it was expected that the measured intensity would increase as the electrospray voltage was increased.

$$E_c = \frac{2V_c}{r_c \ln\frac{4d}{r_c}} \qquad \text{Eq. 3}$$

The effect of changing the electrospray voltage was tested across the following range of values: 1, 2, 3.5, 4, 5, 6 and 7 kV. All other parameters were kept constant (see **Table 5** for

settings). First, an analysis with the voltage set to 7 kV was done (see **Appendix**, section 6.4, Figures 50-52 and section 6.1, Tables 14-19). Shortly after the analyses performed with a voltage of 7 kV were finished, one of the turbomolecular pumps had to be replaced. For this reason, 7 kV was tested again. The average intensity of each compound for the different electrospray voltages is shown in **Figures 14-16**. Tables showing the average intensity of each compound in each sample are shown in **Appendix**, section 6.1 (Tables 21-26).

For the standard mix analyses in negative mode, an electrospray voltage set to 5 kV gave the highest intensity for most standard mix compounds (see **Figure 14**), but only slightly higher than 3.5 kV. In positive mode, the highest intensities were measured using 5 kV (see **Figure 14**). However, 5 kV gave the lowest number of detected standard mix compounds, and 3.5 kV also gave high intensities, indicating that 3.5 kV was a better choice overall.

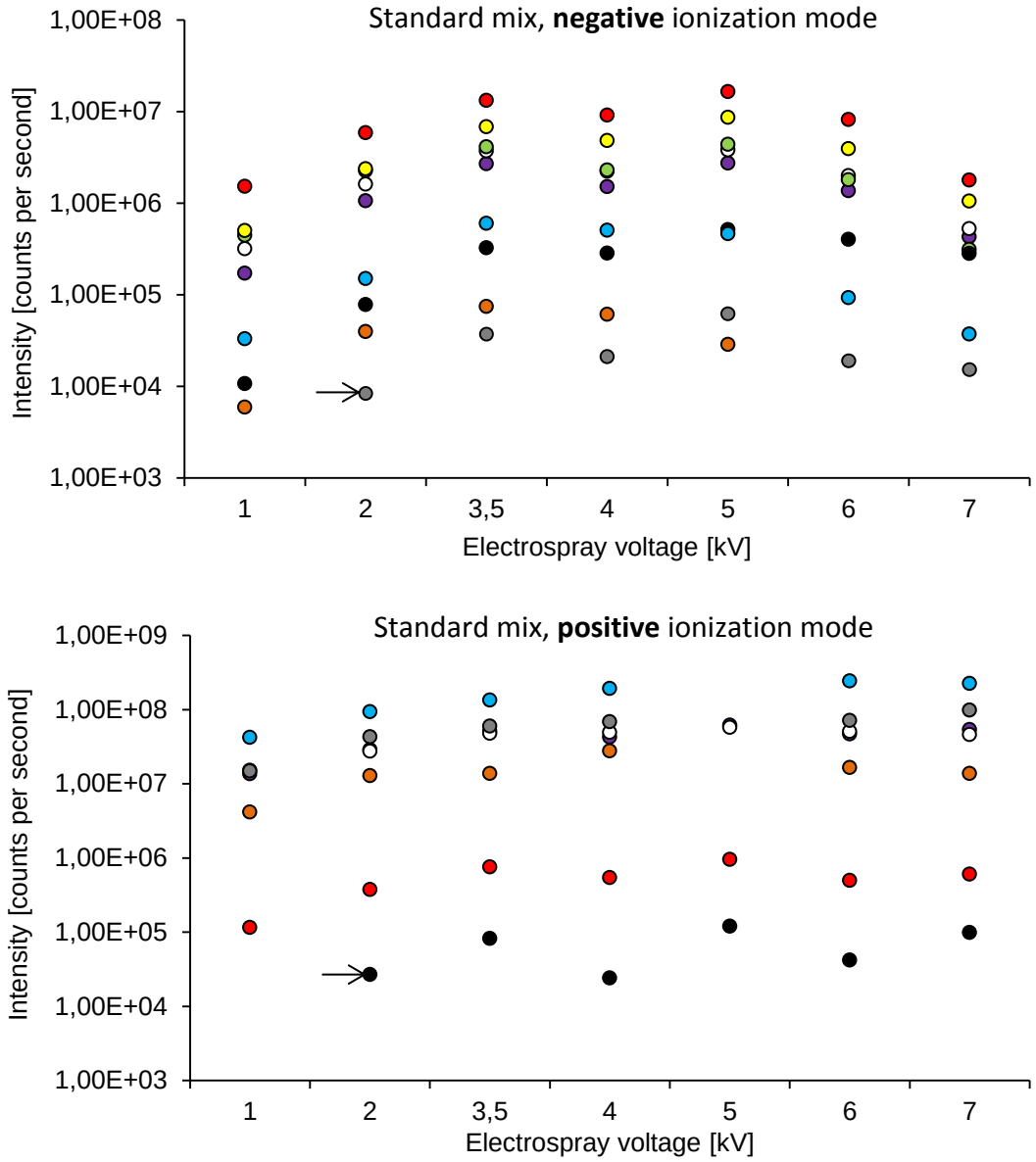


Figure 14 Measured intensity of all compounds in standard mix, negative (top) and positive (bottom) ionization mode, for all electrospray voltage values tested. Intensity shown is the average intensity measured from three injections. Arrows show for which value a compound was first detected. The color code for each compound is shown in **Table 6**.

For the standard mix spot sample analyzed in negative ionization mode, 3.5 kV gave the highest intensity for all compounds except vancomycin. In positive mode, the highest intensities were measured when 3.5 and 5 kV were used (see **Figure 15**). As 5 kV gave a lower number of detected compounds, 3.5 kV was considered to be a better choice.

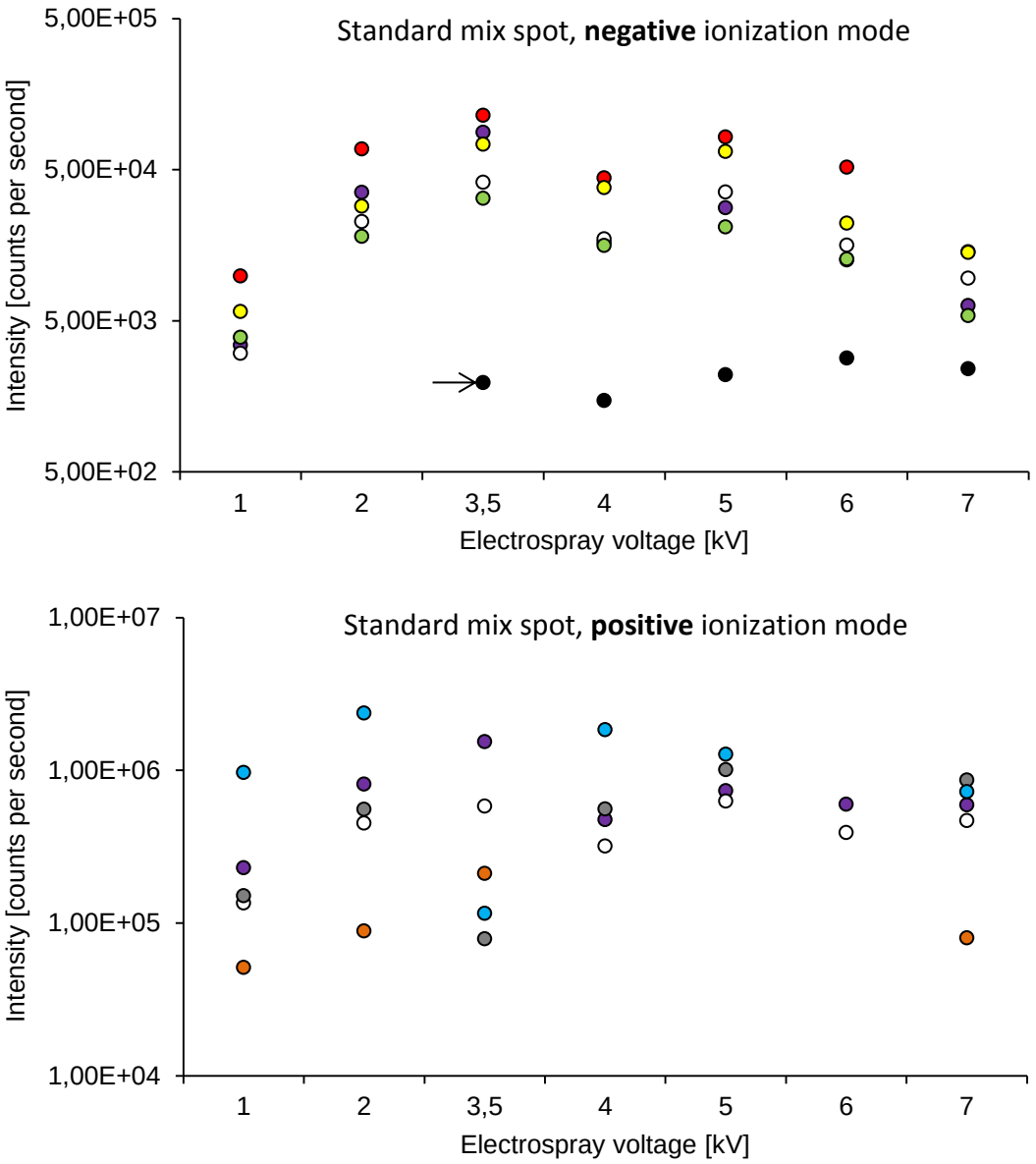


Figure 15 Measured intensity of all compounds in standard mix spot, negative (top) and positive (bottom) ionization mode, for all electrospray voltage values tested. Intensity shown is the average intensity measured from three injections. The arrow shows for which value vancomycin was first detected. The color code for each compound is shown in **Table 6**.

For the standard mix + blood spot samples analyzed in negative mode, the highest intensities were measured with 3.5 and 4 kV (see **Figure 16**). However, vancomycin was only detected with 6 and 7 kV. In positive mode with 3.5 kV, D3 acylcarnitine C12 was not detected. This was unexpected, as this compound was detected in previous analyses using a voltage of 3.5 kV (the dilution experiments, see section **4.2**). The reason is not obvious; however, it might

be caused by matrix effects, such as ion suppression caused by one or many of the other compounds present in whole blood.

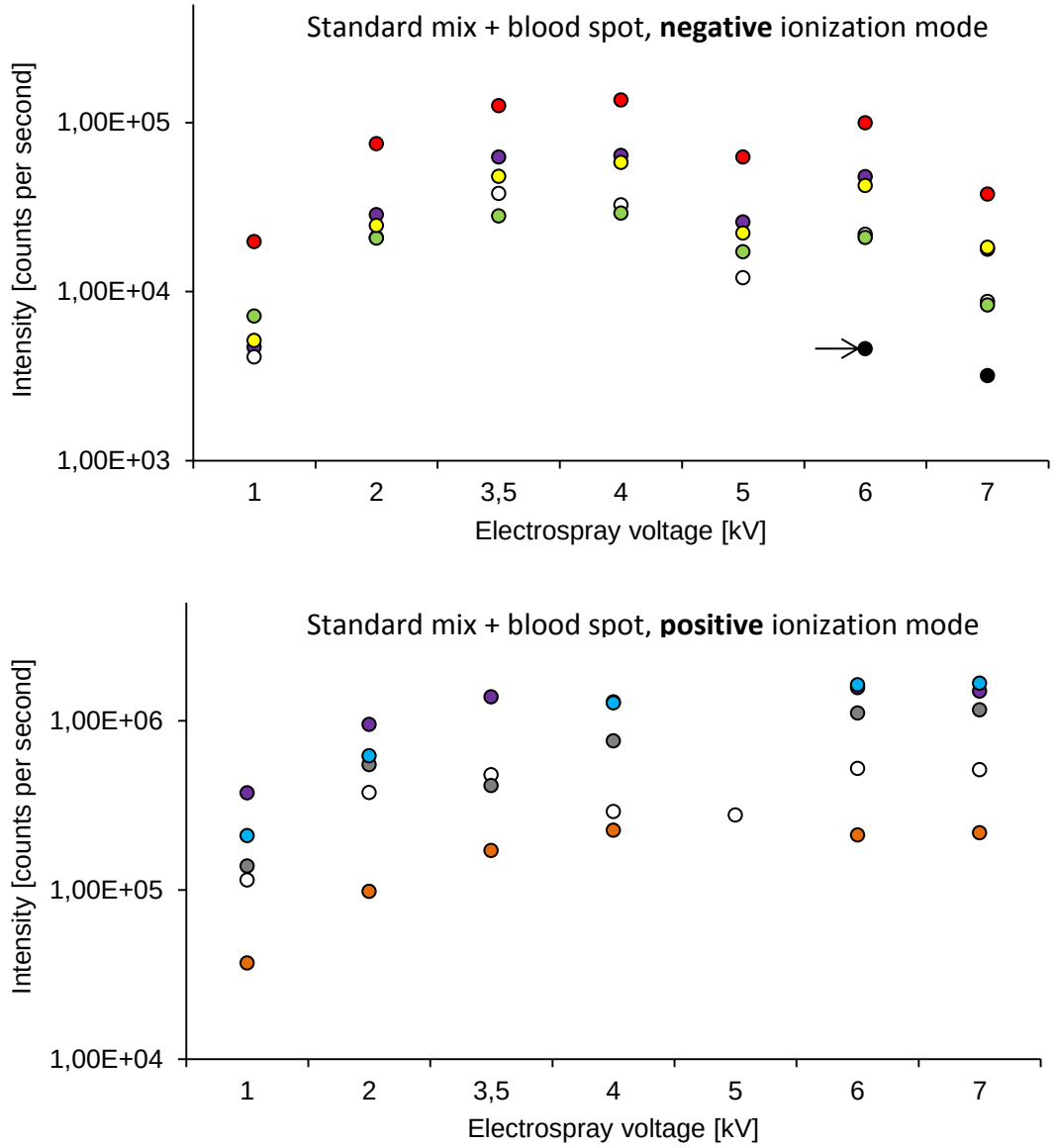


Figure 16 Measured intensity of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all electrospray voltage values tested. Intensity shown is the average intensity measured from three injections. The arrow shows for which value vancomycin was first detected. The color code for each compound is shown in **Table 6**.

Overall, a spray voltage of 3.5 kV was considered to be the best choice, as this gave high intensity, and, with some few exceptions, 3.5 kV was one of the values that gave the highest number of detectable compounds. Using 3.5 kV might give a higher probability of covering more of the metabolome in real samples. The most important sample type was considered to

be the one consisting of standard mix + blood spot, as this was the closest to a patient sample where diagnostic metabolites have accumulated. Based on this, a value of 4 kV for negative mode and 6 or 7 kV for positive mode appear to be the best choices. However, since intensity did not differ much, and 3.5 kV gave relatively high intensities, this value was still considered to be the best choice.

Another reason for choosing 3.5 kV is because the method being developed is meant to be used for untargeted metabolomics. As one does not necessarily know what types of compounds the analytes are in these samples, choosing a value that is either on the lowest or highest end of the scale could be risky. Some compounds could be more affected than others by certain parameters, meaning that it could be safer to choose one of the middle values when the differences in intensities are not that large. It is generally recommended to use lower spray voltage values to increase stability with ESI [57]. 3.5 kV was therefore used in the experiments described subsequently.

Other researchers also use 3-4 kV (such as Lu 2010 [76] and Pirhaji 2016 [77]). In theory (according to **Equation 3**), higher voltage increases the strength of the electric field. This should in turn increase sensitivity. However, above a certain potential between the capillary and the counter electrode, corona discharge is likely to occur [58]. This creates an unstable electrospray, thereby decreasing signal intensity. This is likely to be the reason that the measured intensity in the electrospray voltage experiments in most cases decreased with the highest tested electrospray voltages.

It is also likely that droplet composition affects intensity. At the beginning of the mobile phase gradient, the mobile phase consisted of 95 % water with 0.1 % formic acid, while at the end; it consisted of 100 % methanol with 0.1 % formic acid. As different droplet compositions can have different optimal electrospray voltages, the results may have been different if the voltage was changed throughout the analysis to fit the mobile phase gradient [56], but this was not investigated further in this project.

For most compounds in all sample types, the average intensities increased after the turbomolecular pump was replaced (shown in Table 20 in the **Appendix**, section 6.1). In standard mix with negative ionization mode, D3 acylcarnitine C16 was not detected in the

sample analyzed after the pump was replaced. In positive mode, the same compound's intensity decreased slightly. For standard mix spot (in both ionization modes) and standard mix + blood spot in negative ionization mode the intensities for all standard mix compounds increased, and three compounds that were not detected before were detected after the pump was replaced (see section **4.1.1** for criteria of what was considered a detected compound). For standard mix + blood spot in positive mode, all compound intensities increased, except for acylcarnitines D3 C2 and D3 C16. Overall, the pump replacement had a positive impact on peak intensity. The extreme vacuum conditions in mass spectrometers are important to achieve high sensitivity, as this minimizes interferences caused by the ions colliding with neutral background molecules. This, in turn, facilitates ion transmission to the detector [78]. The results before and after pump replacement serve as an example of the fact that, in a diagnostic routine setting, instrumentation should be regularly checked to see if service or replacement is necessary. Additionally, this points to the importance of standardization using long time quality controls.

In summary, 3.5 kV was considered to be the best electrospray voltage value, as 3.5 kV gave high intensities and was one of the values that gave the highest number of detected compounds.

4.4 Effect of electrospray needle position on sensitivity

Electrospray needle position sets the distance between the electrospray needle and the MS inlet.

As **Equation 3** shows (see section **2.2.2.3** for more details), the strength of the electric field (E_c) increases with decreasing distance (d) from the needle tip to the MS inlet [51]. For higher flow rates, a longer distance between the needle and the MS inlet is generally used, while the opposite is generally used for lower flow rates. A shorter distance increases the amount of spray liquid entering the MS, but, it also decreases the time available for gas phase ions to be released [55]. Based on this, it was expected that a needle position with a short distance to the MS inlet would give the highest peak intensity.

$$E_c = \frac{2V_c}{r_c \ln\frac{4d}{r_c}} \qquad \text{Eq. 3}$$

All four needle positions were tested to see which gave the highest peak intensity and the highest number of detected compounds – A, B, C and D, see **Figures 17-19**. For other settings, see **Table 5**. Position A gives the shortest distance between the ESI needle and the MS inlet, D gives the longest. The difference between the positions is approximately 3.5 mm.

For the standard mix sample in negative mode, position B gave the highest intensities for most compounds, see **Figure 17**. All compounds were detected with all needle positions in negative mode, except for D3 acylcarnitine C16 with position D. In positive mode, the highest intensities for most compounds were measured using position C. All compounds were detected with all needle positions in positive mode (except D2 glycolic acid and D6 glucose, which were not detected in any samples in positive mode), see **Figure 17**.

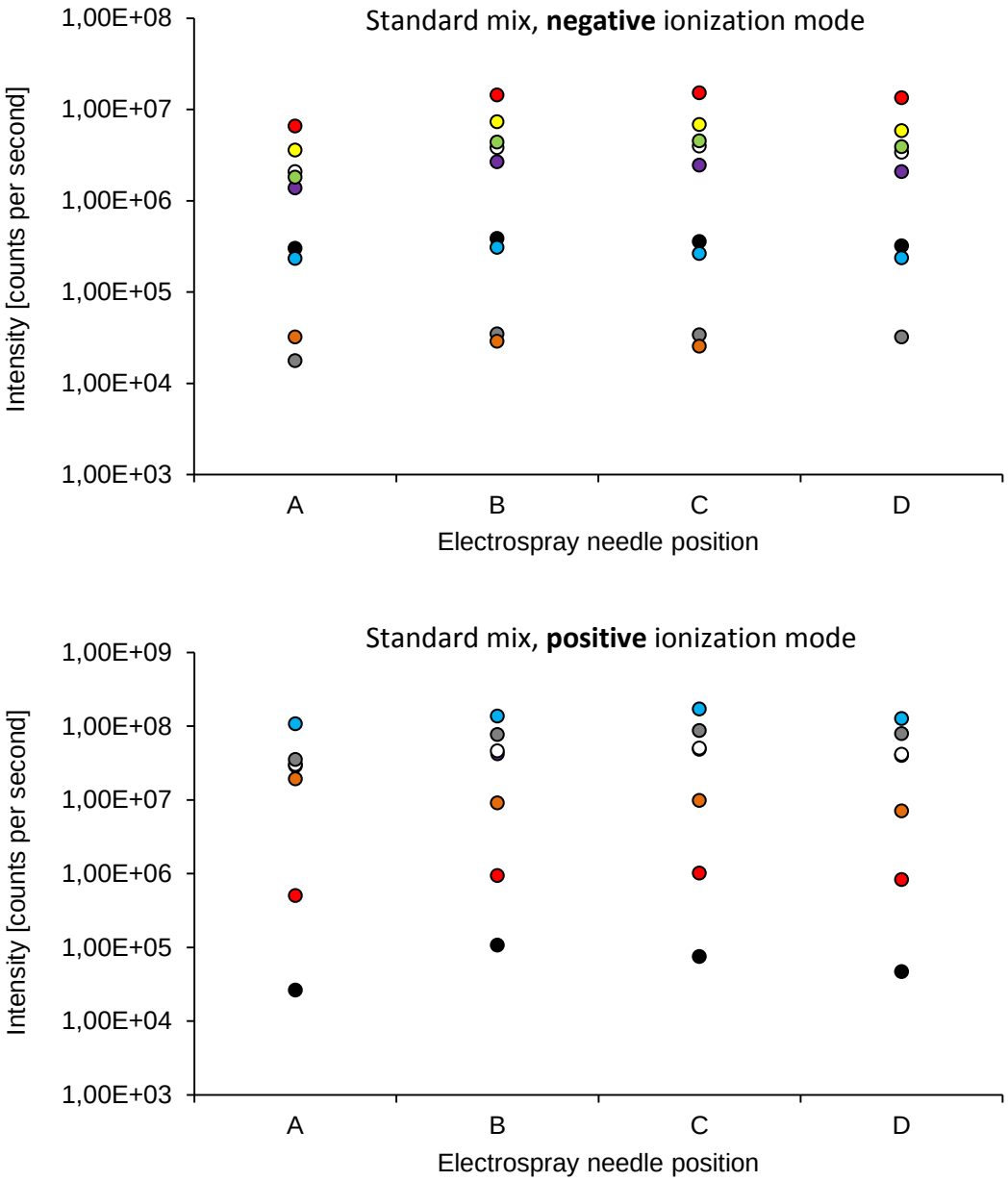


Figure 17 Measured intensity of all compounds in standard mix, negative (top) and positive (bottom) ionization mode, for all electrospray needle positions tested. Intensity shown is the average intensity measured from three injections. The color code for each compound is shown in **Table 6**.

For the standard mix spot sample in negative mode, the highest intensities for most compounds were measured using position D. Position A gave the lowest number of detected compounds, see **Figure 18**. In positive mode, the same number of compounds was detected with all positions, and two of the four detected compounds had the highest intensity with position D, see **Figure 18**.

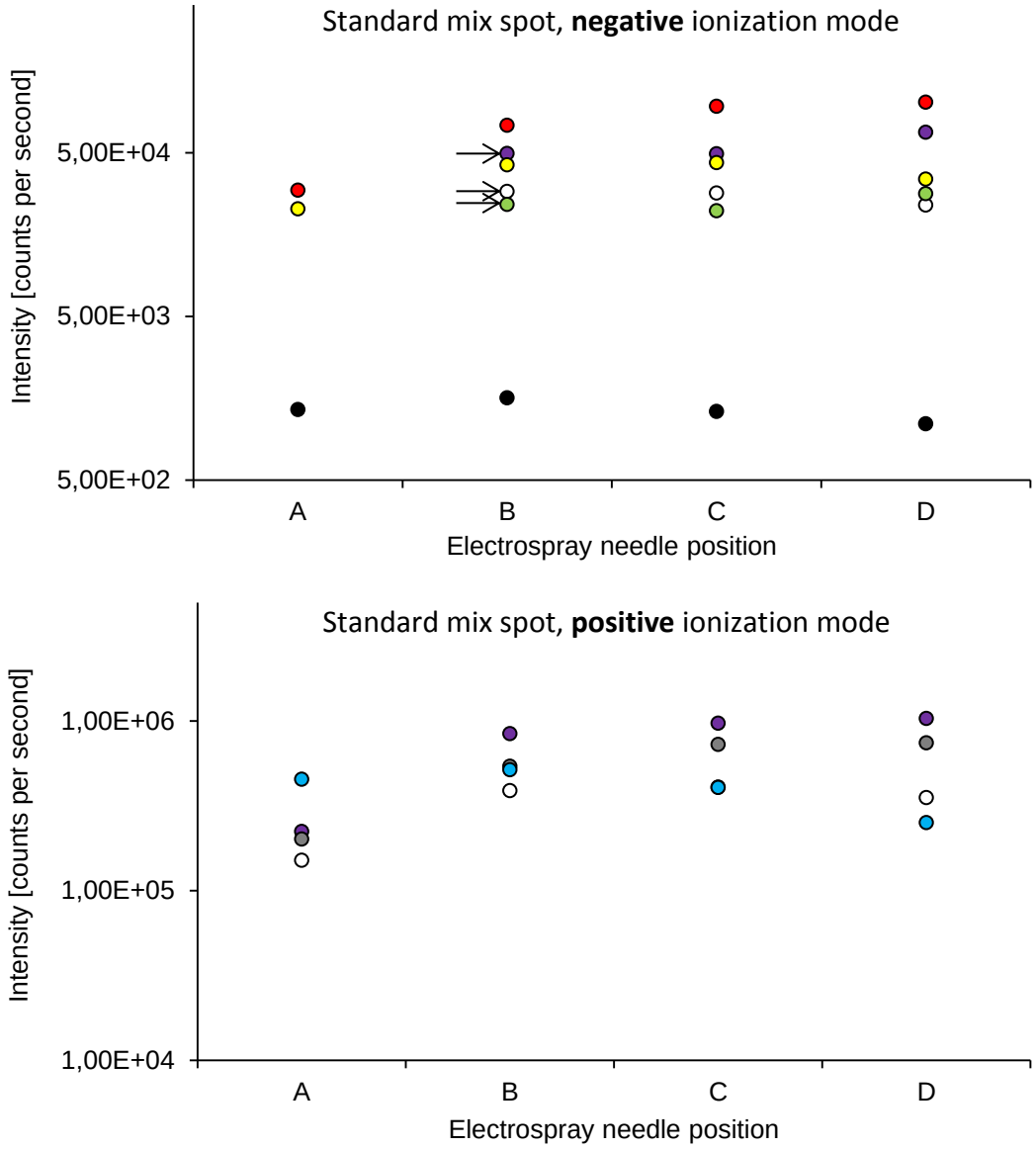


Figure 18 Measured intensity of all compounds in standard mix spot, negative (top) and positive (bottom) ionization mode, for all electrospray needle positions tested. Intensity shown is the average intensity measured from three injections. Arrows show for which value some of the compounds were first detected. The color code for each compound is shown in **Table 6**.

For the standard mix + blood spot sample in negative mode, position B and C gave the highest intensities, see **Figure 19**. Position A gave the lowest number of detected compounds in both ionization modes. In positive mode, position C gave the highest intensity for most compounds, see **Figure 19**.

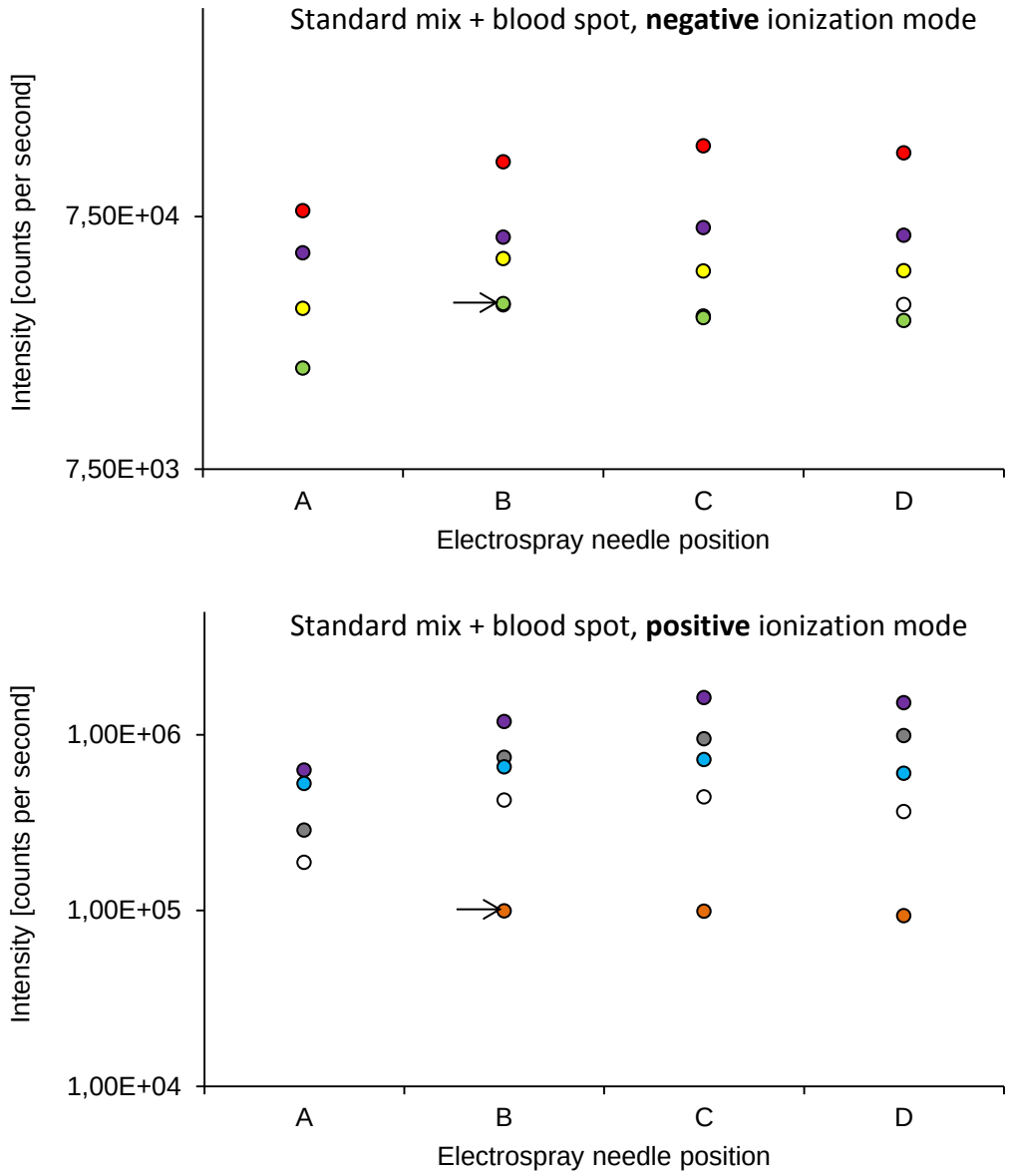


Figure 19 Measured intensity of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all electrospray needle positions tested. Intensity shown is the average intensity measured from three injections. Arrow in the negative ionization mode chart shows where $^{13}C_2$ guanidinoacetate was first detected (white dot, not visible in the figure as the measured intensity was almost exactly the same as for D2 glycolic acid (green dot)). Arrow in the positive ionization mode chart shows where D3 acylcarnitine C16 was first detected. The color code for each compound is shown in **Table 6**.

Position A was not considered to be a suitable choice as it gave the lowest number of detected compounds (see section **4.1.1** for criteria of what was considered a detected compound), which was unexpected, as **Equation 3** indicates that the strength of the electric field should increase with decreasing distance between the needle tip and the MS inlet. Tables showing the

average intensity of each compound in each sample type are shown in the **Appendix**, section 6.1 (Tables 27-32).

As discussed earlier, choosing a value on the lower or higher end of the scale can be risky. Based on this, B or C seemed to be good choices. Additionally, if the distance is too short, there could be a chance that the time is too short for the droplets to evaporate and the ions to be released. If the distance is too long, there could be a risk of the ions not making it into the MS inlet [55]. The advice from the supplier of the MS was to choose B or C, as both positions should work well with the flow rate used (150 µL/min).

As position C gave high intensities, as well as being one of the positions that gave the highest number of detected compounds for all sample types, C was considered to be the best choice, and was used in further experiments.

In summary, an important difference between the electrospray needle positions tested was that the lowest number of compounds was detected with position A. Electrospray needle position C was considered to be the best choice, based on number of compounds detected and measured peak intensities.

4.5 Effect of resolution on sensitivity

For untargeted metabolomics, due to the extreme complexity of the samples to be analyzed, a high resolution is required. However, high resolution can lead to lower sensitivity, as a longer scan time is needed for high resolution [32, 43]. Given this, it was considered reasonable to choose one of the middle values of resolution.

All available resolution values were tested, with different maximum injection times for each value (shown in **Table 7)**, to see which value gave the highest peak intensities. Other settings are shown in **Table 5**. Results are shown in **Figures 20-22**. Tables showing the average intensity of each compound in each sample are shown in the **Appendix**, section 6.1 (Tables 33-38).

Table 7 Maximum injection time for each resolution value.

Resolution (a.u.)	Max IT (ms)
17 500	50
35 000	125
70 000	250
140 000	500

Max IT for each resolution value was chosen based on advice from the supplier.

For the standard mix sample, there were no obvious trends in measured intensity for any of the resolution values, but, for most compounds, the highest intensity was found with resolution set to 35 000 in both ionization modes, see **Figure 20**.

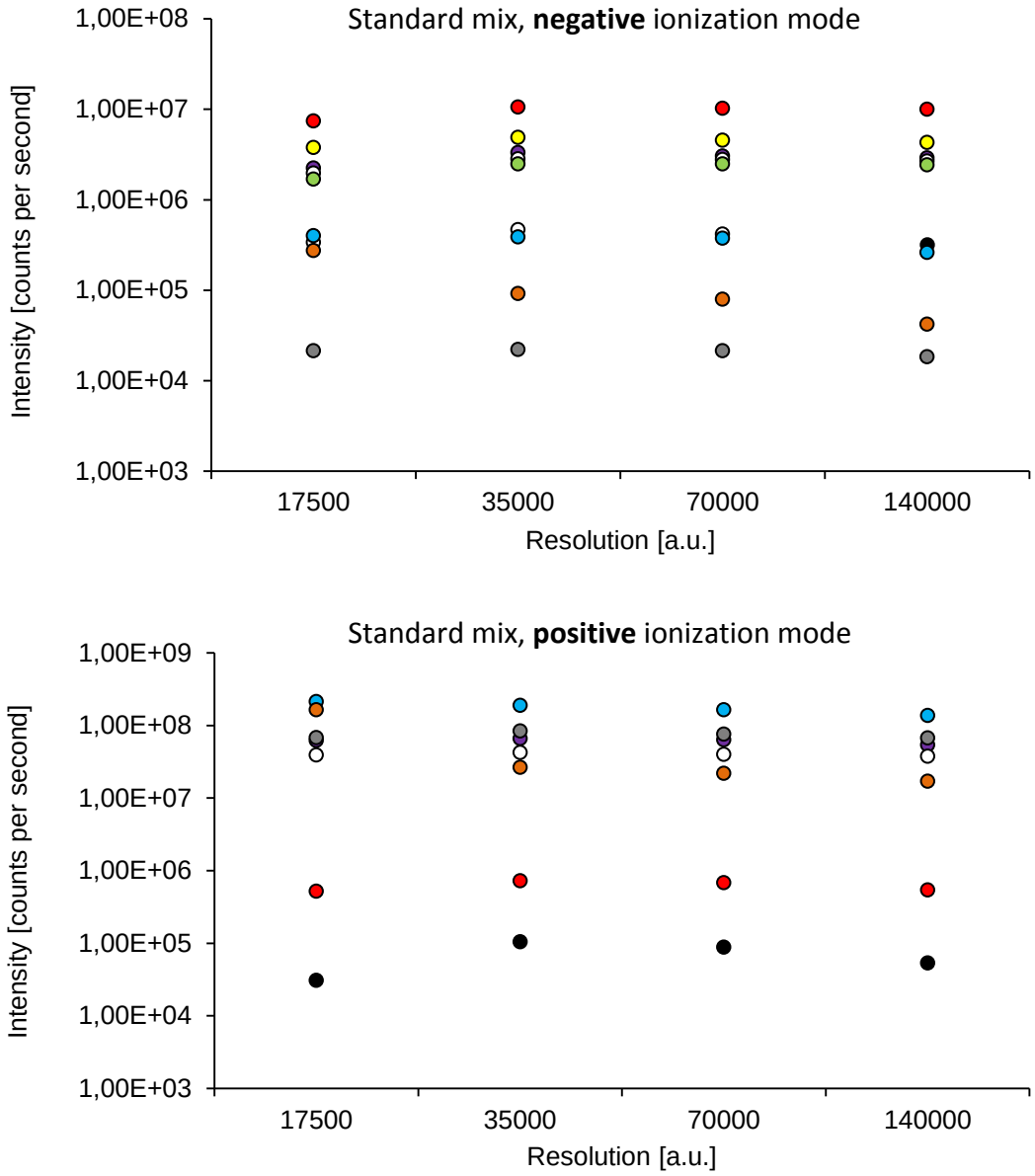


Figure 20 Measured intensity of all compounds in standard mix, negative (top) and positive (bottom) ionization mode, for all resolution values tested. Intensity shown is the average intensity measured from three injections. The color code for each compound is shown in **Table 6**.

For the standard mix spot sample in both ionization modes, more compounds were detected using 70 000 and 140 000 than the lower values, see **Figure 21**. See section **4.1.1** for criteria of what was considered a detected compound.

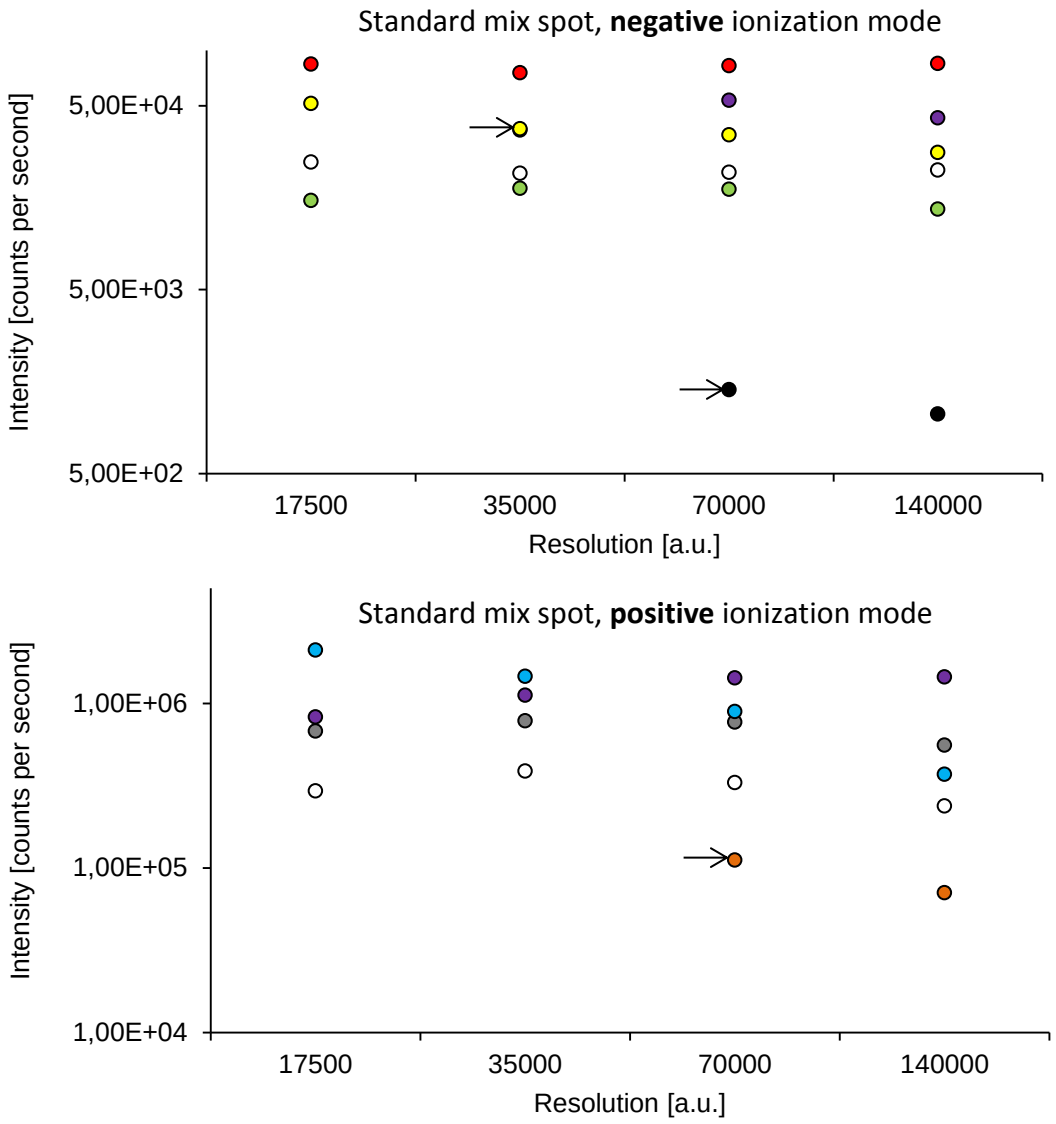


Figure 21 Measured intensity of all compounds in standard mix spot, negative (top) and positive (bottom) ionization mode, for all resolution values tested. Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected (^{13}C creatine was first detected with resolution set to 35 000 in negative mode. The purple dot is not visible behind the yellow dot as ^{13}C creatine had almost exactly the same intensity as D6 glucose). The color code for each compound is shown in **Table 6**.

For the standard mix + blood spot sample, more compounds were detected with resolution set to 70 000 and 140 000 than the lower values in both ionization modes, see **Figure 22**.

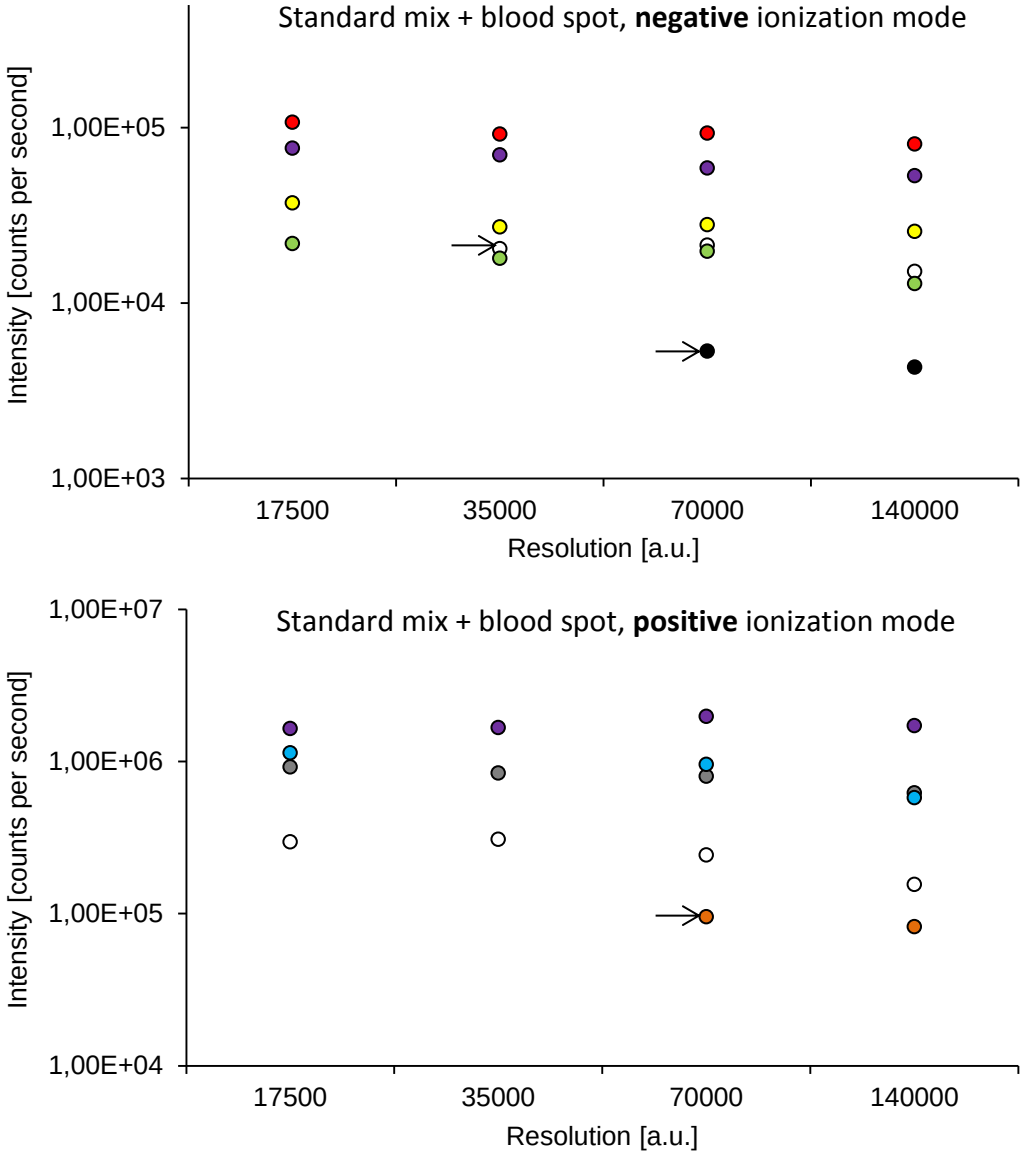


Figure 22 Measured intensity of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all resolution values tested. Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected. The color code for each compound is shown in **Table 6**.

Resolution set to 70 000 and 140 000 gave the highest number of detectable compounds for the spot samples. This was expected as higher resolution makes it more likely that the MS distinguishes between ions of similar masses, thereby detecting more compounds [32]. However, a bigger difference in peak intensities was expected as higher resolution generally leads to lower sensitivity. This is because there is a risk of losing ions when slits and lenses are narrow, and because of the longer scan time, which can decrease the number of data points across the peaks [32, 43]. A possible explanation for this unexpected preservation of sensitivity is that the concentration of the standard mix compounds was too high for the effects of resolution to be seen.

To decide which resolution value would be the best choice – 70 000 or 140 000, both were used to test AGC target values.

To summarize, resolution set to 70 000 or 140 000 appeared to be better choices than lower resolution values because more compounds were detected with resolution set to 70 000 and 140 000 than with 17 500 and 35 000. It was decided that more information was needed to be able to decide between 70 000 and 140 000.

4.6 Effect of AGC target value on sensitivity

Setting the AGC target value too high increases the risk of space charge effects. In addition, the scan rate is slowed as the AGC target value is increased [48]. As both of these scenarios negatively affect the sensitivity, it was expected that the intensity would decrease as the AGC target value was increased.

The available AGC target values were tested to see which gave the highest peak intensities. This was done in two separate experiments – with resolution set to 70 000 (250 ms max IT) and 140 000 (500 ms max IT).

4.6.1 Resolution 70 000

Results for AGC target value optimization experiments with resolution set to 70 000 are shown in **Figures 23-25**. For other settings, see **Table 5**. Tables showing the average intensity of each compound in each sample are shown in the **Appendix**, section 6.1 (Tables 39-44).

For the standard mix samples analyzed in negative ionization mode, all compounds were detected with high intensities using an AGC target value of 1 000 000 ion counts. This was also the case for positive ionization mode, as all compounds except D6 glucose and D2 glycolic acid (which were not detected in any samples using positive ionization) were detected (see **Figure 23**). The number of detected compounds increased as the AGC target value increased.

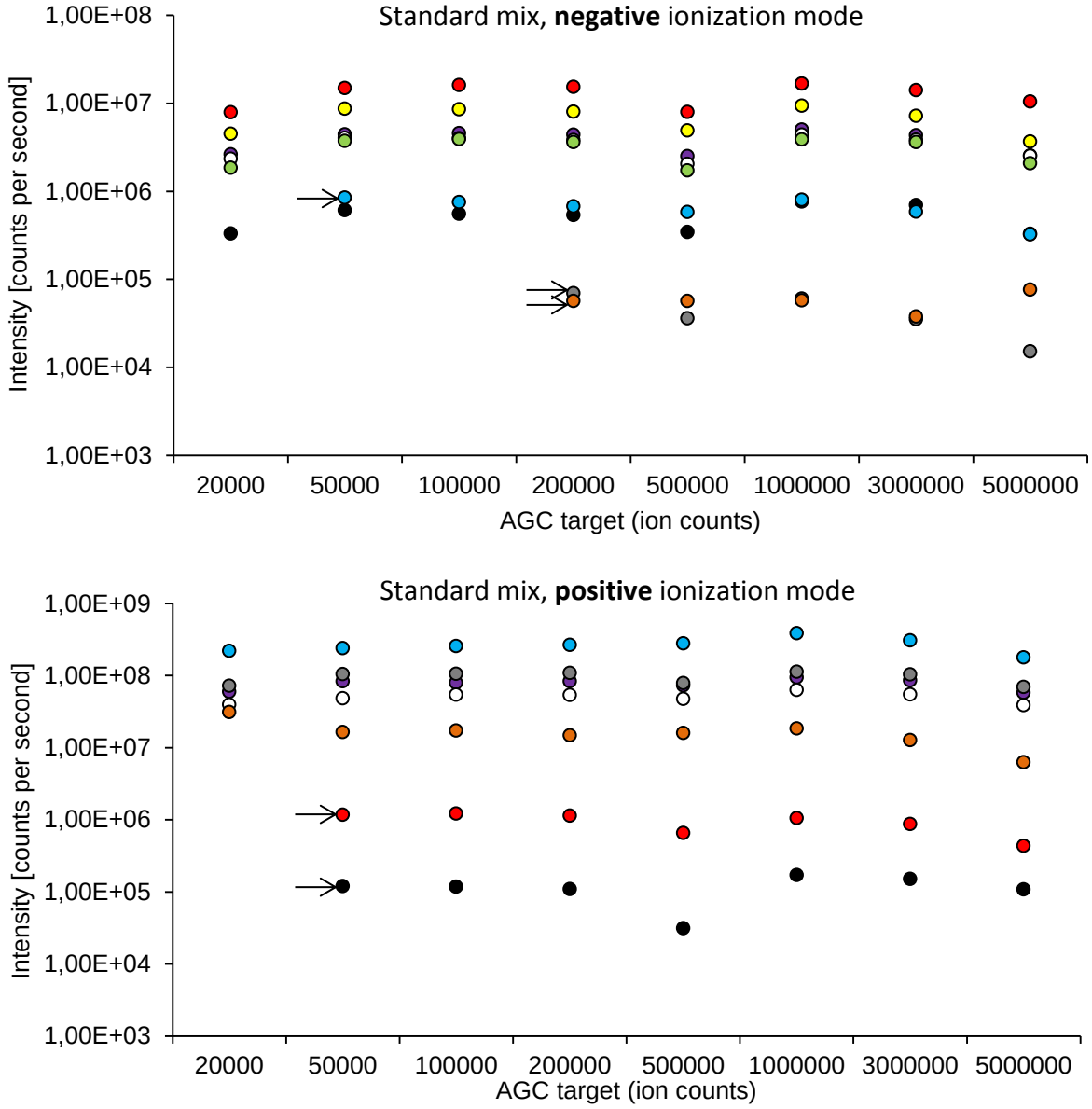


Figure 23 Measured intensity of all compounds in standard mix, negative (top) and positive (bottom) ionization mode, for all AGC target values tested (resolution 70 000). Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected. The color code for each compound is shown in **Table 6**.

For the standard mix spot samples analyzed in negative mode, 1 000 000 ion counts gave the highest intensities for most compounds, see **Figure 24**. D3 acylcarnitine C12 was only detected when an AGC target value of 3 000 000 was used. However, the intensity was low and the peak had a poor chromatographic shape, meaning that it barely met the list of criteria for what was considered a detected compound (see section **4.1.1**). In positive mode, 1 000 000 ion counts also appeared to be the best choice, see **Figure 24**. Again, more compounds were detected with increasing AGC target value.

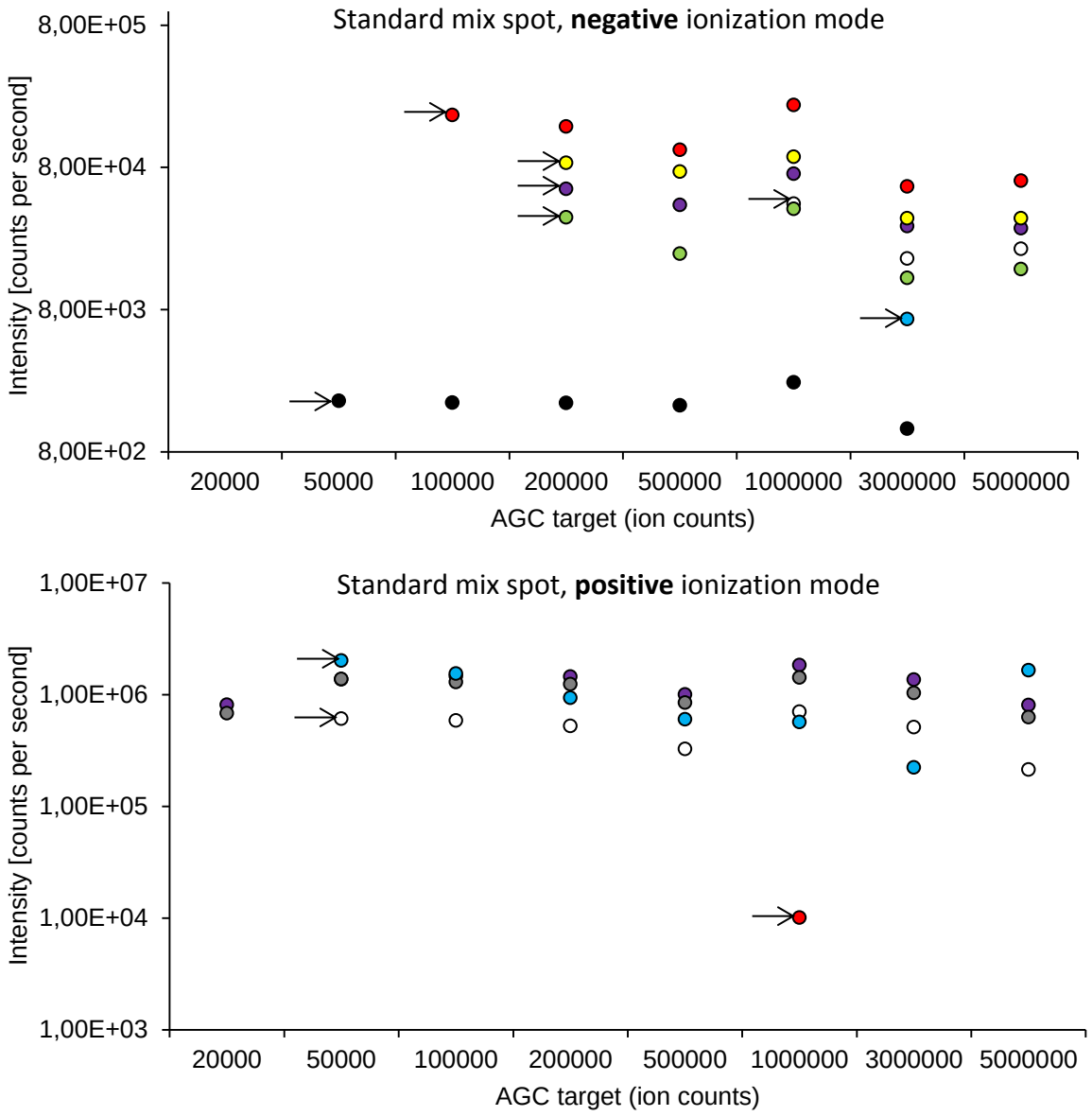


Figure 24 Measured intensity of all compounds in standard mix spot, negative (top) and positive (bottom) ionization mode, for all AGC target values tested (resolution 70 000). Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected. The color code for each compound is shown in **Table 6**.

For the standard mix + blood spot samples, the results coincide with the other samples – an AGC target value of 1 000 000 ion counts gave the highest intensities for most compounds (see **Figure 25**). Also, 1 000 000 ion counts was one of the values where the most compounds were detected. The number of detected compounds increased as the AGC target value increased.

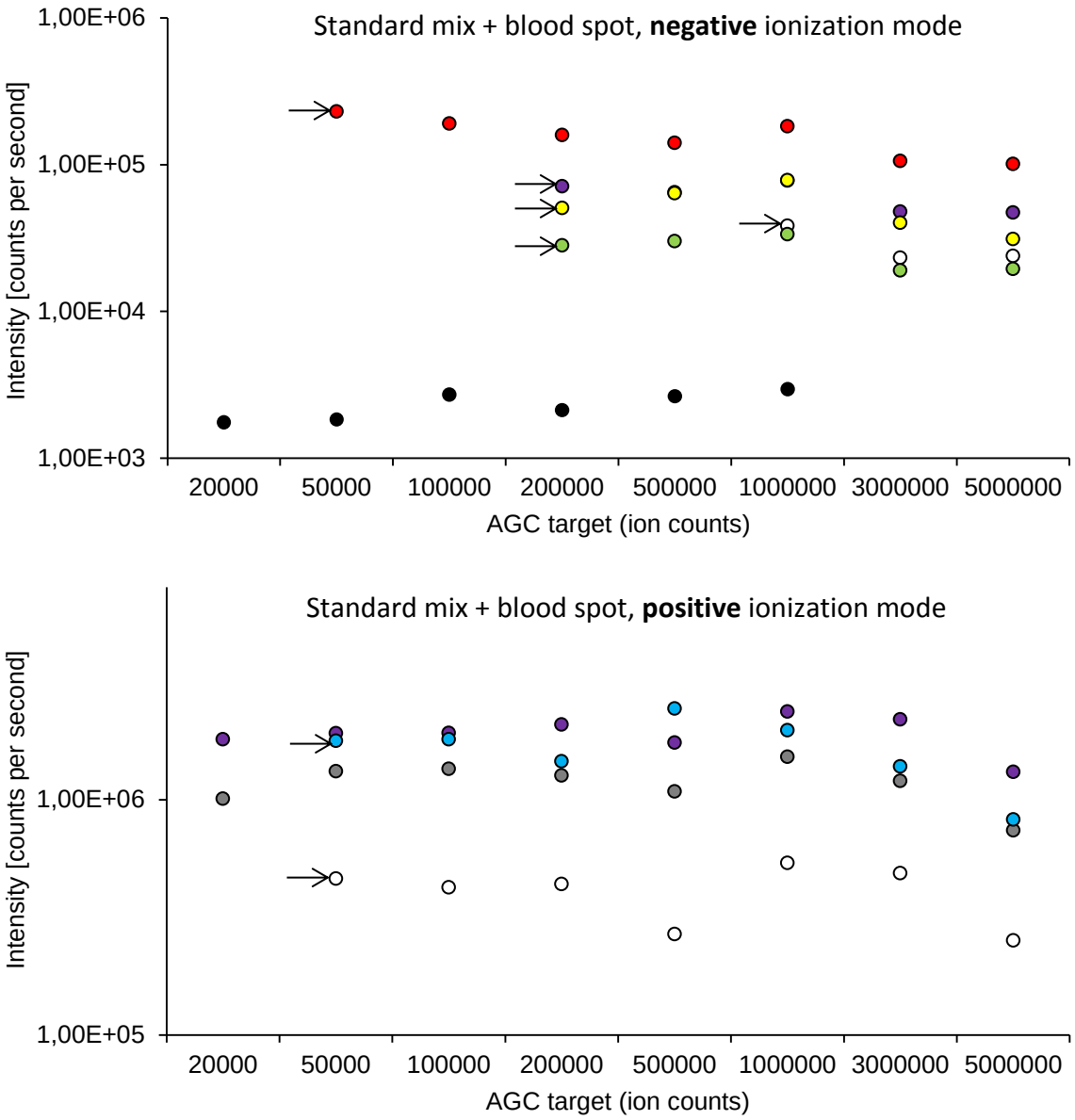


Figure 25 Measured intensity of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all AGC target values tested (resolution 70 000). Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected. The color code for each compound is shown in **Table 6**.

Overall, 1 000 000 ion counts appeared to be the best choice as this gave the highest intensities for most compounds in all sample types. Also, with one exception, 1 000 000 ion counts was one of the values that gave the highest number of detectable compounds. Resolution set to 70 000 and AGC target value of 1 000 000 ion counts seemed to be suitable choices for the method being developed. The results were compared to the AGC target value optimization experiments with resolution set to 140 000 before a final decision was made.

To summarize, with resolution set to 70 000, an AGC target of 1 000 000 ion counts was found to be the best choice. In general, the number of detected compounds increased with increasing AGC target value.

4.6.2 Resolution 140 000

Figures 26-28 show the results of the AGC target value test with resolution set to 140 000. For settings other than resolution, see **Table 5**. Tables showing the average intensity of each compound in each sample are shown in the **Appendix**, section 6.1 (Tables 45-50).

For the standard mix sample in both ionization modes, 1 000 000 ion counts gave the highest intensity for most compounds. Also, 1 000 000 was one of the values which gave the highest number of detected compounds, see **Figure 26**. See section **4.1.1** for criteria of what was considered a detected compound.

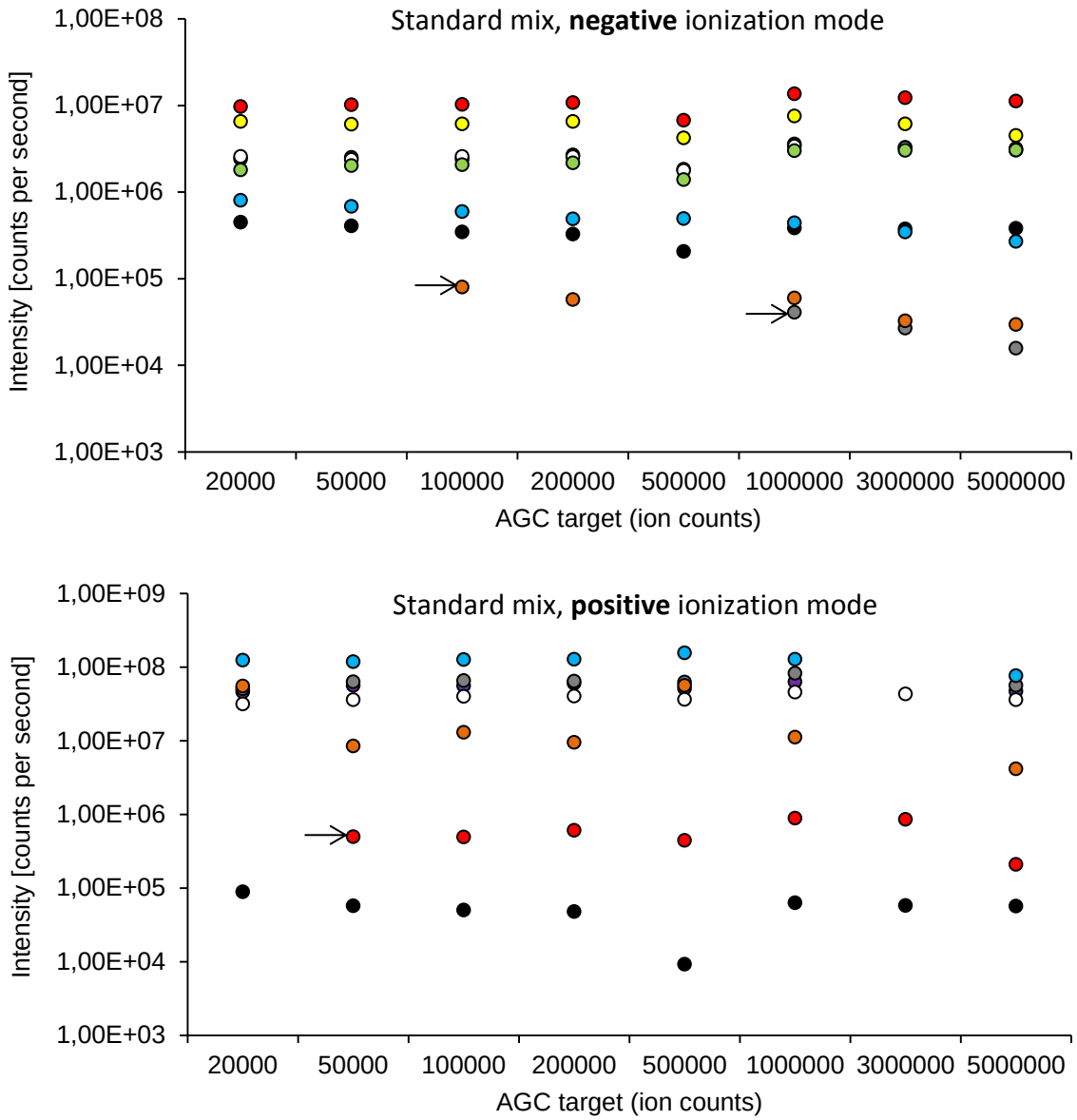


Figure 26 Measured intensity of all compounds in standard mix, negative (top) and positive (bottom) ionization mode, for all AGC target values tested (resolution 140 000). Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected. The color code for each compound is shown in **Table 6**.

For the standard mix spot sample, the highest number of compounds was detected with AGC target set to 3 000 000 ion counts in negative mode. In positive mode, 200 000 and 1 000 000 ion counts gave the highest number of detected compounds, see **Figure 27**. 1 000 000 ion counts gave the highest intensities for most compounds in positive mode.

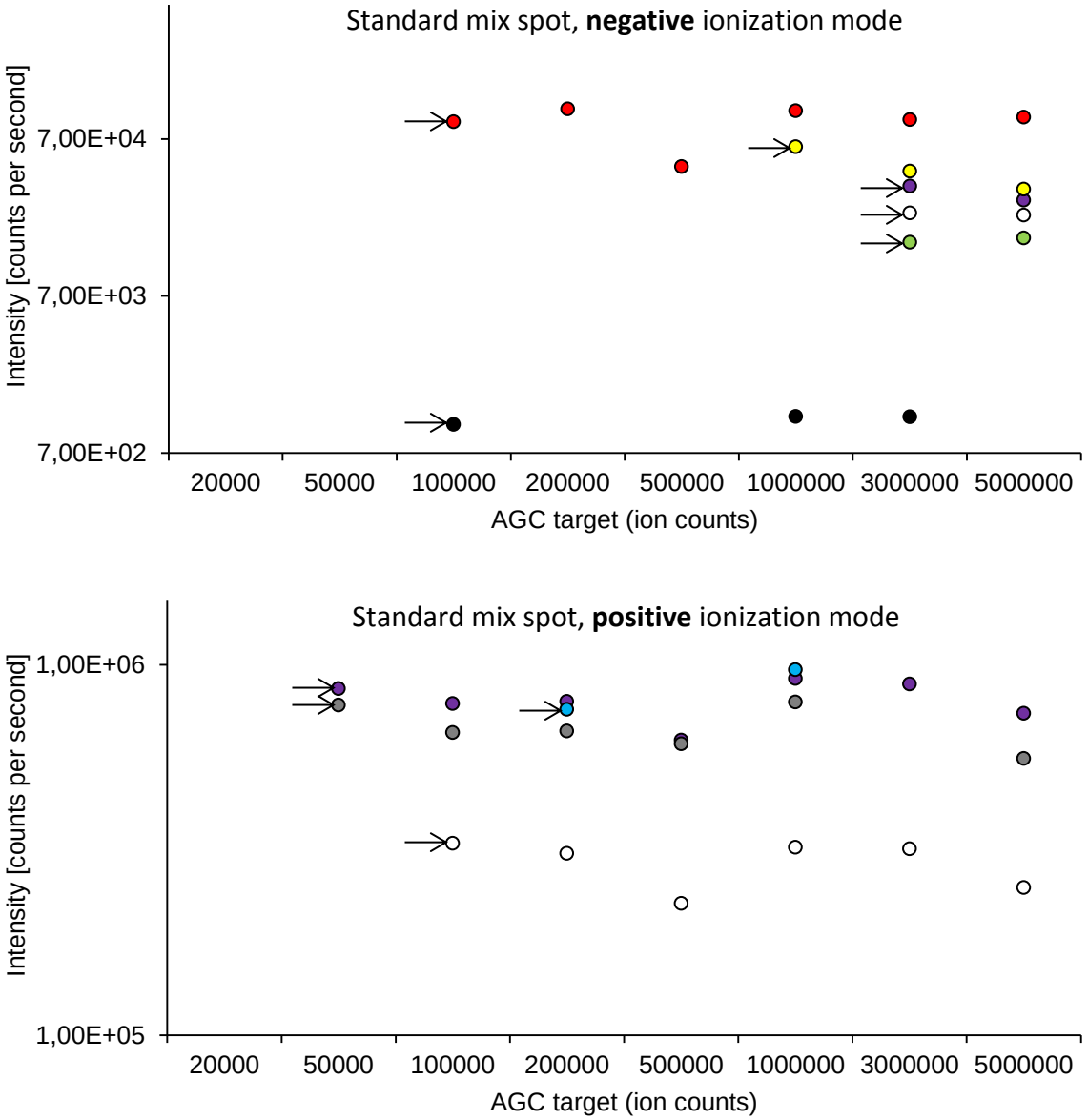


Figure 27 Measured intensity of all compounds in standard mix spot, negative (top) and positive (bottom) ionization mode, for all AGC target values tested (resolution 140 000). Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected. The color code for each compound is shown in **Table 6**.

For the standard mix + blood spot sample in negative mode, 1 000 000 ion counts gave the highest intensities for most compounds. 1 000 000 ion counts was one of the values that gave the highest number of detected compounds in both ionization modes, see **Figure 28**. The number of detected compounds increased as the AGC target value increased.

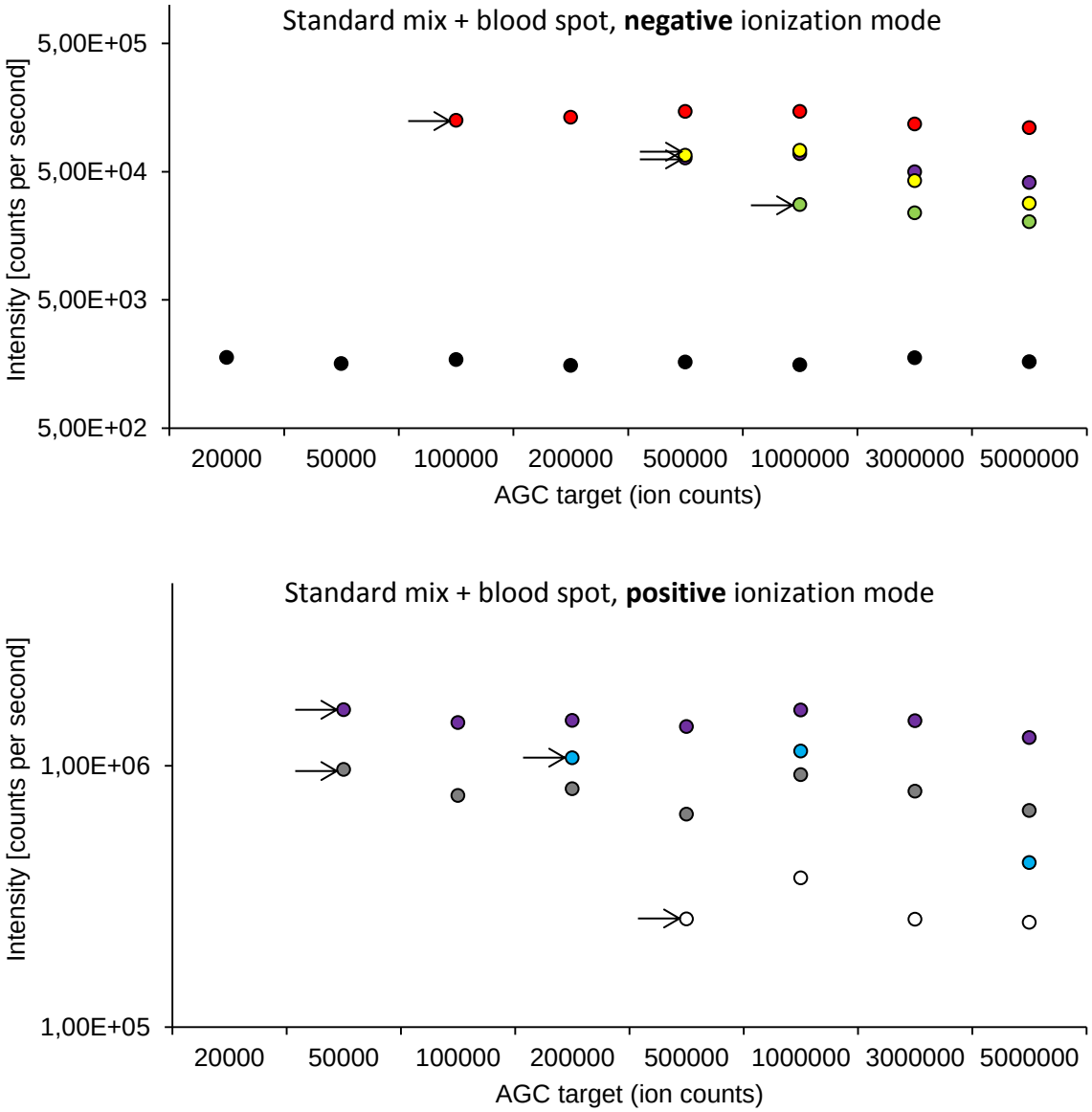


Figure 28 Measured intensity of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all AGC target values tested (resolution 140 000). Intensity shown is the average intensity measured from three injections. Arrows show where some compounds were first detected. The color code for each compound is shown in **Table 6**.

The best choice appeared to be 1 000 000 ion counts, as this gave the highest intensity for most compounds and highest number of detected compounds for most sample types.

In summary, with resolution set to 140 000, an AGC target of 1 000 000 ion counts was considered to be the best choice. Again, the number of detected compounds increased as the AGC target value increased.

4.6.3 Choice of resolution value

To decide the best resolution option, the results with an AGC target value of 1 000 000 ion counts for both resolution values (70 000 and 140 000) were compared (shown in the **Appendix**, section 6.1, Figures 38-43). As 70 000 gave higher intensities for all compounds except one in a single sample type, as well as a higher number of detected compounds, this was considered to be the best choice. The slightly higher intensity with 70 000 was expected as higher resolution generally leads to lower sensitivity [32, 43]. Resolution set to 70 000 and AGC target value set to 1 000 000 ion counts were used in further experiments.

It was expected that the highest AGC target values would give a lower number of detected compounds and lower intensities, as high AGC target values can lead to space charge effects, thereby decreasing sensitivity [48]. However, fewer compounds were detected when lower AGC target values were used. The reason for this is unclear. It may be because the number of collected ions in the C-trap was too low for detection of most compounds to occur. Another study which optimized AGC target value for quantitative bioanalyses reported that detection was improved with increasing number of ions of interest in the Orbitrap, in compliance with the results in this project [79].

In summary, resolution set to 70 000 and AGC target set to 1 000 000 ion counts were considered to be the best choices based on the number of detected compounds and peak intensity.

4.6.4 Chromatograms of analysis performed with resolution set to 70 000 and automatic gain control set to 1 000 000 ion counts

TICs and EICs of all detected compounds in standard mix and standard mix + blood spot, for the analysis using a resolution of 70 000 and AGC target 1 000 000 ion counts are shown in **Figures 29-32**. For standard mix spot, TICs and EICs are shown in the **Appendix**, section 6.3 (Figures 48-49).

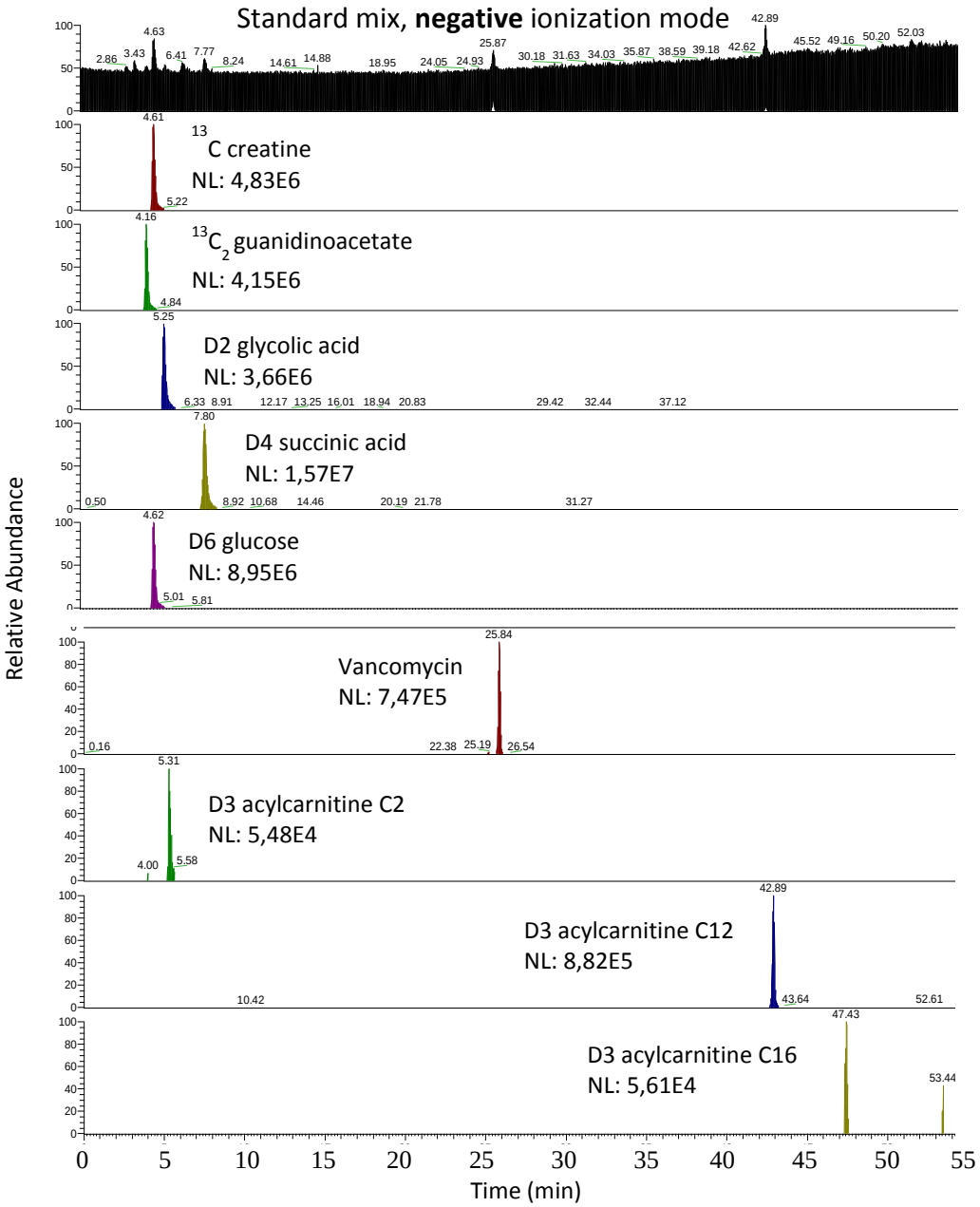


Figure 29 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix, negative ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed. Analysis was performed using the initial settings except for AGC target value, which was set to 1 000 000 ion counts.

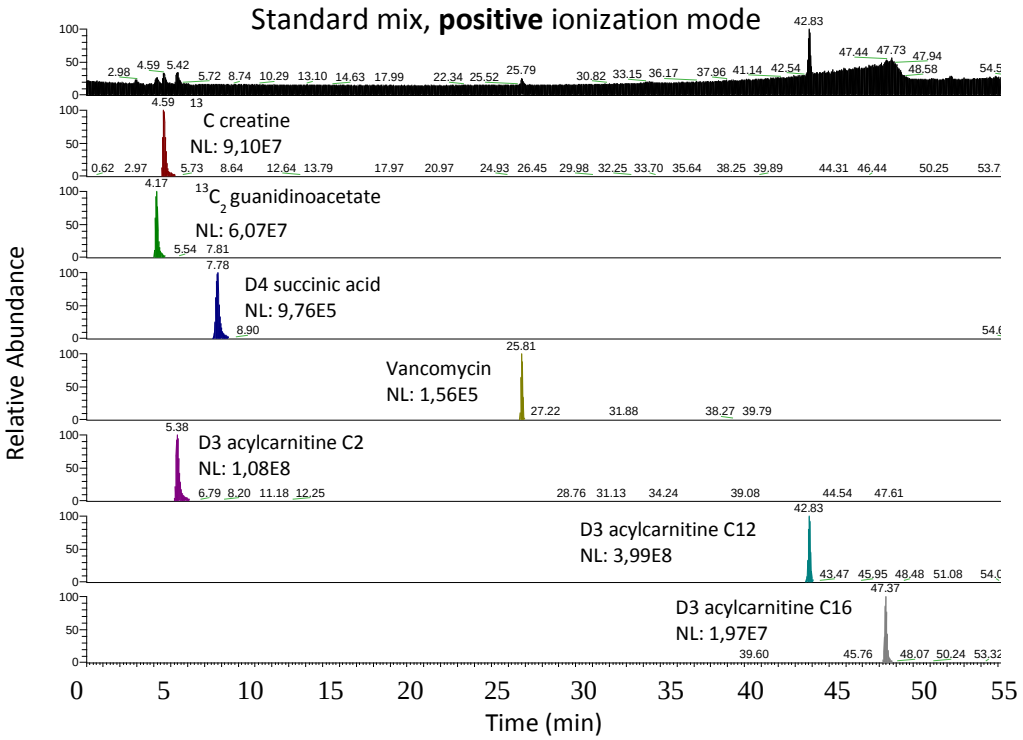


Figure 30 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix, positive ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed. Analysis was performed using the initial settings except for AGC target value, which was set to 1 000 000 ion counts.

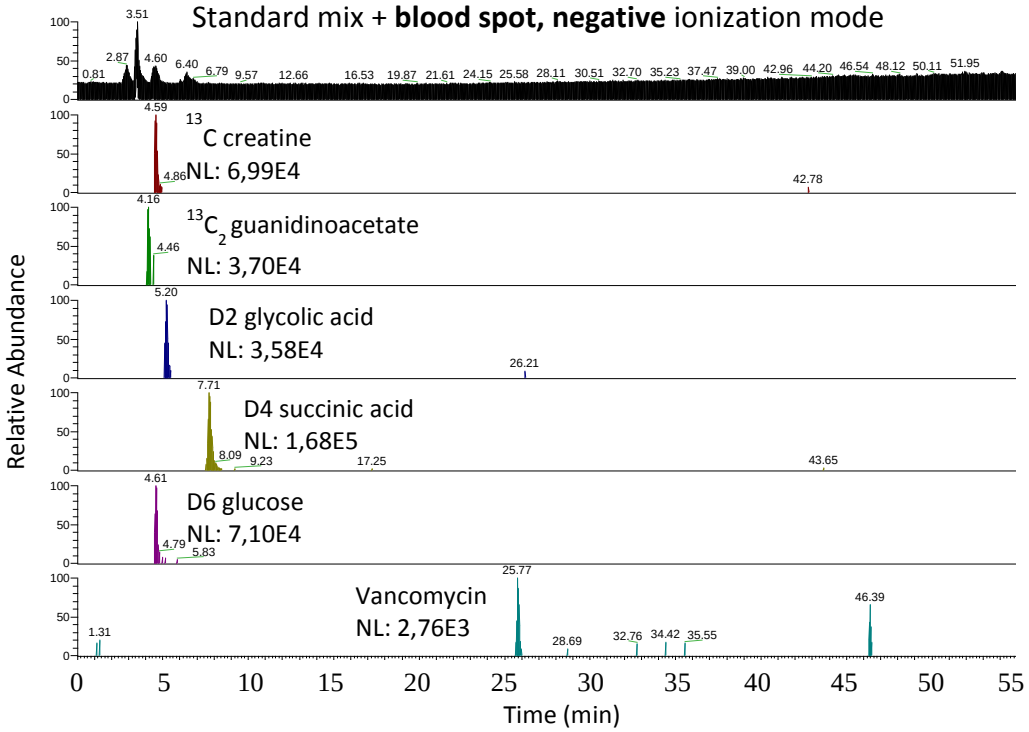


Figure 31 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix + blood spot, negative ionization mode, with a concentration of 5 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each compound peak is listed. Analysis was performed using the initial settings except for AGC target value, which was set to 1 000 000 ion counts.

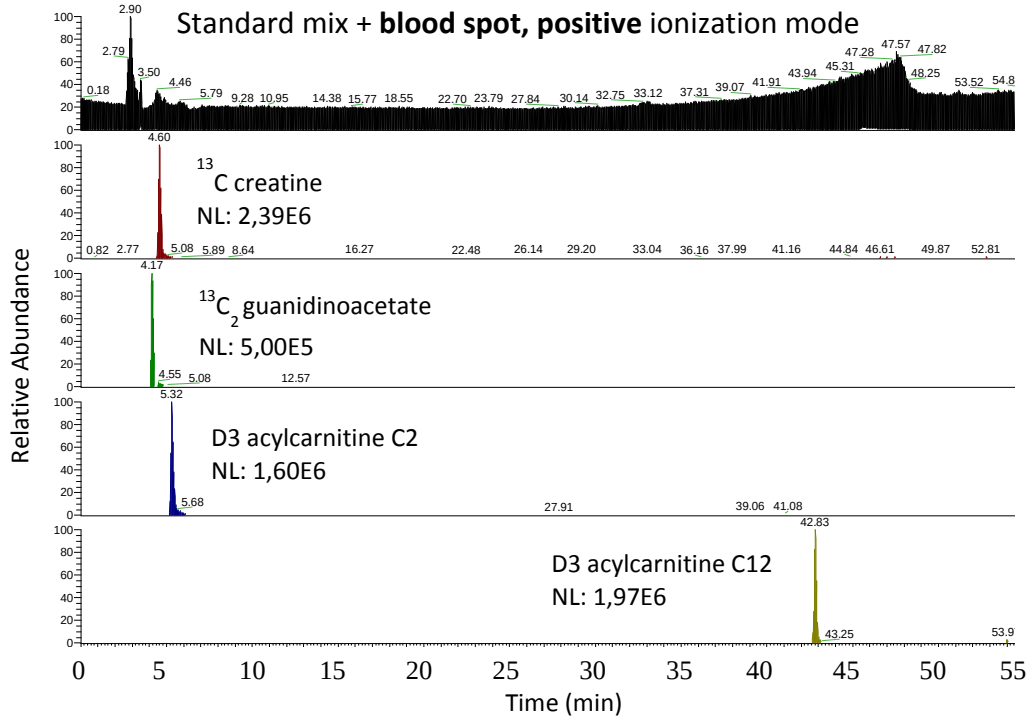


Figure 32 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix + blood spot, positive ionization mode, with a concentration of 5 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each compound peak is listed. Analysis was performed using the initial settings except for AGC target value, which was set to 1 000 000 ion counts.

4.7 Effect of mobile phase flow rate on sensitivity

Parameters that influence electrospray droplet radius, R, are shown in **Equation 4** (see section **2.2.2.3.1** for more details).

$$R \approx \left(\frac{V_f \varepsilon}{K}\right)^{\frac{1}{3}} \qquad \text{Eq. 4}$$

V_f is the flow rate (volume/time) [51]. As **Equation 4** shows, decreasing mobile phase flow rate should decrease the droplet radius. This will in turn increase the sensitivity, because smaller droplets mean that the analyte concentration is increased, which is an advantage for concentration dependent detectors (such as ESI-MS) [62, 63]. Smaller droplets also mean that less solution has to be evaporated for gas phase ions to be released. Based on this, it was expected that signal intensity would decrease as the flow rate was increased.

Several mobile phase flow rates were tested to see which gave the highest peak intensity: 50, 100, 150, 200, 250, and 300 µL/min. Other settings were as shown in **Table 5**, except AGC target (set to 1 000 000 ion counts). **Figure 33** shows the results of the standard mix + blood spot sample, positive ionization mode (the same test was performed for all sample types using both negative and positive ionization, data not shown). As expected, 50 µL/min gave the highest intensity for most compounds, and increasing flow rate resulted in lower intensity. However, the sensitivity drop with increasing flow was less than expected.

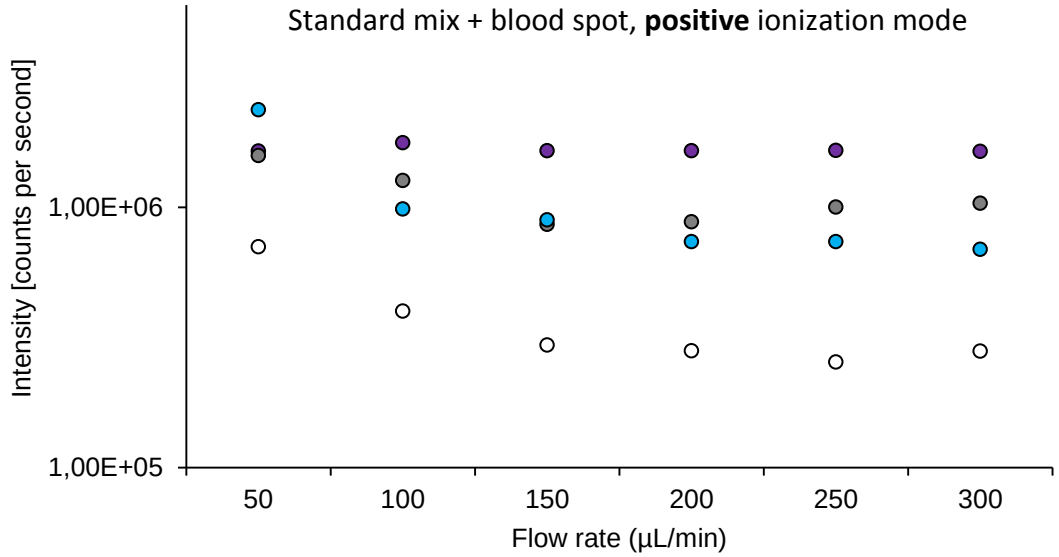


Figure 33 Measured intensity of all compounds in standard mix + blood spot, positive ionization mode, for all mobile phase flow rates tested (with original analysis time, not adjusted to flow rate). Intensity shown is the average intensity measured from three injections. The color code for each compound is shown in **Table 6**.

Initially, the gradient profile was kept constant, and the analysis times were not adjusted to the different flow rates. A more appropriate experiment was done; this time using a constant gradient profile and analysis time adjusted to the flow rates, so that the mobile phase B percentage vs time ratio was the same in all experiments. Three flow rates were tested: 50, 150 and 300 µL/min, and only for the standard mix + blood spot sample (the sample best representing a real patient sample) to save time.

Gradient/analysis times were changed as follows (original gradient shown in **Table 4** and **Figure 8**); this was used for the 150 µL/min experiments): for all 50 µL/min experiments, each original gradient step was prolonged by 2/3. For all 300 µL/min experiments, each original gradient step was halved. **Figure 34** shows the results of the flow rate experiments testing 50, 150 and 300 µL/min. Tables showing the average intensity of each compound are shown in the **Appendix**, section 6.1 (Tables 51-52).

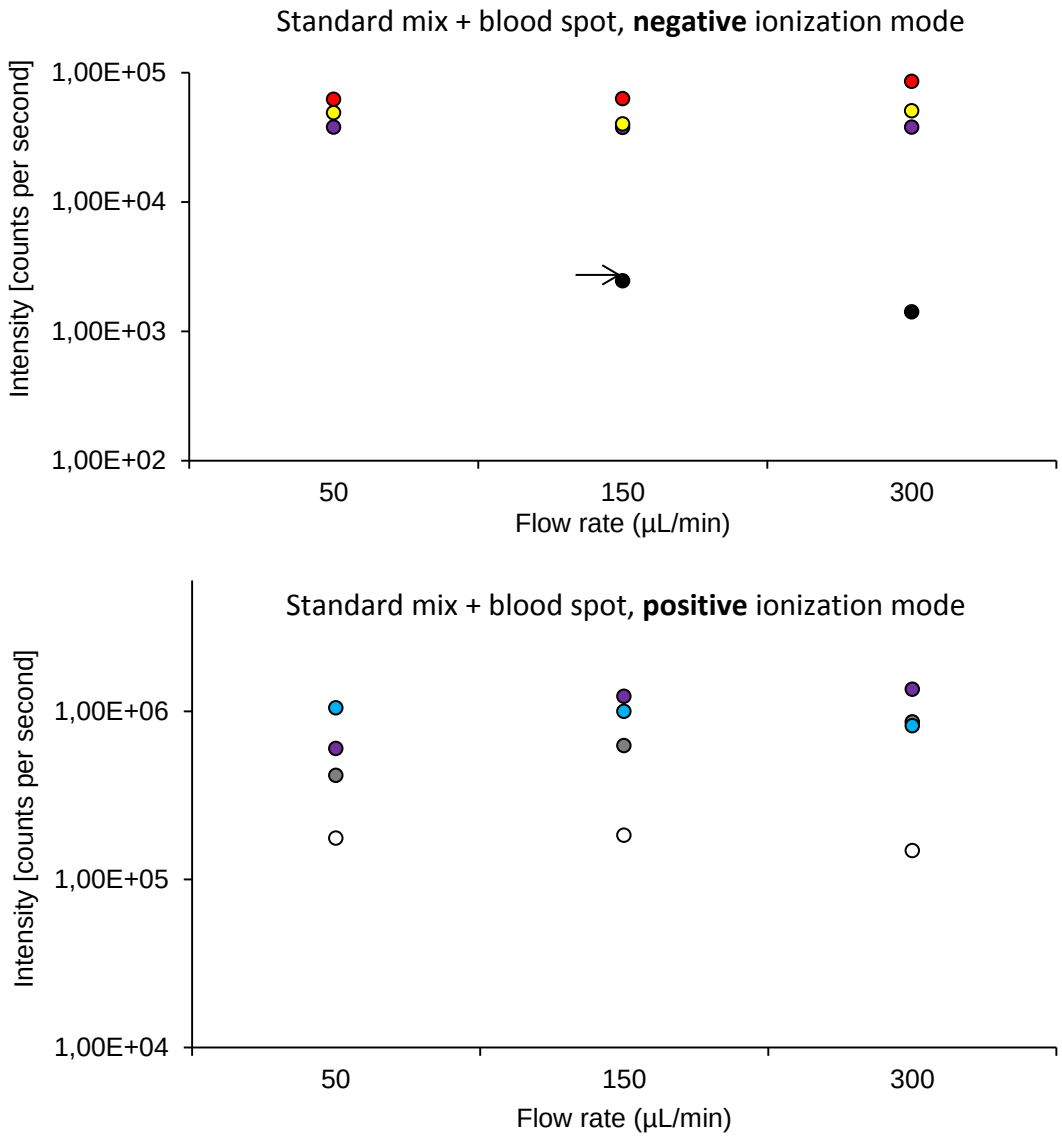


Figure 34 Measured intensity of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all mobile phase flow rates tested (with analysis time adjusted to each flow rate). Intensity shown is the average intensity measured from three injections. The color code for each compound is shown in **Table 6**.

In negative mode, vancomycin was only detected with 150 and 300 µL/min (see section **4.1.1** for criteria of what was considered a detected compound). The highest intensity was found with 300 µL/min for most compounds in both ionization modes. It was unexpected that flow rate did not seem to affect intensity to a larger degree, and that the highest intensities were not found at 50 µL/min as it is generally recommended to use lower flow rates to achieve higher peak intensities using ESI [57]. It is possible that we would have seen a clearer trend if more flow rates had been tested (e.g. higher flow rates). The reason for the unexpected results is not obvious. However, part of the reason could be that the electrospray needle position affects the

results. Position C was used in these experiments. Shorter distances between the needle and the MS inlet are generally used for lower flow rates [55]. If needle position was adjusted for each flow rate, it is possible that we would have seen a larger effect of flow rate.

D3 acylcarnitine C16 was not detected with positive ionization. This compound was detected in previous experiments in positive mode spot samples. The detection problem could potentially be due to stability issues. The stock solution and standard mix used were made 16 and five months respectively prior to flow rate testing, and this could have affected the stability. One study has reported that acylcarnitines are stable on DBS samples stored at -18 °C for at least 330 days. But, as acylcarnitines can hydrolyze to free carnitine and the corresponding fatty acids, correction for sample decay has to be made if the samples are to be used for quantification [80]. DBS samples used in this thesis were either made shortly prior to testing or stored at -80 °C until testing, indicating that the sample compounds should have been stable enough to be used for these types of experiments (where no quantification was done). However, the stock solutions and aqueous standard mix solutions used to make DBS samples were made several months prior to spotting, which could have negatively affected the stability. Long-term stability experiments were not the focus of this thesis, but could have provided useful insights to potential stability issues. Additionally, the RSD % for D3 acylcarnitine C16 was generally high (for examples, see Tables 57, 59, 74 and 87 in the **Appendix**, section 6.2).

To further assess the effect of flow rate on sensitivity, the peak intensity of an endogenous compound (creatinine) originating from the DBS was investigated, see **Figure 35** (and Table 53 in the **Appendix**, section 6.1). Differences in measured peak intensity for creatinine were not considered significant. Peak area was also calculated for the standard mix compounds and creatinine; see Figures 44-45 and Tables 53-55 in the **Appendix**, section 6.1.

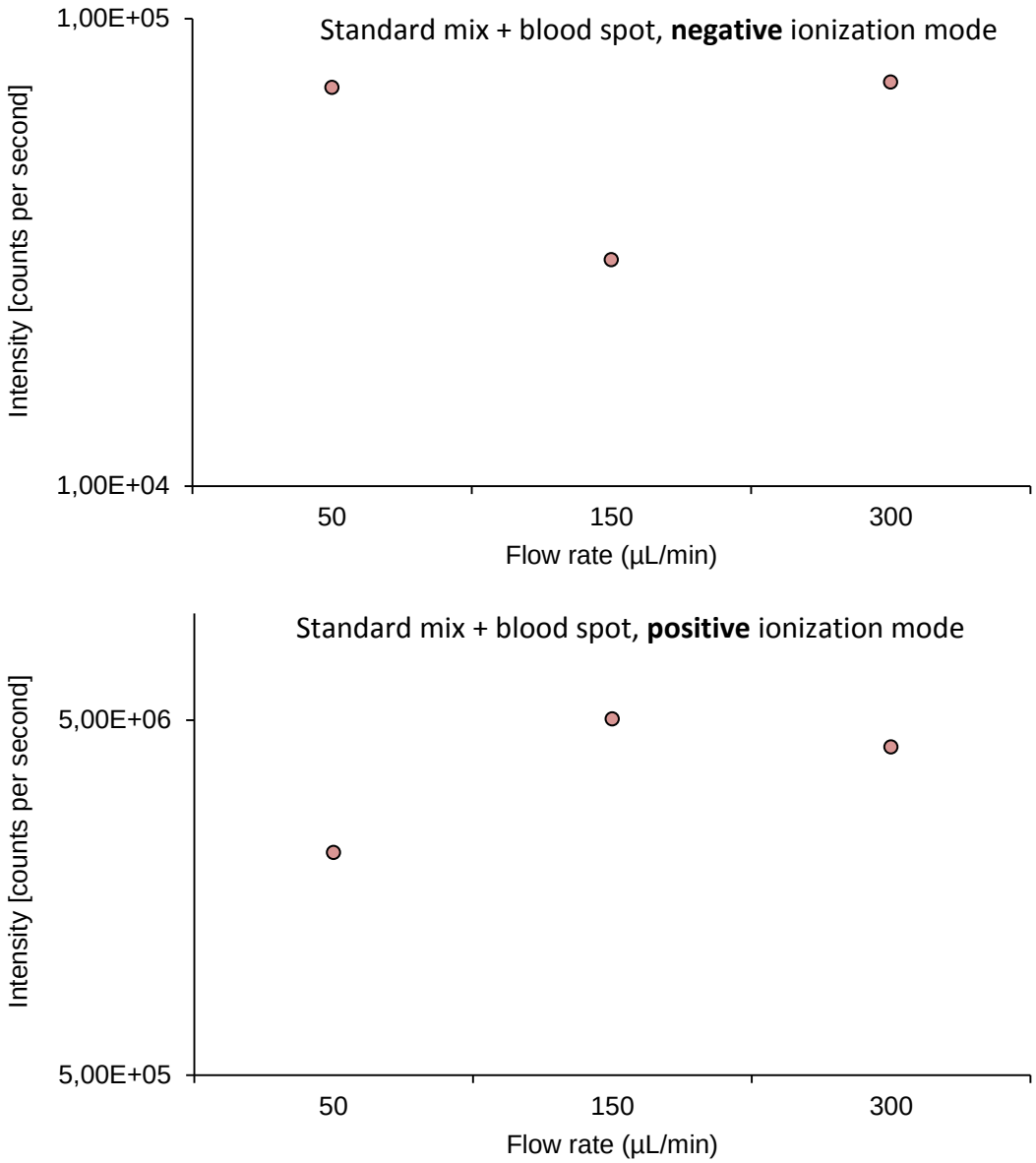


Figure 35 Measured intensity of creatinine in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all mobile phase flow rates tested (with analysis time adjusted to each flow rate). Intensity shown is the average intensity measured from three injections.

Neither peak intensity nor area gave a clear indication of which flow rate should be chosen. 50 µL/min was not considered to be a good option as the chromatographic peaks were very broad (some up to two minutes in length). Also, the total analysis time was considered too long for this flow rate to be of practical use. These flow rates were also tested in another master thesis [74], with regards to peak capacity. There was a large increase in peak capacity from 50 to 150 µL/min, but the peak capacities for 150 and 300 µL/min were similar. Column pressure was also investigated to decide between the two flow rates, see **Figure 36**, as the increased pressure that goes with higher flow rates was a concern. The pressure measured

when using 300 µL/min was not considered to be too high. An important advantage of using 300 µL/min is that this gave an analysis time half that of the original. Based on this, 300 µL/min was chosen as the optimal flow rate option.

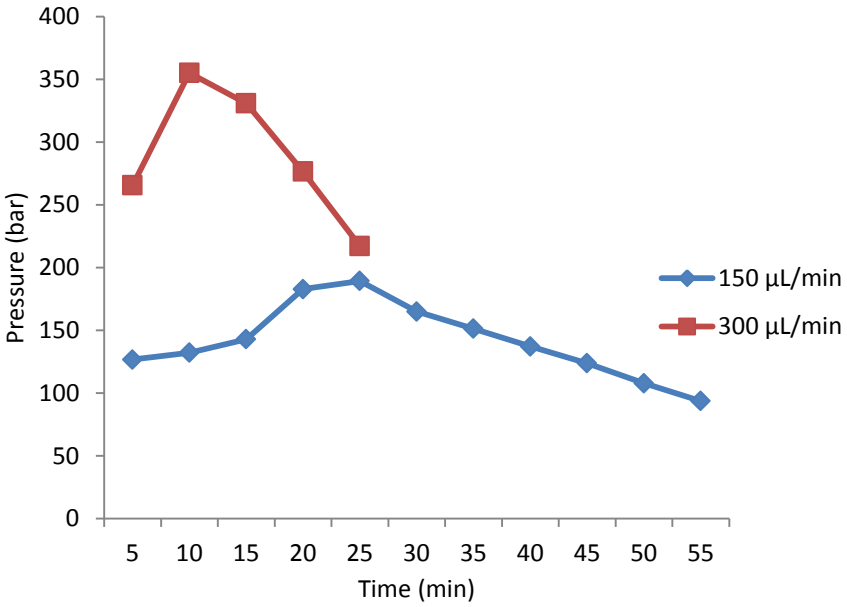


Figure 36 Observed column pressure using mobile phase flow rates 150 and 300 µL/min. Column used was a Pursuit XRs C18 Diphenyl column (250 x 2.0 mm, particle size 3 µm).

To decrease total analysis time even further, it could be argued that higher flow rates should have been tested/chosen, as the results indicated that neither peak intensity, peak area nor peak capacity were negatively affected by higher flow rates. However, experiences from routine analyses performed at Rikshospitalet indicate that column pressure increases over time. It can be risky to operate at the higher limit of tolerable column pressure and reproducibility is adversely affected.

To summarize, an important difference between the flow rates tested was that using 300 µL/min cut the initial analysis time by half. 300 µL/min was considered to be the best choice, although a bigger difference in peak intensity between the flow rates tested was expected.

5 Conclusions and outlooks

In this project, several mass spectrometric parameters were optimized with the purpose of maximizing the number of compounds detected and peak intensity for untargeted metabolomics of dried blood spots. Results of the optimization experiments indicated that the following combination of settings were the best choices: electrospray voltage set to 3.5 kV, electrospray needle position C, resolution set to 70 000, AGC target value set to 1 000 000 ion counts, and mobile phase flow rate 300 µL/min.

Some of the values found to be best were surprising. For electrospray needle position, the low number of detected compounds using the position that gave the shortest distance between the electrospray needle and the MS inlet, was unexpected. For mobile phase flow rate, a bigger difference in measured intensities was expected, as lower flow rates generate smaller electrospray droplets, which should, in theory, increase sensitivity. These findings emphasize the importance of thorough parameter optimization during method development.

Figure 37 illustrates the importance of parameter optimization. Using a suboptimal value can decrease both the number of detected compounds and signal intensity. As an example, the peak intensity for D4 succinic acid increased by a factor of approximately 3.25 when electrospray needle position was changed from position A to C. With an AGC target value of 20 000 ion counts for the standard mix spot sample in negative ionization mode, none of the standard mix compounds were detected. With 100 000 ion counts, only two of the six compounds in standard mix spot, negative mode, were detected. The standard mix compounds were carefully chosen to cover a broad spectrum of compounds from the metabolome. Choosing 100 000 ion counts instead of 1 000 000 ion counts as AGC target would, in this case, mean that only a third of the compounds were detected. This could present a serious problem, especially if the non-detected compounds are diagnostically relevant biomarkers. Results presented in this thesis indicate that if extensive optimization of parameters is done during method development, diagnostic opportunities will be improved.

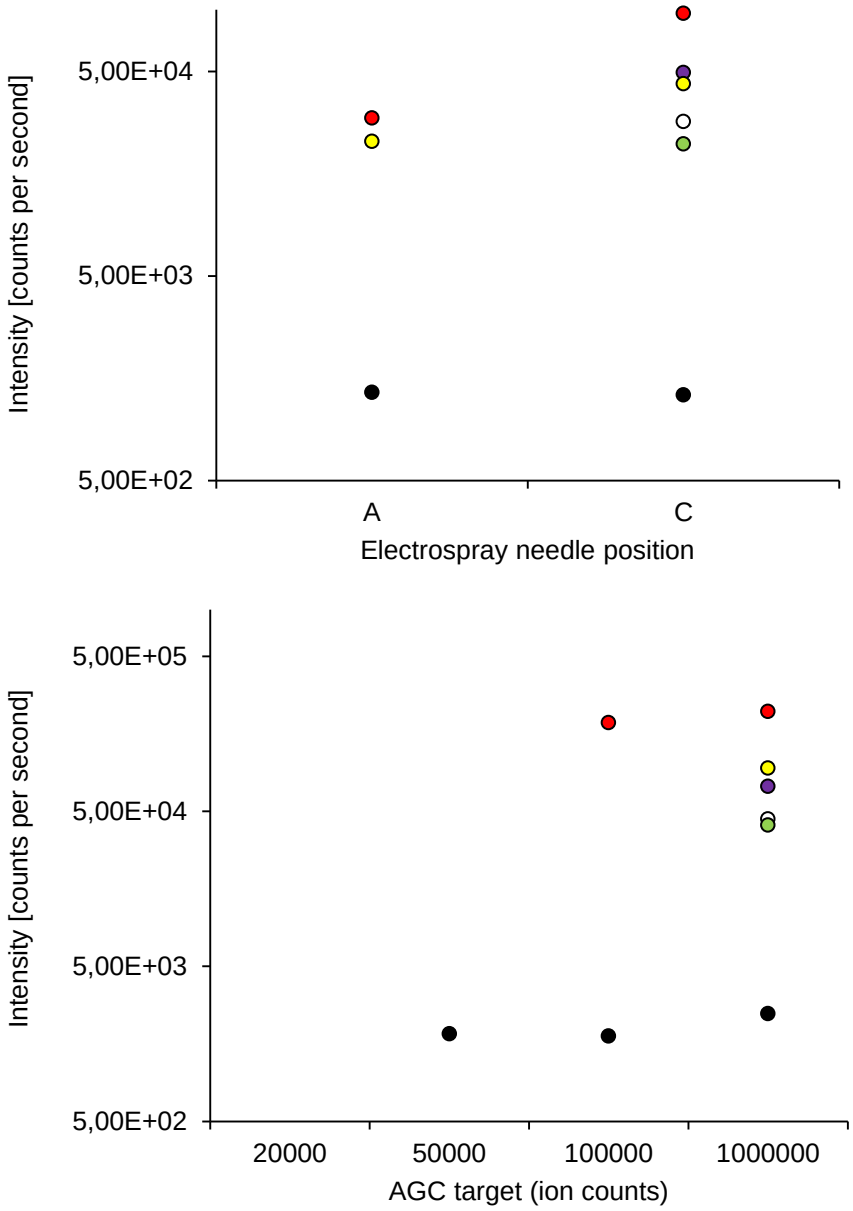


Figure 37 Examples of «worst case» scenarios (within the framework of the conditions tested) compared to analyses with the values found to be the most suitable. For electrospray needle position (top): switching the needle position from A to C lead to three more standard mix compounds being detected in the standard mix spot sample. For AGC target value (bottom): increasing AGC target value lead to an increase in number of detected standard mix compounds. The color code for each compound is shown in **Table 6**.

5.1 Future work

The developed MS method is considered to be suitable for metabolomics studies and will be merged with an LC method developed by Camilla Elene Arnesen [74]. This work is already in progress, and the combined method will be validated. When the combined LC-MS method is

validated, a thorough test of suitability for analyses in a real clinical setting must be done, including analysis of patient samples with known diseases. In this project, some carry-over was observed for acylcarnitines D3 C12 and D3 C16, see **Appendix**, section 6.5), although it was considered not to influence the results of the parameter testing. Nevertheless, evaluation of carry-over will be part of the method validation.

The relative standard deviation of the three injections of each sample was sometimes high. This emphasizes the importance of thorough validation, especially in a clinical setting, as each sample will be injected only once.

If more MS parameters are to be tested, these should be column inner diameter and S-lens RF level. MS/MS analyses can be done to investigate potential effects on sensitivity.

Assessing signal intensity of higher concentration compounds can provide important information on the linearity of the measurements. This can be especially important for quantitative diagnostic analyses.

Using the one-variable-at-a-time strategy, as was done in this thesis, requires many experiments. Using other experiment designs, such as factorial design, can be a good strategy to investigate potential interactions between parameters [49].

Long-term stability experiments of analytes on DBS cards can provide useful information, especially for retrospective analyses of samples that have been stored for longer periods of time.

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6 Appendix

6.1 Average intensities obtained from the optimization experiments

Tables 8-13 show the average intensities of each compound in the dilution experiments.

Table 8 Average intensity (n=3) of each compound in standard mix, negative ionization mode, for all concentrations analyzed.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	4,95E+06	2,68E+06	1,39E+06	1,69E+05	1,44E+04
$^{13}C_2$ guanidinoacetate	5,78E+06	2,98E+06	1,55E+06	1,83E+05	1,47E+04
D2 glycolic acid	5,98E+06	3,05E+06	1,41E+06	1,73E+05	1,06E+04
D4 succinic acid	1,90E+07	1,06E+07	5,66E+06	5,82E+05	3,18E+04
D6 glucose	9,18E+06	4,69E+06	2,40E+06	2,71E+05	3,57E+04
Vancomycin	4,13E+05	1,51E+05	7,31E+04	7,14E+03	ND*
D3 acylcarnitine C2	4,56E+04	2,66E+04	1,19E+04	ND*	ND*
D3 acylcarnitine C12	6,99E+05	3,65E+05	2,01E+05	2,37E+04	ND*
D3 acylcarnitine C16	1,57E+05	7,69E+04	4,92E+04	ND*	ND*

* ND = Not detectable

Table 9 Average intensity (n=3) of each compound in standard mix, positive ionization mode, for all concentrations analyzed.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,38E+08	2,15E+07	3,22E+07	4,46E+06	2,53E+05
$^{13}C_2$ guanidinoacetate	1,08E+08	1,76E+07	2,61E+07	3,65E+06	1,46E+05
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	7,93E+05	1,55E+05	2,63E+05	3,57E+04	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*
Vancomycin	5,07E+04	ND*	3,00E+03	ND*	ND*
D3 acylcarnitine C2	1,50E+08	2,56E+07	3,60E+07	5,93E+06	4,87E+05
D3 acylcarnitine C12	2,85E+08	3,63E+07	5,72E+07	8,53E+06	4,01E+05
D3 acylcarnitine C16	7,43E+07	6,52E+06	1,07E+07	9,52E+05	ND*

* ND = Not detectable

Table 10 Average intensity (n=3) of each compound in standard mix spot, negative ionization mode, for all concentrations analyzed.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	6,06E+04	2,80E+04	1,51E+04	ND*	ND*
$^{13}C_2$ guanidinoacetate	5,51E+04	3,17E+04	1,27E+04	ND*	ND*
D2 glycolic acid	3,34E+04	1,65E+04	5,43E+03	ND*	ND*
D4 succinic acid	1,06E+05	5,61E+04	3,25E+04	ND*	ND*
D6 glucose	6,84E+04	3,73E+04	1,94E+04	ND*	ND*
Vancomycin	1,70E+03	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 11 Average intensity (n=3) of each compound in standard mix spot, positive ionization mode, for all concentrations analyzed.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,04E+06	9,53E+05	1,32E+06	1,22E+05	1,16E+05
$^{13}C_2$ guanidinoacetate	1,16E+06	4,82E+05	2,76E+05	3,15E+04	ND*
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	1,38E+06	5,70E+05	5,13E+05	2,44E+05	ND*
D3 acylcarnitine C12	2,82E+06	7,03E+05	6,55E+05	5,62E+04	ND*
D3 acylcarnitine C16	4,11E+05	1,32E+05	1,27E+05	0,00E+00	ND*

* ND = Not detectable

Table 12 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all concentrations analyzed.

Compound	Concentration (µmol/L)				
	5	2,5	1,25	0,125	0,0125
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,11E+05	7,28E+04	5,51E+04	4,25E+04	3,59E+04
$^{13}C_2$ guanidinoacetate	5,83E+04	2,83E+04	1,68E+04	ND*	ND*
D2 glycolic acid	4,62E+04	2,37E+04	1,35E+04	ND*	ND*
D4 succinic acid	1,80E+05	8,23E+04	4,95E+04	ND*	ND*
D6 glucose	6,81E+04	3,75E+04	1,93E+04	ND*	ND*
Vancomycin	2,05E+03	8,93E+02	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 13 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all concentrations analyzed.

Compound	Concentration (µmol/L)				
	5	2,5	1,25	0,125	0,0125
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	3,57E+06	1,29E+06	1,39E+06	1,41E+06	1,06E+06
$^{13}C_2$ guanidinoacetate	1,27E+06	1,72E+05	2,56E+05	2,22E+04	ND*
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	1,90E+06	3,91E+05	3,60E+05	4,08E+04	9,76E+04
D3 acylcarnitine C12	2,43E+06	2,36E+05	3,53E+05	3,84E+04	ND*
D3 acylcarnitine C16	8,79E+05	ND*	4,22E+04	ND*	ND*

* ND = Not detectable

An initial electrospray voltage experiment was performed to investigate the effect of high electrospray voltage on intensity. **Tables 14-19** show the average intensities of each compound in the electrospray voltage optimization experiment comparing 3.5 kV to 7 kV. The results for 3.5 kV are obtained from the dilution experiments (see **Tables 8-13**).

Table 14 Average intensity (n=3) of each compound in standard mix, negative ionization mode, for electrospray voltages 3.5 and 7 kV.

Compound	Electrospray voltage (kV)	
	3,5	7
	NL (counts per second)	NL (counts per second)
^{13}C creatine	4,95E+06	7,78E+04
$^{13}C_2$ guanidinoacetate	5,78E+06	9,76E+04
D2 glycolic acid	5,98E+06	1,35E+05
D4 succinic acid	1,90E+07	8,10E+05
D6 glucose	9,18E+06	3,11E+05
Vancomycin	4,13E+05	1,31E+05
D3 acylcarnitine C2	4,56E+04	3,56E+03
D3 acylcarnitine C12	6,99E+05	2,76E+04
D3 acylcarnitine C16	1,57E+05	3,91E+03

Table 15 Average intensity (n=3) of each compound in standard mix, positive ionization mode, for electrospray voltages 3.5 and 7 kV.

Compound	Electrospray voltage (kV)	
	3,5	7
	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,38E+08	4,39E+07
$^{13}C_2$ guanidinoacetate	1,08E+08	3,54E+07
D2 glycolic acid	ND*	ND*
D4 succinic acid	7,93E+05	4,01E+05
D6 glucose	ND*	ND*
Vancomycin	5,07E+04	1,67E+04
D3 acylcarnitine C2	1,50E+08	8,27E+07
D3 acylcarnitine C12	2,85E+08	1,59E+08
D3 acylcarnitine C16	7,43E+07	3,36E+07

* ND = Not detectable

Table 16 Average intensity (n=3) of each compound in standard mix spot, negative ionization mode, for electrospray voltages 3.5 and 7 kV.

Compound	Electrospray voltage (kV)	
	3,5	7
	NL (counts per second)	NL (counts per second)
^{13}C creatine	6,06E+04	ND*
$^{13}C_2$ guanidinoacetate	5,51E+04	ND*
D2 glycolic acid	3,34E+04	ND*
D4 succinic acid	1,06E+05	3,93E+03
D6 glucose	6,84E+04	2,26E+03
Vancomycin	1,70E+03	1,20E+03
D3 acylcarnitine C2	ND*	ND*
D3 acylcarnitine C12	ND*	ND*
D3 acylcarnitine C16	ND*	ND*
	ND*	ND*

* ND = Not detectable

Table 17 Average intensity (n=3) of each compound in standard mix spot, positive ionization mode, for electrospray voltages 3.5 and 7 kV.

Compound	Electrospray voltage (kV)	
	3,5	7
	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,04E+06	5,01E+05
$^{13}C_2$ guanidinoacetate	1,16E+06	3,31E+05
D2 glycolic acid	ND*	ND*
D4 succinic acid	ND*	ND*
D6 glucose	ND*	ND*
Vancomycin	ND*	ND*
D3 acylcarnitine C2	1,38E+06	ND*
D3 acylcarnitine C12	2,82E+06	ND*
D3 acylcarnitine C16	4,11E+05	ND*

* ND = Not detectable

Table 18 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for electrospray voltages 3.5 and 7 kV.

Compound	Electrospray voltage (kV)	
	3,5	7
	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,11E+05	ND*
$^{13}C_2$ guanidinoacetate	5,83E+04	ND*
D2 glycolic acid	4,62E+04	ND*
D4 succinic acid	1,80E+05	5,58E+03
D6 glucose	6,81E+04	3,25E+03
Vancomycin	2,05E+03	2,24E+03
D3 acylcarnitine C2	ND*	ND*
D3 acylcarnitine C12	ND*	ND*
D3 acylcarnitine C16	ND*	ND*

* ND = Not detectable

Table 19 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for electrospray voltages 3.5 and 7 kV.

Compound	Electrospray voltage (kV)	
	3,5	7
	NL (counts per second)	NL (counts per second)
^{13}C creatine	3,57E+06	1,35E+06
$^{13}C_2$ guanidinoacetate	1,27E+06	3,79E+05
D2 glycolic acid	ND*	ND*
D4 succinic acid	ND*	ND*
D6 glucose	ND*	ND*
Vancomycin	ND*	ND*
D3 acylcarnitine C2	1,90E+06	1,17E+06
D3 acylcarnitine C12	2,43E+06	1,48E+06
D3 acylcarnitine C16	8,79E+05	1,02E+06

* ND = Not detectable

Table 20 shows the average intensities of each compound before and after the turbomolecular pump was replaced.

Table 20 Comparison of average intensities (n=3) of each compound with an electrospray voltage of 7 kV, before and after the turbomolecular pump was replaced.

Sample Type	Standard mix, negative ionization mode		Standard mix, positive ionization mode		Standard mix spot, negative ionization mode		Standard mix spot, positive ionization mode		Standard mix + blood spot, negative ionization mode		Standard mix + blood spot, positive ionization mode	
Compound	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	7,78E+04	4,30E+05	4,39E+07	5,34E+07	ND*	6,32E+03	5,01E+05	5,93E+05	ND*	1,78E+04	1,35E+06	1,50E+06
$^{13}C_2$ guanidinoacetate	9,76E+04	5,26E+05	3,54E+07	4,61E+07	ND*	9,55E+03	3,31E+05	4,69E+05	ND*	8,73E+03	3,79E+05	5,14E+05
D2 glycolic acid	1,35E+05	3,13E+05	ND*	ND*	ND*	5,40E+03	ND*	ND*	ND*	8,35E+03	ND*	ND*
D4 succinic acid	8,10E+05	1,78E+06	4,01E+05	6,07E+05	3,93E+03	1,43E+04	ND*	ND*	5,58E+03	3,77E+04	ND*	ND*
D6 glucose	3,11E+05	1,06E+06	ND*	ND*	2,26E+03	1,42E+04	ND*	ND*	3,25E+03	1,83E+04	ND*	ND*
Vancomycin	1,31E+05	2,82E+05	1,67E+04	9,84E+04	1,20E+03	2,41E+03	ND*	ND*	2,24E+03	3,18E+03	ND*	ND*
D3 acylcarnitine C2	3,56E+03	1,53E+04	8,27E+07	9,78E+07	ND*	ND*	ND*	8,61E+05	ND*	ND*	1,17E+06	1,16E+06
D3 acylcarnitine C12	2,76E+04	3,71E+04	1,59E+08	2,25E+08	ND*	ND*	ND*	7,23E+05	ND*	ND*	1,48E+06	1,67E+06
D3 acylcarnitine C16	3,91E+03	ND*	3,36E+07	1,37E+07	ND*	ND*	ND*	8,00E+04	ND*	ND*	1,02E+06	2,18E+05

* ND = Not detectable

Tables 21-26 show the average intensities of each compound in the electrospray voltage optimization experiments using 1, 2, 3.5, 4, 5, 6 and 7 kV.

Table 21 Average intensity (n=3) of each compound in standard mix, negative ionization mode, for all electrospray voltages tested.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,72E+05	1,06E+06	2,69E+06	1,52E+06	2,74E+06	1,36E+06	4,30E+05
$^{13}C_2$ guanidinoacetate	3,18E+05	1,61E+06	3,73E+06	2,23E+06	3,80E+06	2,00E+06	5,26E+05
D2 glycolic acid	4,47E+05	2,26E+06	4,12E+06	2,30E+06	4,37E+06	1,80E+06	3,13E+05
D4 succinic acid	1,53E+06	5,86E+06	1,32E+07	9,10E+06	1,64E+07	8,16E+06	1,78E+06
D6 glucose	5,01E+05	2,37E+06	6,83E+06	4,84E+06	8,63E+06	3,92E+06	1,06E+06
Vancomycin	1,08E+04	7,82E+04	3,26E+05	2,84E+05	5,15E+05	4,03E+05	2,82E+05
D3 acylcarnitine C2	ND*	8,39E+03	3,73E+04	2,12E+04	6,21E+04	1,91E+04	1,53E+04
D3 acylcarnitine C12	3,32E+04	1,50E+05	6,01E+05	5,08E+05	4,65E+05	9,28E+04	3,71E+04
D3 acylcarnitine C16	5,92E+03	3,95E+04	7,45E+04	6,11E+04	2,87E+04	ND*	ND*

* ND = Not detectable

Table 22 Average intensity (n=3) of each compound in standard mix, positive ionization mode, for all electrospray voltages tested.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,37E+07	2,87E+07	5,08E+07	4,23E+07	6,15E+07	4,70E+07	5,34E+07
$^{13}C_2$ guanidinoacetate	1,51E+07	2,74E+07	4,79E+07	4,91E+07	5,77E+07	5,05E+07	4,61E+07
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	1,15E+05	3,76E+05	7,54E+05	5,44E+05	9,53E+05	4,96E+05	6,07E+05
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	2,69E+04	8,23E+04	2,42E+04	1,20E+05	4,19E+04	9,84E+04
D3 acylcarnitine C2	1,48E+07	4,30E+07	6,00E+07	6,86E+07	ND*	7,13E+07	9,78E+07
D3 acylcarnitine C12	4,21E+07	9,35E+07	1,33E+08	1,93E+08	ND*	2,44E+08	2,25E+08
D3 acylcarnitine C16	4,16E+06	1,28E+07	1,37E+07	2,77E+07	ND*	1,66E+07	1,37E+07

* ND = Not detectable

Table 23 Average intensity (n=3) of each compound in standard mix spot, negative ionization mode, for all electrospray voltages tested.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	3,44E+03	3,54E+04	8,85E+04	1,65E+04	2,80E+04	1,27E+04	6,32E+03
$^{13}C_2$ guanidinoacetate	3,05E+03	2,27E+04	4,13E+04	1,74E+04	3,56E+04	1,58E+04	9,55E+03
D2 glycolic acid	3,89E+03	1,81E+04	3,23E+04	1,58E+04	2,09E+04	1,28E+04	5,40E+03
D4 succinic acid	9,90E+03	6,85E+04	1,15E+05	4,41E+04	8,23E+04	5,21E+04	1,43E+04
D6 glucose	5,77E+03	2,87E+04	7,36E+04	3,80E+04	6,61E+04	2,22E+04	1,42E+04
Vancomycin	ND*	ND*	1,95E+03	1,48E+03	2,19E+03	2,83E+03	2,41E+03
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 24 Average intensity (n=3) of each compound in standard mix spot, positive ionization mode, for all electrospray voltages tested.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,30E+05	8,11E+05	1,54E+06	4,74E+05	7,34E+05	5,99E+05	5,93E+05
$^{13}C_2$ guanidinoacetate	1,35E+05	4,50E+05	5,83E+05	3,18E+05	6,28E+05	3,90E+05	4,69E+05
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	1,51E+05	5,55E+05	7,88E+04	5,57E+05	1,01E+06	ND*	8,61E+05
D3 acylcarnitine C12	9,65E+05	2,36E+06	1,15E+05	1,84E+06	1,27E+06	ND*	7,23E+05
D3 acylcarnitine C16	5,11E+04	8,88E+04	2,11E+05	ND*	ND*	ND*	8,00E+04

* ND = Not detectable

Table 25 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all electrospray voltages tested.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	4,70E+03	2,85E+04	6,26E+04	6,38E+04	2,58E+04	4,79E+04	1,78E+04
$^{13}C_2$ guanidinoacetate	4,11E+03	2,09E+04	3,81E+04	3,26E+04	1,21E+04	2,18E+04	8,73E+03
D2 glycolic acid	7,16E+03	2,07E+04	2,81E+04	2,92E+04	1,72E+04	2,09E+04	8,35E+03
D4 succinic acid	1,98E+04	7,51E+04	1,26E+05	1,36E+05	6,25E+04	9,96E+04	3,77E+04
D6 glucose	5,15E+03	2,46E+04	4,80E+04	5,80E+04	2,22E+04	4,24E+04	1,83E+04
Vancomycin	ND*	ND*	ND*	ND*	ND*	4,59E+03	3,18E+03
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 26 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all electrospray voltages tested.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	3,75E+05	9,52E+05	1,38E+06	1,29E+06	ND*	1,57E+06	1,50E+06
$^{13}C_2$ guanidinoacetate	1,15E+05	3,76E+05	4,80E+05	2,90E+05	2,78E+05	5,24E+05	5,14E+05
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	1,38E+05	5,49E+05	4,13E+05	7,61E+05	ND*	1,11E+06	1,16E+06
D3 acylcarnitine C12	2,10E+05	6,21E+05	ND*	1,28E+06	ND*	1,63E+06	1,67E+06
D3 acylcarnitine C16	3,70E+04	9,82E+04	1,71E+05	2,25E+05	ND*	2,11E+05	2,18E+05

* ND = Not detectable

Tables 27-32 show the average intensities of each compound in the electrospray needle position optimization experiments.

Table 27 Average intensity (n=3) of each compound in standard mix, negative ionization mode, for all electrospray needle positions tested.

Compound	Needle Position			
	A	B	C	D
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,38E+06	2,67E+06	2,46E+06	2,07E+06
$^{13}C_2$ guanidinoacetate	2,08E+06	3,82E+06	3,96E+06	3,41E+06
D2 glycolic acid	1,81E+06	4,40E+06	4,51E+06	3,92E+06
D4 succinic acid	6,59E+06	1,44E+07	1,52E+07	1,34E+07
D6 glucose	3,59E+06	7,34E+06	6,85E+06	5,86E+06
Vancomycin	3,02E+05	3,86E+05	3,56E+05	3,19E+05
D3 acylcarnitine C2	1,76E+04	3,46E+04	3,38E+04	3,22E+04
D3 acylcarnitine C12	2,33E+05	3,09E+05	2,63E+05	2,37E+05
D3 acylcarnitine C16	3,21E+04	2,88E+04	2,54E+04	ND*

* ND = Not detectable

Table 28 Average intensity (n=3) of each compound in standard mix, positive ionization mode, for different all electrospray needle positions tested.

Compound	Needle Position			
	A	B	C	D
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,90E+07	4,21E+07	4,86E+07	4,00E+07
$^{13}C_2$ guanidinoacetate	3,02E+07	4,63E+07	5,03E+07	4,16E+07
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	5,06E+05	9,39E+05	1,02E+06	8,33E+05
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	2,62E+04	1,08E+05	7,53E+04	4,69E+04
D3 acylcarnitine C2	3,54E+07	7,69E+07	8,73E+07	7,96E+07
D3 acylcarnitine C12	1,08E+08	1,38E+08	1,71E+08	1,27E+08
D3 acylcarnitine C16	1,94E+07	9,08E+06	9,82E+06	7,11E+06

* ND = Not detectable

Table 29 Average intensity (n=3) of each compound in standard mix spot, negative ionization mode, for all electrospray needle positions tested.

Compound	Needle Position			
	A	B	C	D
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	4,95E+04	4,93E+04	6,66E+04
$^{13}C_2$ guanidinoacetate	ND*	2,90E+04	2,84E+04	2,39E+04
D2 glycolic acid	ND*	2,41E+04	2,21E+04	2,80E+04
D4 succinic acid	2,95E+04	7,36E+04	9,60E+04	1,02E+05
D6 glucose	2,27E+04	4,22E+04	4,35E+04	3,44E+04
Vancomycin	1,35E+03	1,59E+03	1,31E+03	1,10E+03
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 30 Average intensity (n=3) of each compound in standard mix spot, positive ionization mode, for all electrospray needle positions tested.

Compound	Needle Position			
	A	B	C	D
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,23E+05	8,41E+05	9,70E+05	1,04E+06
$^{13}C_2$ guanidinoacetate	1,51E+05	3,88E+05	4,07E+05	3,54E+05
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	2,01E+05	5,40E+05	7,28E+05	7,42E+05
D3 acylcarnitine C12	4,53E+05	5,16E+05	4,06E+05	2,52E+05
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 31 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all electrospray needle positions tested.

Compound	Needle Position			
	A	B	C	D
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	5,38E+04	6,21E+04	6,78E+04	6,31E+04
$^{13}C_2$ guanidinoacetate	ND*	3,36E+04	3,03E+04	3,36E+04
D2 glycolic acid	1,88E+04	3,39E+04	2,98E+04	2,91E+04
D4 succinic acid	7,90E+04	1,23E+05	1,43E+05	1,34E+05
D6 glucose	3,24E+04	5,11E+04	4,56E+04	4,58E+04
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 32 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all electrospray needle positions tested.

Compound	Needle Position			
	A	B	C	D
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	6,30E+05	1,19E+06	1,63E+06	1,52E+06
$^{13}C_2$ guanidinoacetate	1,88E+05	4,24E+05	4,42E+05	3,66E+05
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	2,87E+05	7,44E+05	9,48E+05	9,88E+05
D3 acylcarnitine C12	5,28E+05	6,57E+05	7,22E+05	6,05E+05
D3 acylcarnitine C16	ND*	9,96E+04	9,93E+04	9,36E+04

* ND = Not detectable

Tables 33-38 show the average intensities of each compound in the resolution optimization experiments.

Table 33 Average intensity (n=3) of each compound in standard mix, negative ionization mode, for all resolution values tested.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,24E+06	3,31E+06	3,04E+06	2,90E+06
$^{13}C_2$ guanidinoacetate	1,96E+06	2,81E+06	2,77E+06	2,67E+06
D2 glycolic acid	1,69E+06	2,48E+06	2,47E+06	2,42E+06
D4 succinic acid	7,44E+06	1,05E+07	1,02E+07	1,00E+07
D6 glucose	3,77E+06	4,88E+06	4,54E+06	4,29E+06
Vancomycin	3,37E+05	4,65E+05	4,16E+05	3,18E+05
D3 acylcarnitine C2	2,13E+04	2,20E+04	2,13E+04	1,84E+04
D3 acylcarnitine C12	3,99E+05	3,87E+05	3,75E+05	2,62E+05
D3 acylcarnitine C16	2,73E+05	9,18E+04	7,91E+04	4,20E+04

Table 34 Average intensity (n=3) of each compound in standard mix, positive ionization mode, for all resolution values tested.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	6,18E+07	6,58E+07	6,30E+07	5,39E+07
$^{13}C_2$ guanidinoacetate	3,88E+07	4,24E+07	3,97E+07	3,74E+07
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	5,19E+05	7,25E+05	6,82E+05	5,42E+05
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	3,08E+04	1,04E+05	8,77E+04	5,34E+04
D3 acylcarnitine C2	6,77E+07	8,39E+07	7,59E+07	6,68E+07
D3 acylcarnitine C12	2,12E+08	1,89E+08	1,64E+08	1,36E+08
D3 acylcarnitine C16	1,63E+08	2,65E+07	2,18E+07	1,70E+07

* ND = Not detectable

Table 35 Average intensity (n=3) of each compound in standard mix spot, negative ionization mode, for all resolution values tested.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	3,69E+04	5,36E+04	4,29E+04
$^{13}C_2$ guanidinoacetate	2,47E+04	2,15E+04	2,18E+04	2,23E+04
D2 glycolic acid	1,53E+04	1,78E+04	1,76E+04	1,37E+04
D4 succinic acid	8,44E+04	7,56E+04	8,25E+04	8,49E+04
D6 glucose	5,14E+04	3,75E+04	3,48E+04	2,79E+04
Vancomycin	ND*	ND*	1,43E+03	1,06E+03
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 36 Average intensity (n=3) of each compound in standard mix spot, positive ionization mode, for all resolution values tested.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	8,26E+05	1,12E+06	1,43E+06	1,45E+06
$^{13}C_2$ guanidinoacetate	2,93E+05	3,86E+05	3,30E+05	2,38E+05
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	6,78E+05	7,82E+05	7,68E+05	5,57E+05
D3 acylcarnitine C12	2,10E+06	1,46E+06	8,90E+05	3,70E+05
D3 acylcarnitine C16	ND*	ND*	1,12E+05	7,06E+04

* ND = Not detectable

Table 37 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all resolution values tested.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	7,62E+04	6,99E+04	5,86E+04	5,31E+04
$^{13}C_2$ guanidinoacetate	ND*	2,04E+04	2,14E+04	1,51E+04
D2 glycolic acid	2,18E+04	1,79E+04	1,97E+04	1,29E+04
D4 succinic acid	1,07E+05	9,18E+04	9,27E+04	8,05E+04
D6 glucose	3,72E+04	2,71E+04	2,79E+04	2,55E+04
Vancomycin	ND*	ND*	5,31E+03	4,30E+03
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 38 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all resolution values tested.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,64E+06	1,67E+06	1,98E+06	1,72E+06
$^{13}C_2$ guanidinoacetate	2,96E+05	3,07E+05	2,43E+05	1,55E+05
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	9,20E+05	8,38E+05	8,00E+05	6,20E+05
D3 acylcarnitine C12	1,14E+06	ND*	9,56E+05	5,77E+05
D3 acylcarnitine C16	ND*	ND*	9,52E+04	8,18E+04

* ND = Not detectable

Tables 39-44 show the average intensities of each compound in the AGC target optimization experiments (with resolution set to 70 000).

Table 39 Average intensity (n=3) of each compound in standard mix, negative ionization mode, for all AGC target values tested (resolution 70 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,63E+06	4,43E+06	4,60E+06	4,43E+06	2,51E+06	5,06E+06	4,35E+06	2,55E+06
$^{13}C_2$ guanidinoacetate	2,34E+06	4,04E+06	4,03E+06	3,83E+06	2,06E+06	4,42E+06	3,87E+06	2,54E+06
D2 glycolic acid	1,86E+06	3,75E+06	3,94E+06	3,64E+06	1,73E+06	3,90E+06	3,64E+06	2,09E+06
D4 succinic acid	7,96E+06	1,49E+07	1,62E+07	1,54E+07	7,97E+06	1,68E+07	1,41E+07	1,05E+07
D6 glucose	4,51E+06	8,73E+06	8,57E+06	8,08E+06	4,91E+06	9,41E+06	7,25E+06	3,67E+06
Vancomycin	3,32E+05	6,12E+05	5,58E+05	5,39E+05	3,45E+05	7,70E+05	6,99E+05	3,30E+05
D3 acylcarnitine C2	ND*	ND*	ND*	6,95E+04	3,62E+04	6,07E+04	3,53E+04	1,52E+04
D3 acylcarnitine C12	ND*	8,46E+05	7,53E+05	6,74E+05	5,82E+05	8,05E+05	5,86E+05	3,25E+05
D3 acylcarnitine C16	ND*	ND*	ND*	5,67E+04	5,67E+04	5,77E+04	3,80E+04	7,68E+04

* ND = Not detectable

Table 40 Average intensity (n=3) of each compound in standard mix, positive ionization mode, for all AGC target values tested (resolution 70 000).

Compound	AGC target value (ion counts)							
	2,00E+04	**5,00E+04**	**1,00E+05**	**2,00E+05**	**5,00E+05**	**1,00E+06**	**3,00E+06**	**5,00E+06**
	NL (counts per second)	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**
^{13}C creatine	5,98E+07	8,31E+07	7,99E+07	8,33E+07	7,30E+07	9,47E+07	8,61E+07	5,83E+07
$^{13}C_2$ guanidinoacetate	3,95E+07	4,84E+07	5,45E+07	5,37E+07	4,73E+07	6,33E+07	5,51E+07	3,91E+07
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	1,18E+06	1,23E+06	1,14E+06	6,59E+05	1,06E+06	8,72E+05	4,34E+05
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	1,21E+05	1,18E+05	1,10E+05	3,13E+04	1,71E+05	1,51E+05	1,09E+05
D3 acylcarnitine C2	7,20E+07	1,05E+08	1,06E+08	1,09E+08	7,84E+07	1,14E+08	1,04E+08	6,97E+07
D3 acylcarnitine C12	2,21E+08	2,40E+08	2,56E+08	2,66E+08	2,81E+08	3,88E+08	3,08E+08	1,79E+08
D3 acylcarnitine C16	3,12E+07	1,64E+07	1,73E+07	1,48E+07	1,61E+07	1,86E+07	1,27E+07	6,28E+06

* ND = Not detectable

Table 41 Average intensity (n=3) of each compound in standard mix spot, negative ionization mode, for all AGC target values tested (resolution 70 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	ND*	ND*	5,65E+04	4,36E+04	7,23E+04	3,09E+04	2,99E+04
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	4,45E+04	1,83E+04	2,14E+04
D2 glycolic acid	ND*	ND*	ND*	3,56E+04	1,98E+04	4,08E+04	1,34E+04	1,54E+04
D4 succinic acid	ND*	ND*	1,86E+05	1,55E+05	1,06E+05	2,20E+05	5,86E+04	6,45E+04
D6 glucose	ND*	ND*	ND*	8,63E+04	7,46E+04	9,50E+04	3,51E+04	3,51E+04
Vancomycin	ND*	1,83E+03	1,77E+03	1,77E+03	1,70E+03	2,47E+03	1,17E+03	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	6,86E+03	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 42 Average intensity (n=3) of each compound in standard mix spot, positive ionization mode, for all AGC target values tested (resolution 70 000).

Compound	AGC target value (ion counts)							
	2,00E+04	**5,00E+04**	**1,00E+05**	**2,00E+05**	**5,00E+05**	**1,00E+06**	**3,00E+06**	**5,00E+06**
	NL (counts per second)	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**	**NL (counts per second)**
^{13}C creatine	8,13E+05	1,37E+06	1,47E+06	1,45E+06	1,00E+06	1,84E+06	1,37E+06	8,09E+05
$^{13}C_2$ guanidinoacetate	ND*	6,07E+05	5,88E+05	5,24E+05	3,27E+05	7,03E+05	5,10E+05	2,14E+05
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	1,01E+04	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	6,81E+05	1,38E+06	1,30E+06	1,24E+06	8,50E+05	1,43E+06	1,03E+06	6,28E+05
D3 acylcarnitine C12	ND*	2,02E+06	1,54E+06	9,37E+05	6,03E+05	5,68E+05	2,23E+05	1,66E+06
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 43 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all AGC target values tested (resolution 70 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	ND*	ND*	7,11E+04	6,48E+04	7,77E+04	4,78E+04	4,72E+04
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	3,83E+04	2,31E+04	2,39E+04
D2 glycolic acid	ND*	ND*	ND*	2,82E+04	3,00E+04	3,34E+04	1,90E+04	1,94E+04
D4 succinic acid	ND*	2,30E+05	1,90E+05	1,59E+05	1,40E+05	1,82E+05	1,06E+05	1,01E+05
D6 glucose	ND*	ND*	ND*	5,06E+04	6,38E+04	7,82E+04	4,01E+04	3,11E+04
Vancomycin	1,75E+03	1,83E+03	2,71E+03	2,12E+03	2,64E+03	2,95E+03	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 44 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all AGC target values tested (resolution 70 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	1,81E+06	1,92E+06	1,92E+06	2,09E+06	1,75E+06	2,37E+06	2,20E+06	1,31E+06
$^{13}C_2$ guanidinoacetate	ND*	4,62E+05	4,24E+05	4,39E+05	2,69E+05	5,39E+05	4,87E+05	2,52E+05
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	1,01E+06	1,32E+06	1,35E+06	1,27E+06	1,08E+06	1,52E+06	1,20E+06	7,40E+05
D3 acylcarnitine C12	ND*	1,78E+06	1,80E+06	1,46E+06	2,44E+06	1,97E+06	1,38E+06	8,25E+05
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Tables 45-50 show the average intensities of each compound in the AGC target optimization experiments (with resolution set to 140 000).

Table 45 Average intensity (n=3) of each compound in standard mix, negative ionization mode, for all AGC target values tested (resolution 140 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	2,40E+06	2,51E+06	2,39E+06	2,67E+06	1,83E+06	3,58E+06	3,27E+06	3,16E+06
$^{13}C_2$ guanidinoacetate	2,57E+06	2,37E+06	2,58E+06	2,55E+06	1,77E+06	3,39E+06	3,22E+06	3,05E+06
D2 glycolic acid	1,80E+06	2,01E+06	2,07E+06	2,17E+06	1,39E+06	3,00E+06	3,01E+06	3,05E+06
D4 succinic acid	9,71E+06	1,02E+07	1,03E+07	1,08E+07	6,72E+06	1,36E+07	1,23E+07	1,12E+07
D6 glucose	6,52E+06	6,07E+06	6,09E+06	6,49E+06	4,20E+06	7,51E+06	6,10E+06	4,49E+06
Vancomycin	4,48E+05	4,05E+05	3,45E+05	3,25E+05	2,05E+05	3,83E+05	3,74E+05	3,80E+05
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	4,07E+04	2,68E+04	1,57E+04
D3 acylcarnitine C12	8,01E+05	6,84E+05	5,92E+05	4,90E+05	4,92E+05	4,42E+05	3,45E+05	2,68E+05
D3 acylcarnitine C16	ND*	ND*	8,01E+04	5,72E+04	ND*	5,99E+04	3,25E+04	2,94E+04

* ND = Not detectable

Table 46 Average intensity (n=3) of each compound in standard mix, positive ionization mode, for all AGC target values tested (resolution 140 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	4,62E+07	5,62E+07	5,57E+07	6,03E+07	5,12E+07	6,29E+07	ND*	4,70E+07
$^{13}C_2$ guanidinoacetate	3,13E+07	3,58E+07	3,99E+07	4,05E+07	3,62E+07	4,57E+07	4,32E+07	3,61E+07
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	4,98E+05	4,90E+05	6,04E+05	4,41E+05	8,84E+05	8,54E+05	2,09E+05
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	8,89E+04	5,73E+04	5,01E+04	4,77E+04	9,19E+03	6,30E+04	5,76E+04	5,65E+04
D3 acylcarnitine C2	5,07E+07	6,28E+07	6,57E+07	6,43E+07	6,22E+07	8,25E+07	ND*	5,62E+07
D3 acylcarnitine C12	1,23E+08	1,18E+08	1,26E+08	1,28E+08	1,55E+08	1,27E+08	ND*	7,62E+07
D3 acylcarnitine C16	5,50E+07	8,42E+06	1,30E+07	9,52E+06	5,60E+07	1,11E+07	ND*	4,13E+06

* ND = Not detectable

Table 47 Average intensity (n=3) of each compound in standard mix spot, negative ionization mode, for all AGC target values tested (resolution 140 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	ND*	ND*	ND*	ND*	ND*	3,49E+04	2,85E+04
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	ND*	2,36E+04	2,28E+04
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	1,54E+04	1,63E+04
D4 succinic acid	ND*	ND*	9,00E+04	1,08E+05	4,65E+04	1,06E+05	9,26E+04	9,60E+04
D6 glucose	ND*	ND*	ND*	ND*	ND*	6,24E+04	4,35E+04	3,35E+04
Vancomycin	ND*	ND*	1,06E+03	ND*	ND*	1,19E+03	1,19E+03	
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 48 Average intensity (n=3) of each compound in standard mix spot, positive ionization mode, for all AGC target values tested (resolution 140 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	8,62E+05	7,86E+05	7,97E+05	6,26E+05	9,18E+05	8,88E+05	7,39E+05
$^{13}C_2$ guanidinoacetate	ND*	ND*	3,29E+05	3,10E+05	2,27E+05	3,21E+05	3,18E+05	2,50E+05
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	7,78E+05	6,56E+05	6,63E+05	6,12E+05	7,94E+05	ND*	5,58E+05
D3 acylcarnitine C12	ND*	ND*	ND*	7,58E+05	ND*	9,71E+05	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 49 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all AGC target values tested (resolution 140 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	ND*	ND*	ND*	6,38E+04	6,89E+04	4,98E+04	4,10E+04
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	2,76E+04	2,38E+04	2,03E+04
D4 succinic acid	ND*	ND*	1,26E+05	1,33E+05	1,47E+05	1,47E+05	1,17E+05	1,10E+05
D6 glucose	ND*	ND*	ND*	ND*	6,70E+04	7,33E+04	4,25E+04	2,82E+04
Vancomycin	1,78E+03	1,59E+03	1,71E+03	1,54E+03	1,64E+03	1,56E+03	1,77E+03	1,64E+03
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 50 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all AGC target values tested (resolution 140 000).

Compound	AGC target value (ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	ND*	1,64E+06	1,46E+06	1,49E+06	1,41E+06	1,63E+06	1,49E+06	1,28E+06
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	2,59E+05	3,72E+05	2,59E+05	2,52E+05
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	9,67E+05	7,69E+05	8,16E+05	6,53E+05	9,23E+05	7,98E+05	6,74E+05
D3 acylcarnitine C12	ND*	ND*	ND*	1,07E+06	ND*	1,14E+06	ND*	4,26E+05
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Figures 38-43 show the comparison of measured intensities in the experiments with AGC target value set to 1 000 000 ion counts with resolution set to 70 000 and resolution set to 140 000. The standard mix compounds are listed as the following numbers in the figures:

Compound 1 = ^{13}C creatine

Compound 2 = $^{13}C_2$ guanidinoacetate

Compound 3 = D2 glycolic acid

Compound 4 = D4 succinic acid

Compound 5 = D6 glucose

Compound 6 = Vancomycin

Compound 7 = D3 acylcarnitine C2

Compound 8 = D3 acylcarnitine C12

Compound 9 = D3 acylcarnitine C16

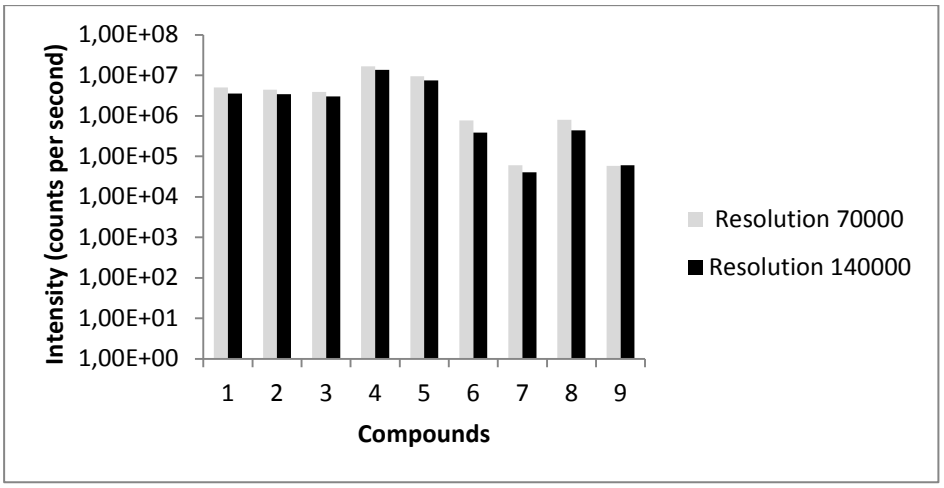

Figure 38 Comparison of measured intensities of standard mix compounds with resolution set to 70 000 and 140 000 (AGC target value 1 000 000 ion counts), in standard mix, negative ionization mode.

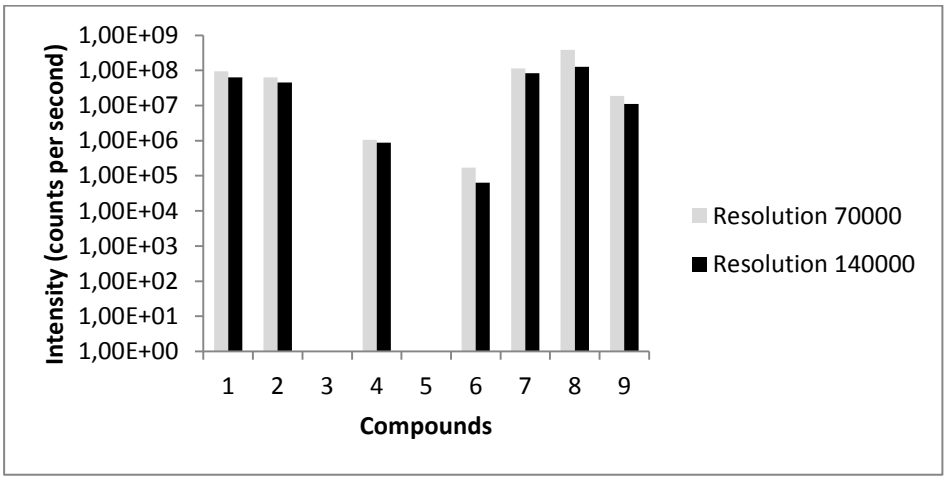

Figure 39 Comparison of measured intensities of standard mix compounds with resolution set to 70 000 and 140 000 (AGC target value 1 000 000 ion counts), in standard mix, positive ionization mode.

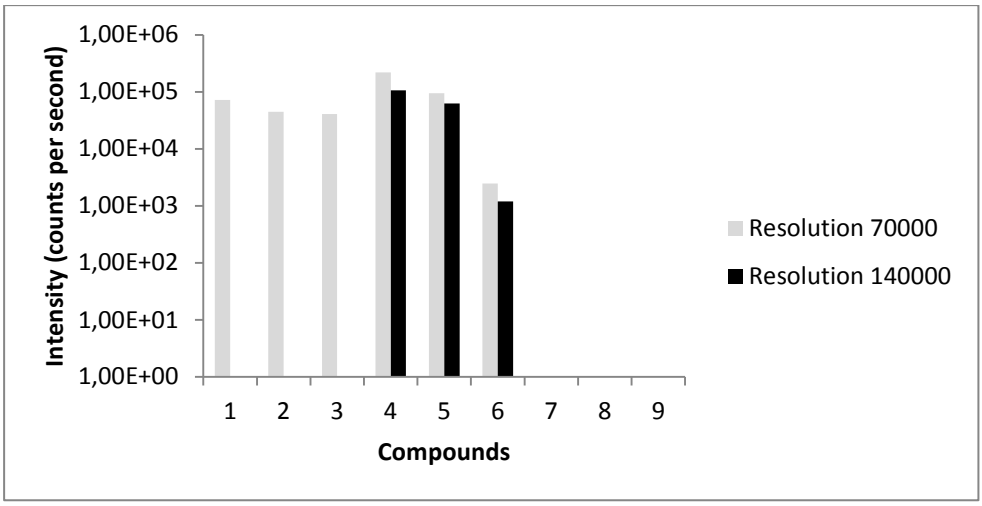

Figure 40 Comparison of measured intensities of standard mix compounds with resolution set to 70 000 and 140 000 (AGC target value 1 000 000 ion counts), in standard mix spot, negative ionization mode.

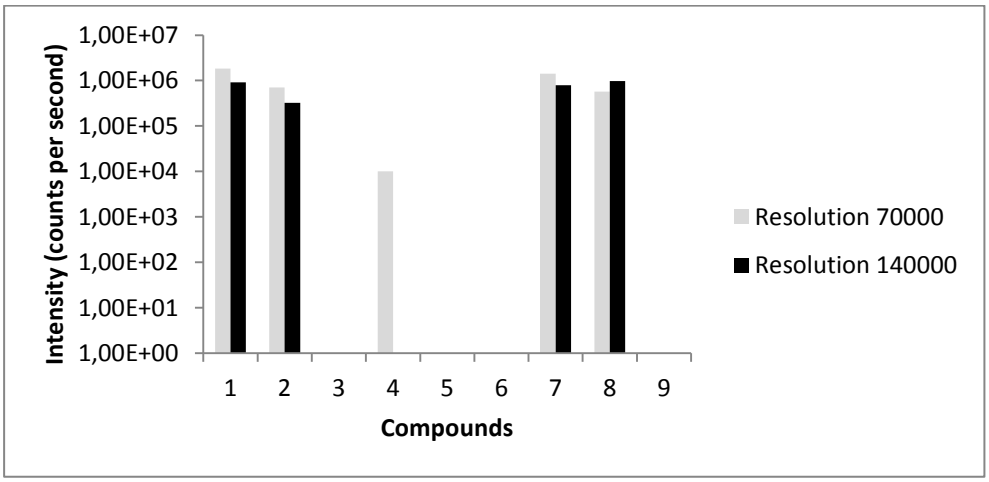

Figure 41 Comparison of measured intensities of standard mix compounds with resolution set to 70 000 and 140 000 (AGC target value 1 000 000 ion counts), in standard mix spot, positive ionization mode.

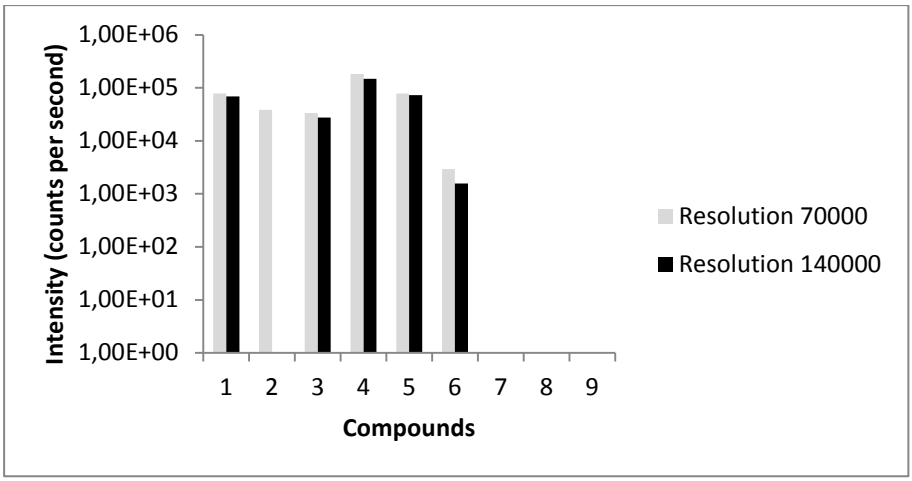

Figure 42 Comparison of measured intensities of standard mix compounds with resolution set to 70 000 and 140 000 (AGC target value 1 000 000 ion counts), in standard mix + blood spot, negative ionization mode.

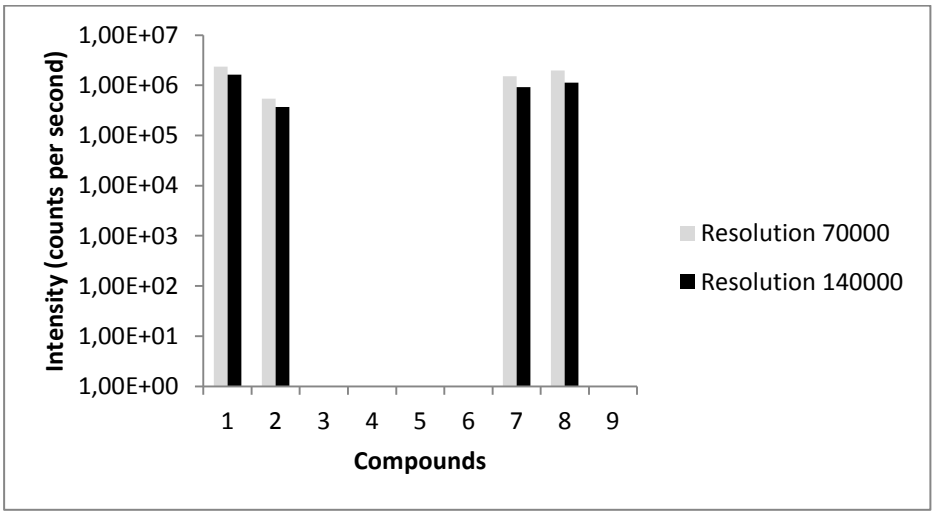

Figure 43 Comparison of measured intensities of standard mix compounds with resolution set to 70 000 and 140 000 (AGC target value 1 000 000 ion counts), in standard mix + blood spot, positive ionization mode.

As shown in **Figures 38-43**, resolution set to 70 000 gave a slightly higher intensity for most compounds in all sample types, and was considered to be the best choice.

Tables 51-52 show the average intensities of each compound in the mobile phase flow rate testing experiments (with analysis time adjusted to each flow rate).

Table 51 Average intensity (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all mobile phase flow rates tested.

Compound	Mobile phase flow rate (µL/min)		
	50	150	300
	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	3,80E+04	3,76E+04	3,80E+04
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*
D2 glycolic acid	ND*	ND*	ND*
D4 succinic acid	6,21E+04	6,31E+04	8,59E+04
D6 glucose	4,89E+04	4,01E+04	5,07E+04
Vancomycin	ND*	2,45E+03	1,41E+03
D3 acylcarnitine C2	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*

* ND = Not detectable

Table 52 Average intensity (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all mobile phase flow rates tested.

Compound	Mobile phase flow rate (µL/min)		
	50	150	300
	NL (counts per second)	NL (counts per second)	NL (counts per second)
^{13}C creatine	5,99E+05	1,22E+06	1,35E+06
$^{13}C_2$ guanidinoacetate	1,76E+05	1,83E+05	1,48E+05
D2 glycolic acid	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*
D3 acylcarnitine C2	4,15E+05	6,23E+05	8,61E+05
D3 acylcarnitine C12	1,05E+06	9,97E+05	8,19E+05
D3 acylcarnitine C16	ND*	ND*	ND*

* ND = Not detectable

Table 53 shows the average intensity and average area of creatinine measured in standard mix + blood spot, in the flow rate experiments (with analysis time adjusted to each flow rate).

Table 53 Average intensity and average area (n=3) of creatinine in standard mix + blood spot, from the flow rate experiment.

	Mobile phase flow rate (µL/min)		
	50	**150**	**300**
Average intensity (negative ionization mode), NL (counts per second)	7,12E+04	3,05E+04	7,31E+04
Average intensity (positive ionization mode), NL (counts per second)	2,12E+06	5,04E+06	4,20E+06
Average area (negative ionization mode), a.u.	9,67E+05	8,09E+04	1,19E+05
Average area (positive ionization mode), a.u.	6,46E+07	2,67E+07	1,30E+07

Tables 54-55 shows the average area of each compound in standard mix + blood spot, for the flow rate testing experiments (with analysis time adjusted to each flow rate).

Table 54 Average area (n=3) of each compound in standard mix + blood spot, negative ionization mode, for all mobile phase flow rates tested.

Compound	**Mobile phase flow rate (µL/min)**		
	50	**150**	**300**
	Area (a.u.)	**Area (a.u.)**	**Area (a.u.)**
^{13}C creatine	1,71E+05	1,05E+05	7,69E+04
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*
D2 glycolic acid	ND*	ND*	ND*
D4 succinic acid	8,18E+05	3,50E+05	2,72E+05
D6 glucose	2,87E+05	1,26E+05	8,77E+04
Vancomycin	ND*	4,50E+03	3,20E+03
D3 acylcarnitine C2	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*

* ND = Not detectable

Table 55 Average area (n=3) of each compound in standard mix + blood spot, positive ionization mode, for all mobile phase flow rates tested.

Compound	Mobile phase flow rate (µL/min)		
	50	150	300
	Area (a.u.)	Area (a.u.)	Area (a.u.)
^{13}C creatine	1,10E+07	5,66E+06	3,26E+06
$^{13}C_2$ guanidinoacetate	1,68E+06	9,60E+05	4,00E+05
D2 glycolic acid	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*
D3 acylcarnitine C2	4,92E+06	4,85E+06	3,32E+06
D3 acylcarnitine C12	1,26E+07	5,92E+06	2,95E+06
D3 acylcarnitine C16	ND*	ND*	ND*

* ND = Not detectable

Figures 44-45 show the average area of the standard mix compounds and creatinine in standard mix + blood spot, in the flow rate experiments (with analysis time adjusted to each flow rate).

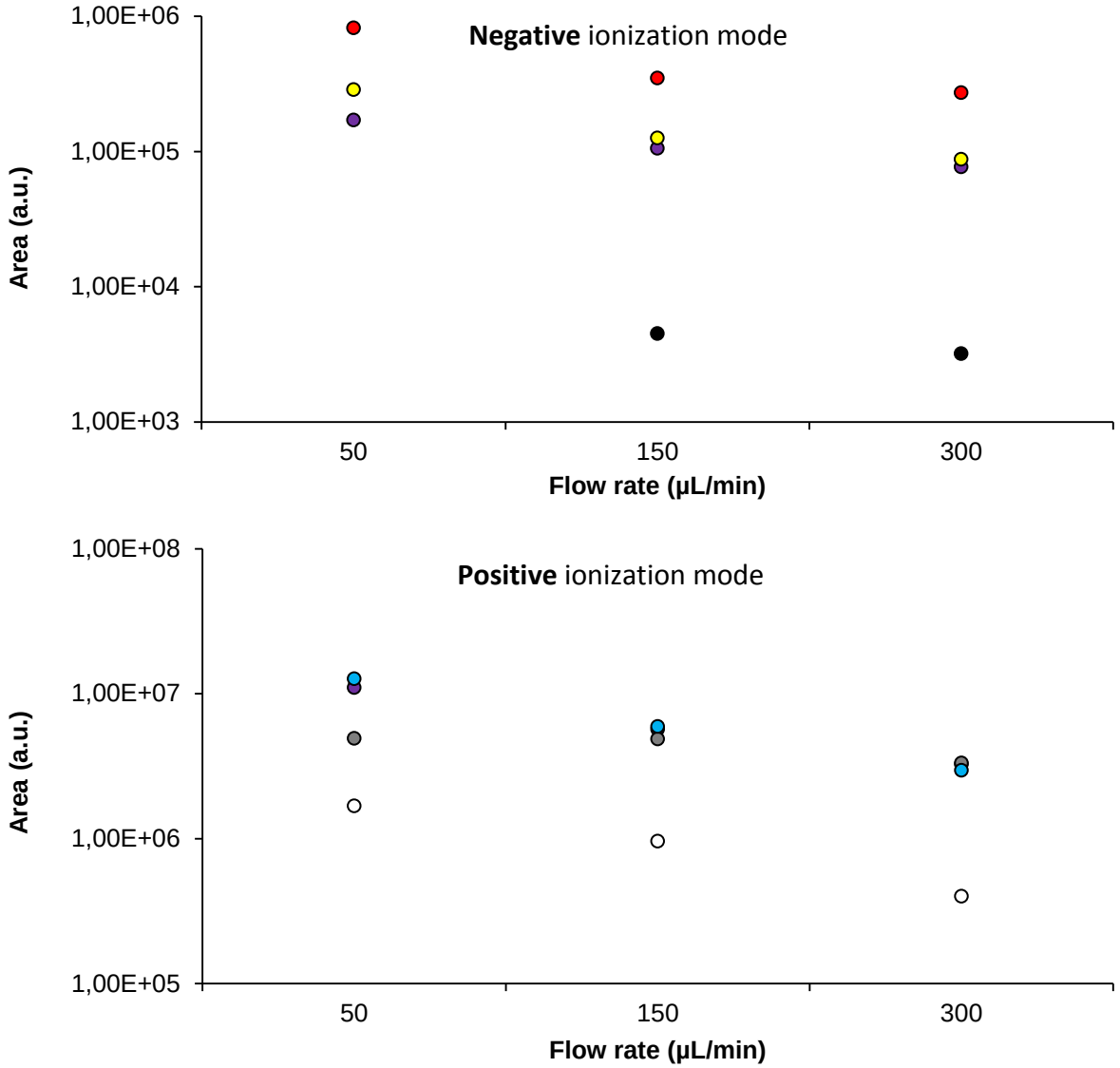

Figure 44 Average area of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all mobile phase flow rates tested (with analysis time adjusted to each flow rate). Area shown is the average area measured from three injections.

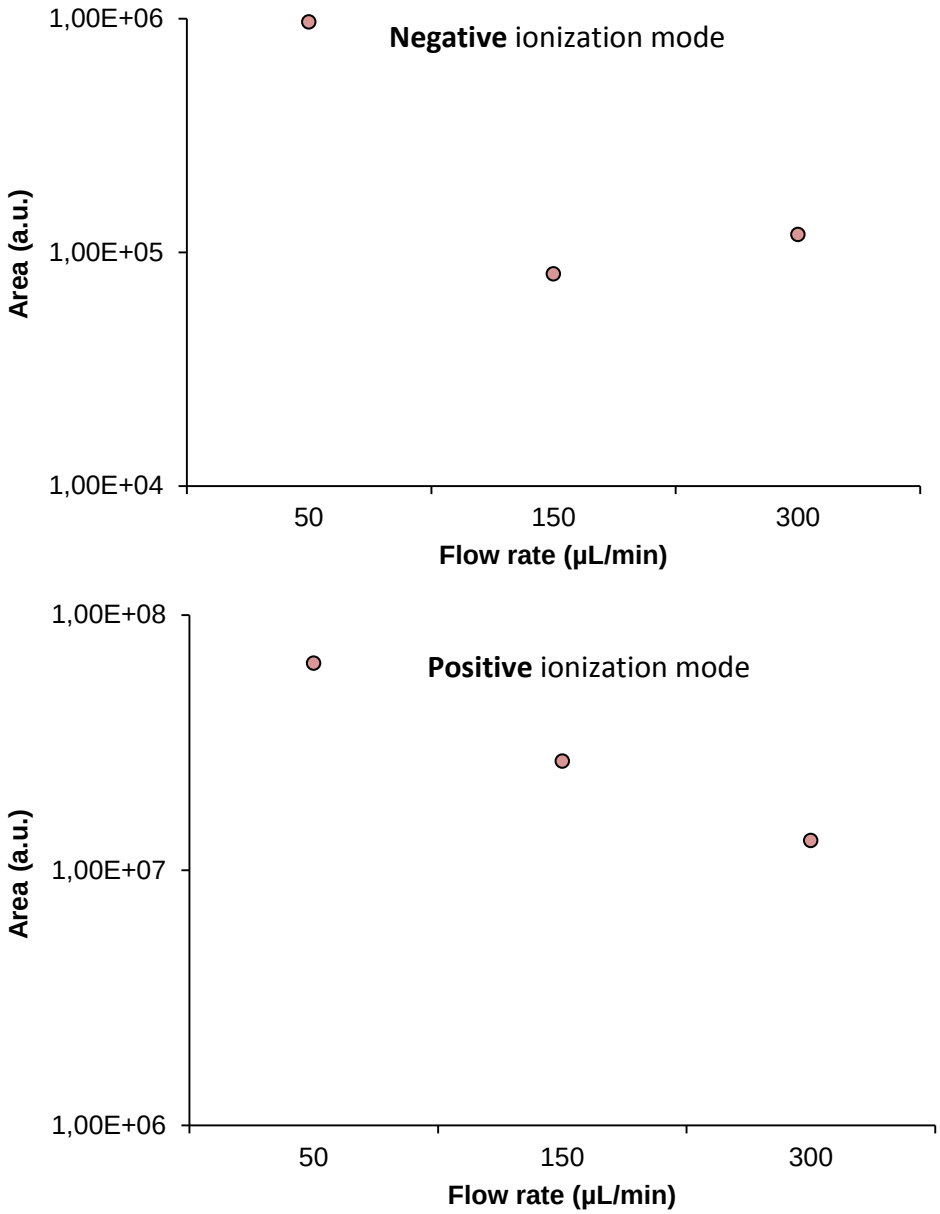

Figure 45 Average area of creatinine in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for all mobile phase flow rates tested (with analysis time adjusted to each flow rate). Area shown is the average area measured from three injections.

6.2 RSD % in the optimization experiments

Tables 56-61 show the relative standard deviation (RSD) % of all compounds in all sample types in the dilution experiments. N=3 for all experiments.

Table 56 RSD % for all compounds in standard mix, negative ionization mode, in the dilution experiments.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	6 %	7 %	4 %	9 %	27 %
$^{13}C_2$ guanidinoacetate	7 %	5 %	4 %	9 %	14 %
D2 glycolic acid	7 %	5 %	4 %	7 %	30 %
D4 succinic acid	6 %	2 %	4 %	14 %	11 %
D6 glucose	8 %	1 %	3 %	12 %	32 %
Vancomycin	11 %	3 %	4 %	9 %	ND*
D3 acylcarnitine C2	9 %	13 %	12 %	ND*	ND*
D3 acylcarnitine C12	10 %	5 %	9 %	13 %	ND*
D3 acylcarnitine C16	40 %	13 %	7 %	ND*	ND*

* ND = Not detectable

Table 57 RSD % for all compounds in standard mix, positive ionization mode, in the dilution experiments.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	5 %	29 %	8 %	4 %	10 %
$^{13}C_2$ guanidinoacetate	5 %	26 %	5 %	9 %	13 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	4 %	24 %	11 %	7 %	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*
Vancomycin	9 %	ND*	25 %	ND*	ND*
D3 acylcarnitine C2	5 %	23 %	2 %	4 %	19 %
D3 acylcarnitine C12	1 %	33 %	9 %	5 %	1 %
D3 acylcarnitine C16	91 %	21 %	19 %	17 %	ND*

* ND = Not detectable

Table 58 RSD % for all compounds in standard mix spot, negative ionization mode, in the dilution experiments.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	13 %	10 %	23 %	ND*	ND*
$^{13}C_2$ guanidinoacetate	15 %	2 %	8 %	ND*	ND*
D2 glycolic acid	14 %	14 %	24 %	ND*	ND*
D4 succinic acid	11 %	9 %	15 %	ND*	ND*
D6 glucose	13 %	7 %	13 %	ND*	ND*
Vancomycin	6 %	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 59 RSD % for all compounds in standard mix spot, positive ionization mode, in the dilution experiments.

Compound	Concentration (µmol/L)				
	10	5	2,5	0,25	0,025
	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	7 %	32 %	97 %	29 %	56 %
$^{13}C_2$ guanidinoacetate	1 %	24 %	8 %	6 %	ND*
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	4 %	27 %	57 %	3 %	ND*
D3 acylcarnitine C12	5 %	20 %	15 %	11 %	ND*
D3 acylcarnitine C16	44 %	43 %	40 %	ND*	ND*

* ND = Not detectable

Table 60 RSD % for all compounds in standard mix + blood spot, negative ionization mode, in the dilution experiments.

Compound	Concentration (µmol/L)				
	5	2,5	1,25	0,125	0,0125
	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	7 %	16 %	12 %	5 %	23 %
$^{13}C_2$ guanidinoacetate	12 %	10 %	8 %	ND*	ND*
D2 glycolic acid	10 %	15 %	13 %	ND*	ND*
D4 succinic acid	5 %	1 %	6 %	ND*	ND*
D6 glucose	6 %	16 %	12 %	ND*	ND*
Vancomycin	10 %	11 %	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 61 RSD % for all compounds in standard mix + blood spot, positive ionization mode, in the dilution experiments.

Compound	Concentration (µmol/L)				
	5	2,5	1,25	0,125	0,0125
	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	4 %	10 %	2 %	4 %	29 %
$^{13}C_2$ guanidinoacetate	8 %	23 %	6 %	8 %	ND*
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	3 %	14 %	6 %	15 %	8 %
D3 acylcarnitine C12	6 %	30 %	2 %	18 %	ND*
D3 acylcarnitine C16	6 %	ND*	12 %	ND*	ND*

* ND = Not detectable

Tables 62-67 show the RSD % of all compounds in all sample types in the electrospray voltage experiments. N=3 for all experiments.

Table 62 RSD % for all compounds in standard mix, negative ionization mode, in the electrospray voltage experiments.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	7 %	5 %	7 %	4 %	1 %	3 %	10 %
$^{13}C_2$ guanidinoacetate	14 %	4 %	10 %	4 %	4 %	3 %	22 %
D2 glycolic acid	14 %	7 %	5 %	6 %	6 %	6 %	21 %
D4 succinic acid	10 %	4 %	10 %	6 %	3 %	4 %	4 %
D6 glucose	7 %	5 %	13 %	2 %	2 %	4 %	29 %
Vancomycin	10 %	6 %	5 %	10 %	6 %	9 %	4 %
D3 acylcarnitine C2	ND*	21 %	11 %	15 %	13 %	9 %	10 %
D3 acylcarnitine C12	7 %	4 %	4 %	10 %	9 %	19 %	15 %
D3 acylcarnitine C16	12 %	6 %	17 %	2 %	36 %	ND*	ND*

* ND = Not detectable

Table 63 RSD % for all compounds in standard mix, positive ionization mode, in the electrospray voltage experiments.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	2 %	5 %	2 %	2 %	3 %	3 %	1 %
$^{13}C_2$ guanidinoacetate	2 %	5 %	1 %	4 %	4 %	6 %	1 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	7 %	8 %	10 %	4 %	3 %	5 %	8 %
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	8 %	9 %	16 %	6 %	8 %	6 %
D3 acylcarnitine C2	2 %	6 %	37 %	3 %	ND*	14 %	1 %
D3 acylcarnitine C12	3 %	8 %	87 %	4 %	ND*	5 %	5 %
D3 acylcarnitine C16	29 %	5 %	76 %	5 %	ND*	16 %	12 %

* ND = Not detectable

Table 64 RSD % for all compounds in standard mix spot, negative ionization mode, in the electrospray voltage experiments.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	21 %	14 %	9 %	13 %	8 %	38 %	104 %
$^{13}C_2$ guanidinoacetate	20 %	17 %	4 %	18 %	25 %	20 %	103 %
D2 glycolic acid	24 %	10 %	2 %	11 %	5 %	14 %	106 %
D4 succinic acid	23 %	20 %	11 %	5 %	7 %	5 %	63 %
D6 glucose	5 %	14 %	1 %	17 %	5 %	33 %	95 %
Vancomycin	ND*	ND*	13 %	10 %	11 %	14 %	7 %
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 65 RSD % for all compounds in standard mix spot, positive ionization mode, in the electrospray voltage experiments.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	10 %	3 %	1 %	4 %	5 %	12 %	8 %
$^{13}C_2$ guanidinoacetate	5 %	3 %	6 %	3 %	8 %	11 %	5 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	5 %	4 %	35 %	8 %	6 %	ND*	5 %
D3 acylcarnitine C12	3 %	9 %	148 %	5 %	6 %	ND*	9 %
D3 acylcarnitine C16	16 %	23 %	41 %	ND*	ND*	ND*	53 %

* ND = Not detectable

Table 66 RSD % for all compounds in standard mix + blood spot, negative ionization mode, in the electrospray voltage experiments.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	23 %	10 %	6 %	24 %	15 %	4 %	84 %
$^{13}C_2$ guanidinoacetate	37 %	10 %	9 %	13 %	7 %	8 %	95 %
D2 glycolic acid	11 %	13 %	11 %	19 %	9 %	8 %	115 %
D4 succinic acid	30 %	20 %	5 %	16 %	9 %	5 %	101 %
D6 glucose	4 %	7 %	8 %	3 %	11 %	5 %	111 %
Vancomycin	ND*	ND*	ND*	ND*	ND*	12 %	15 %
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 67 RSD % for all compounds in standard mix + blood spot, positive ionization mode, in the electrospray voltage experiments.

Compound	Electrospray voltage (kV)						
	1	2	3,5	4	5	6	7
	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	3 %	4 %	4 %	6 %	ND*	2 %	10 %
$^{13}C_2$ guanidinoacetate	15 %	10 %	9 %	15 %	5 %	9 %	12 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	4 %	7 %	125 %	4 %	ND*	6 %	10 %
D3 acylcarnitine C12	11 %	1 %	ND*	5 %	ND*	2 %	7 %
D3 acylcarnitine C16	11 %	12 %	8 %	10 %	ND*	3 %	11 %

* ND = Not detectable

Tables 68-73 show the RSD % of all compounds in all sample types in the electrospray needle position experiments. N=3 for all experiments.

Table 68 RSD % for all compounds in standard mix, negative ionization mode, in the electrospray needle position experiments.

Compound	Electrospray needle position			
	A	B	C	D
	RSD	RSD	RSD	RSD
^{13}C creatine	7 %	3 %	2 %	3 %
$^{13}C_2$ guanidinoacetate	7 %	5 %	1 %	3 %
D2 glycolic acid	5 %	5 %	6 %	4 %
D4 succinic acid	6 %	5 %	2 %	3 %
D6 glucose	6 %	2 %	2 %	2 %
Vancomycin	9 %	3 %	5 %	9 %
D3 acylcarnitine C2	11 %	10 %	13 %	11 %
D3 acylcarnitine C12	5 %	6 %	8 %	5 %
D3 acylcarnitine C16	20 %	4 %	14 %	ND*

* ND = Not detectable

Table 69 RSD % for all compounds in standard mix, positive ionization mode, in the electrospray needle position experiments.

Compound	Electrospray needle position			
	A	B	C	D
	RSD	RSD	RSD	RSD
^{13}C creatine	22 %	3 %	1 %	5 %
$^{13}C_2$ guanidinoacetate	3 %	5 %	2 %	0 %
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	9 %	5 %	4 %	5 %
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	6 %	3 %	8 %	2 %
D3 acylcarnitine C2	3 %	2 %	0 %	2 %
D3 acylcarnitine C12	1 %	6 %	7 %	3 %
D3 acylcarnitine C16	54 %	5 %	5 %	1 %

* ND = Not detectable

Table 70 RSD % for all compounds in standard mix spot, negative ionization mode, in the electrospray needle position experiments.

Compound	Electrospray needle position			
	A	B	C	D
	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	38 %	32 %	31 %
$^{13}C_2$ guanidinoacetate	ND*	9 %	18 %	7 %
D2 glycolic acid	ND*	7 %	9 %	10 %
D4 succinic acid	8 %	7 %	16 %	11 %
D6 glucose	10 %	14 %	1 %	8 %
Vancomycin	9 %	13 %	12 %	15 %
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 71 RSD % for all compounds in standard mix spot, positive ionization mode, in the electrospray needle position experiments.

Compound	Electrospray needle position			
	A	B	C	D
	RSD	RSD	RSD	RSD
^{13}C creatine	5 %	42 %	14 %	13 %
$^{13}C_2$ guanidinoacetate	3 %	8 %	3 %	6 %
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	4 %	6 %	3 %	3 %
D3 acylcarnitine C12	9 %	3 %	10 %	12 %
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 72 RSD % for all compounds in standard mix + blood spot, negative ionization mode, in the electrospray needle position experiments.

Compound	Electrospray needle position			
	A	B	C	D
	RSD	RSD	RSD	RSD
^{13}C creatine	13 %	6 %	12 %	17 %
$^{13}C_2$ guanidinoacetate	ND*	9 %	10 %	20 %
D2 glycolic acid	9 %	8 %	16 %	5 %
D4 succinic acid	17 %	7 %	6 %	13 %
D6 glucose	8 %	14 %	8 %	17 %
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 73 RSD % for all compounds in standard mix + blood spot, positive ionization mode, in the electrospray needle position experiments.

Compound	Electrospray needle position			
	A	B	C	D
	RSD	RSD	RSD	RSD
^{13}C creatine	10 %	4 %	3 %	4 %
$^{13}C_2$ guanidinoacetate	7 %	1 %	5 %	4 %
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	6 %	2 %	8 %	2 %
D3 acylcarnitine C12	2 %	21 %	6 %	5 %
D3 acylcarnitine C16	ND*	13 %	24 %	6 %

* ND = Not detectable

Tables 74-79 show the RSD % of all compounds in all sample types in the resolution experiments. N=3 for all experiments.

Table 74 RSD % for all compounds in standard mix, negative ionization mode, in the resolution experiments.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	RSD	RSD	RSD	RSD
^{13}C creatine	3 %	1 %	4 %	1 %
$^{13}C_2$ guanidinoacetate	5 %	35 %	6 %	10 %
D2 glycolic acid	9 %	48 %	6 %	4 %
D4 succinic acid	11 %	5 %	5 %	3 %
D6 glucose	4 %	3 %	6 %	4 %
Vancomycin	2 %	2 %	5 %	4 %
D3 acylcarnitine C2	5 %	11 %	14 %	12 %
D3 acylcarnitine C12	2 %	61 %	6 %	4 %
D3 acylcarnitine C16	43 %	781 %	3 %	18 %

Table 75 RSD % for all compounds in standard mix, positive ionization mode, in the resolution experiments.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	RSD	RSD	RSD	RSD
^{13}C creatine	3 %	6 %	1 %	2 %
$^{13}C_2$ guanidinoacetate	1 %	3 %	2 %	1 %
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	6 %	3 %	2 %	8 %
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	18 %	8 %	12 %	12 %
D3 acylcarnitine C2	2 %	1 %	3 %	1 %
D3 acylcarnitine C12	4 %	1 %	5 %	10 %
D3 acylcarnitine C16	2 %	19 %	11 %	8 %

* ND = Not detectable

Table 76 RSD % for all compounds in standard mix spot, negative ionization mode, in the resolution experiments.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	2 %	6 %	10 %
$^{13}C_2$ guanidinoacetate	1 %	6 %	7 %	19 %
D2 glycolic acid	29 %	2 %	10 %	11 %
D4 succinic acid	22 %	12 %	7 %	18 %
D6 glucose	4 %	7 %	8 %	5 %
Vancomycin	ND*	ND*	6 %	4 %
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 77 RSD % for all compounds in standard mix spot, positive ionization mode, in the resolution experiments.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	RSD	RSD	RSD	RSD
^{13}C creatine	9 %	3 %	23 %	13 %
$^{13}C_2$ guanidinoacetate	4 %	4 %	3 %	3 %
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	7 %	5 %	5 %	9 %
D3 acylcarnitine C12	8 %	7 %	1 %	5 %
D3 acylcarnitine C16	ND*	ND*	31 %	18 %

* ND = Not detectable

Table 78 RSD % for all compounds in standard mix + blood spot, negative ionization mode, in the resolution experiments.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	RSD	RSD	RSD	RSD
^{13}C creatine	76 %	29 %	13 %	5 %
$^{13}C_2$ guanidinoacetate	ND*	11 %	3 %	14 %
D2 glycolic acid	5 %	11 %	14 %	9 %
D4 succinic acid	11 %	7 %	11 %	13 %
D6 glucose	14 %	4 %	16 %	11 %
Vancomycin	ND*	ND*	17 %	4 %
D3 acylcarnitine C2	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 79 RSD % for all compounds in standard mix + blood spot, positive ionization mode, in the resolution experiments.

Compound	Resolution (a.u.)			
	17500	35000	70000	140000
	RSD	RSD	RSD	RSD
^{13}C creatine	5 %	8 %	14 %	7 %
$^{13}C_2$ guanidinoacetate	3 %	2 %	3 %	14 %
D2 glycolic acid	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	4 %	9 %	8 %	7 %
D3 acylcarnitine C12	3 %	ND*	9 %	3 %
D3 acylcarnitine C16	ND*	ND*	6 %	2 %

* ND = Not detectable

Tables 80-85 show the RSD % of all compounds in all sample types in the AGC target experiments with resolution set to 70 000. N=3 for all experiments.

Table 80 RSD % for all compounds in standard mix, negative ionization mode, in the AGC target experiments with resolution set to 70 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	10 %	4 %	2 %	5 %	3 %	4 %	1 %	5 %
$^{13}C_2$ guanidinoacetate	17 %	6 %	4 %	4 %	6 %	5 %	5 %	4 %
D2 glycolic acid	14 %	5 %	9 %	5 %	10 %	6 %	8 %	0 %
D4 succinic acid	10 %	4 %	4 %	6 %	10 %	6 %	3 %	1 %
D6 glucose	4 %	4 %	1 %	3 %	2 %	5 %	4 %	5 %
Vancomycin	7 %	3 %	5 %	7 %	5 %	4 %	4 %	1 %
D3 acylcarnitine C2	ND*	ND*	ND*	16 %	5 %	9 %	3 %	24 %
D3 acylcarnitine C12	ND*	13 %	5 %	9 %	3 %	10 %	6 %	6 %
D3 acylcarnitine C16	ND*	ND*	ND*	29 %	9 %	5 %	5 %	41 %

* ND = Not detectable

Table 81 RSD % for all compounds in standard mix, positive ionization mode, in the AGC target experiments with resolution set to 70 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	4 %	2 %	3 %	3 %	2 %	4 %	2 %	47 %
$^{13}C_2$ guanidinoacetate	6 %	7 %	3 %	1 %	2 %	4 %	1 %	46 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	9 %	5 %	11 %	5 %	7 %	6 %	52 %
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	14 %	20 %	14 %	13 %	9 %	8 %	73 %
D3 acylcarnitine C2	9 %	2 %	4 %	4 %	0 %	6 %	4 %	48 %
D3 acylcarnitine C12	1 %	6 %	1 %	5 %	2 %	4 %	8 %	64 %
D3 acylcarnitine C16	31 %	10 %	6 %	0 %	3 %	7 %	6 %	63 %

* ND = Not detectable

Table 82 RSD % for all compounds in standard mix spot, negative ionization mode, in the AGC target experiments with resolution set to 70 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	ND*	ND*	39 %	3 %	9 %	8 %	9 %
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	10 %	28 %	8 %
D2 glycolic acid	ND*	ND*	ND*	20 %	34 %	8 %	2 %	9 %
D4 succinic acid	ND*	ND*	5 %	9 %	24 %	14 %	15 %	7 %
D6 glucose	ND*	ND*	ND*	9 %	17 %	11 %	14 %	9 %
Vancomycin	ND*	7 %	8 %	8 %	25 %	4 %	6 %	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	13 %	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 83 RSD % for all compounds in standard mix spot, positive ionization mode, in the AGC target experiments with resolution set to 70 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	2 %	7 %	3 %	1 %	8 %	4 %	30 %	4 %
$^{13}C_2$ guanidinoacetate	ND*	9 %	1 %	5 %	10 %	3 %	20 %	6 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	2 %	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	22 %	9 %	3 %	2 %	12 %	5 %	28 %	9 %
D3 acylcarnitine C12	ND*	11 %	9 %	18 %	3 %	21 %	63 %	15 %
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 84 RSD % for all compounds in standard mix + blood spot, negative ionization mode, in the AGC target experiments with resolution set to 70 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	ND*	ND*	28 %	14 %	9 %	14 %	8 %
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	4 %	8 %	5 %
D2 glycolic acid	ND*	ND*	ND*	15 %	10 %	6 %	10 %	6 %
D4 succinic acid	ND*	21 %	21 %	8 %	26 %	7 %	4 %	13 %
D6 glucose	ND*	ND*	ND*	6 %	15 %	11 %	8 %	3 %
Vancomycin	45 %	5 %	3 %	4 %	5 %	9 %	ND*	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 85 RSD % for all compounds in standard mix + blood spot, positive ionization mode, in the AGC target experiments with resolution set to 70 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	7 %	4 %	3 %	7 %	8 %	1 %	5 %	8 %
$^{13}C_2$ guanidinoacetate	ND*	18 %	11 %	3 %	9 %	6 %	8 %	10 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	11 %	5 %	14 %	3 %	1 %	5 %	8 %	4 %
D3 acylcarnitine C12	ND*	6 %	5 %	2 %	4 %	4 %	4 %	21 %
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Tables 86-91 show the RSD % of all compounds in all sample types in the AGC target experiments with resolution set to 140 000. N=3 for all experiments.

Table 86 RSD % for all compounds in standard mix, negative ionization mode, in the AGC target experiments with resolution set to 140 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	6 %	2 %	7 %	4 %	7 %	6 %	3 %	4 %
$^{13}C_2$ guanidinoacetate	8 %	7 %	6 %	7 %	7 %	7 %	2 %	6 %
D2 glycolic acid	7 %	3 %	4 %	9 %	3 %	7 %	5 %	6 %
D4 succinic acid	7 %	4 %	7 %	10 %	10 %	5 %	4 %	10 %
D6 glucose	6 %	6 %	5 %	2 %	3 %	6 %	4 %	8 %
Vancomycin	6 %	5 %	5 %	3 %	9 %	5 %	2 %	4 %
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	17 %	6 %	13 %
D3 acylcarnitine C12	9 %	6 %	5 %	2 %	6 %	2 %	1 %	2 %
D3 acylcarnitine C16	ND*	ND*	31 %	12 %	ND*	10 %	8 %	13 %

* ND = Not detectable

Table 87 RSD % for all compounds in standard mix, positive ionization mode, in the AGC target experiments with resolution set to 140 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	21 %	2 %	1 %	2 %	7 %	3 %	ND*	4 %
$^{13}C_2$ guanidinoacetate	6 %	3 %	3 %	7 %	5 %	5 %	2 %	5 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	14 %	12 %	8 %	6 %	4 %	6 %	23 %
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	5 %	21 %	23 %	14 %	9 %	13 %	33 %	18 %
D3 acylcarnitine C2	10 %	7 %	5 %	4 %	4 %	2 %	ND*	6 %
D3 acylcarnitine C12	3 %	5 %	4 %	4 %	5 %	3 %	ND*	3 %
D3 acylcarnitine C16	83 %	10 %	26 %	3 %	48 %	22 %	ND*	14 %

* ND = Not detectable

Table 88 RSD % for all compounds in standard mix spot, negative ionization mode, in the AGC target experiments with resolution set to 140 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	ND*	ND*	ND*	ND*	ND*	20 %	6 %
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	ND*	8 %	7 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	11 %	13 %
D4 succinic acid	ND*	ND*	12 %	26 %	30 %	4 %	8 %	11 %
D6 glucose	ND*	ND*	ND*	ND*	ND*	14 %	13 %	9 %
Vancomycin	ND*	ND*	4 %	ND*	ND*	6 %	7 %	ND*
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 89 RSD % for all compounds in standard mix spot, positive ionization mode, in the AGC target experiments with resolution set to 140 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	10 %	12 %	11 %	17 %	7 %	5 %	5 %
$^{13}C_2$ guanidinoacetate	ND*	ND*	17 %	14 %	17 %	8 %	11 %	5 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	9 %	16 %	10 %	3 %	8 %	ND*	10 %
D3 acylcarnitine C12	ND*	ND*	ND*	16 %	ND*	17 %	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 90 RSD % for all compounds in standard mix + blood spot, negative ionization mode, in the AGC target experiments with resolution set to 140 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	ND*	ND*	ND*	6 %	6 %	7 %	3 %
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	30 %	5 %	10 %
D4 succinic acid	ND*	ND*	18 %	14 %	9 %	5 %	14 %	7 %
D6 glucose	ND*	ND*	ND*	ND*	19 %	14 %	9 %	6 %
Vancomycin	13 %	4 %	6 %	10 %	6 %	11 %	11 %	4 %
D3 acylcarnitine C2	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Table 91 RSD % for all compounds in standard mix + blood spot, positive ionization mode, in the AGC target experiments with resolution set to 140 000.

Compound	AGC target value (number of ion counts)							
	2,00E+04	5,00E+04	1,00E+05	2,00E+05	5,00E+05	1,00E+06	3,00E+06	5,00E+06
	RSD	RSD	RSD	RSD	RSD	RSD	RSD	RSD
^{13}C creatine	ND*	6 %	5 %	8 %	20 %	1 %	5 %	5 %
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*	ND*	16 %	9 %	5 %	7 %
D2 glycolic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
D3 acylcarnitine C2	ND*	10 %	9 %	2 %	27 %	4 %	10 %	4 %
D3 acylcarnitine C12	ND*	ND*	ND*	8 %	ND*	9 %	ND*	10 %
D3 acylcarnitine C16	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

* ND = Not detectable

Tables 92-93 show the RSD % of all compounds in the mobile phase flow rate experiments (with analysis time adjusted to each flow rate). N=3 for all experiments.

Table 92 RSD % for all compounds in standard mix + blood spot, negative ionization mode, in the mobile phase flow rate experiments.

Compound	Mobile phase flow rate (µL/min)		
	50	150	300
	RSD	RSD	RSD
^{13}C creatine	23 %	15 %	11 %
$^{13}C_2$ guanidinoacetate	ND*	ND*	ND*
D2 glycolic acid	ND*	ND*	ND*
D4 succinic acid	31 %	13 %	25 %
D6 glucose	19 %	7 %	9 %
Vancomycin	ND*	73 %	18 %
D3 acylcarnitine C2	ND*	ND*	ND*
D3 acylcarnitine C12	ND*	ND*	ND*
D3 acylcarnitine C16	ND*	ND*	ND*

* ND = Not detectable

Table 93 RSD % for all compounds in standard mix + blood spot, positive ionization mode, in the mobile phase flow rate experiments.

Compound	Mobile phase flow rate (µL/min)		
	50	150	300
	RSD	RSD	RSD
^{13}C creatine	19 %	15 %	10 %
$^{13}C_2$ guanidinoacetate	3 %	10 %	6 %
D2 glycolic acid	ND*	ND*	ND*
D4 succinic acid	ND*	ND*	ND*
D6 glucose	ND*	ND*	ND*
Vancomycin	ND*	ND*	ND*
D3 acylcarnitine C2	9 %	2 %	9 %
D3 acylcarnitine C12	5 %	6 %	7 %
D3 acylcarnitine C16	ND*	ND*	ND*

* ND = Not detectable

6.3 Total ion chromatograms and extracted ion chromatograms

Figures 46-47 show the TICs and EICs of all detected compounds in standard mix spot, using initial settings (see **Table 5**) (before any optimization was done).

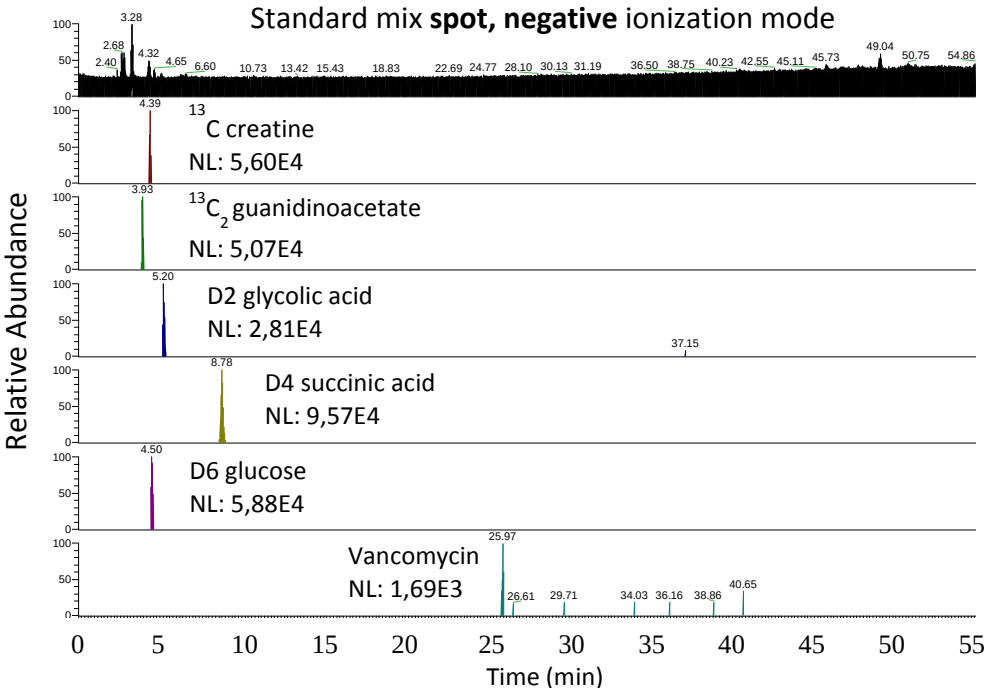

Figure 46 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix spot, negative ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed.

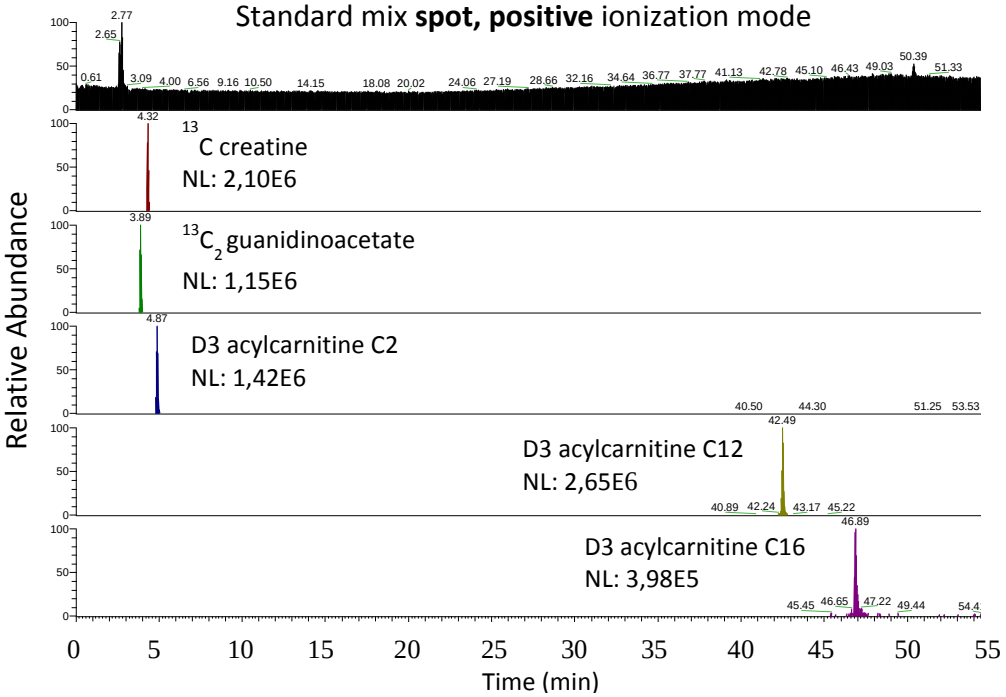

Figure 47 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix spot, positive ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed.

Figures 48-49 show the TICs and EICs of all detected compounds in standard mix spot, with initial settings (see **Table 5**) for all parameters except AGC target value (set to 1 000 000 ion counts).

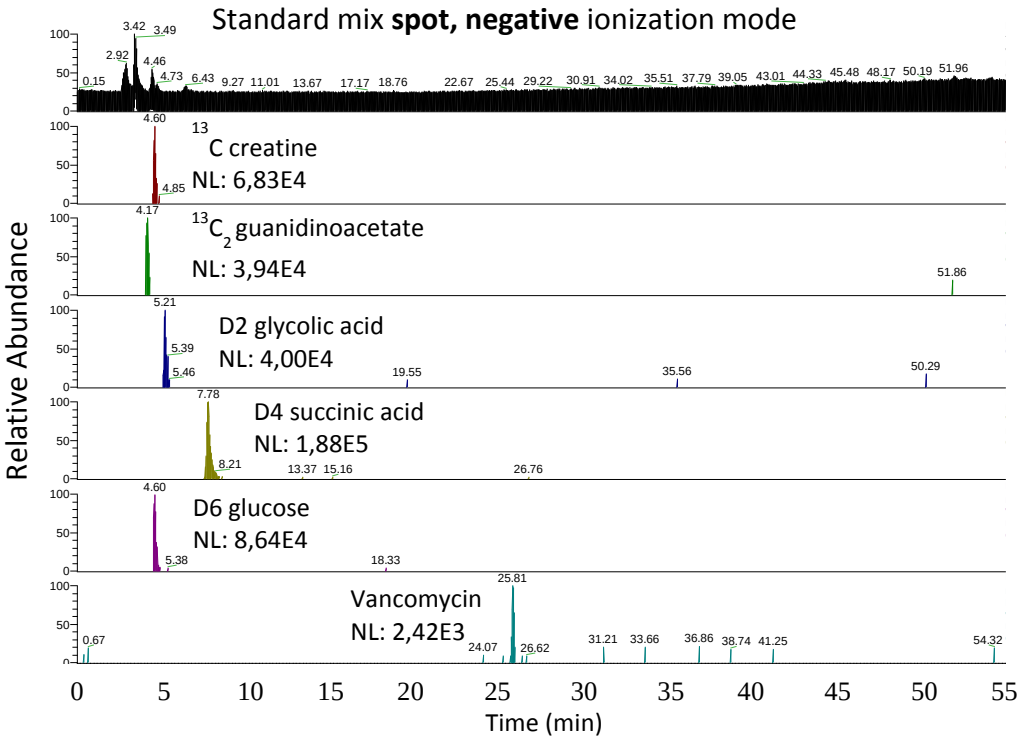

Figure 48 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix spot, negative ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed. Analysis was performed using the initial settings except for AGC target value, which was set to 1 000 000 ion counts.

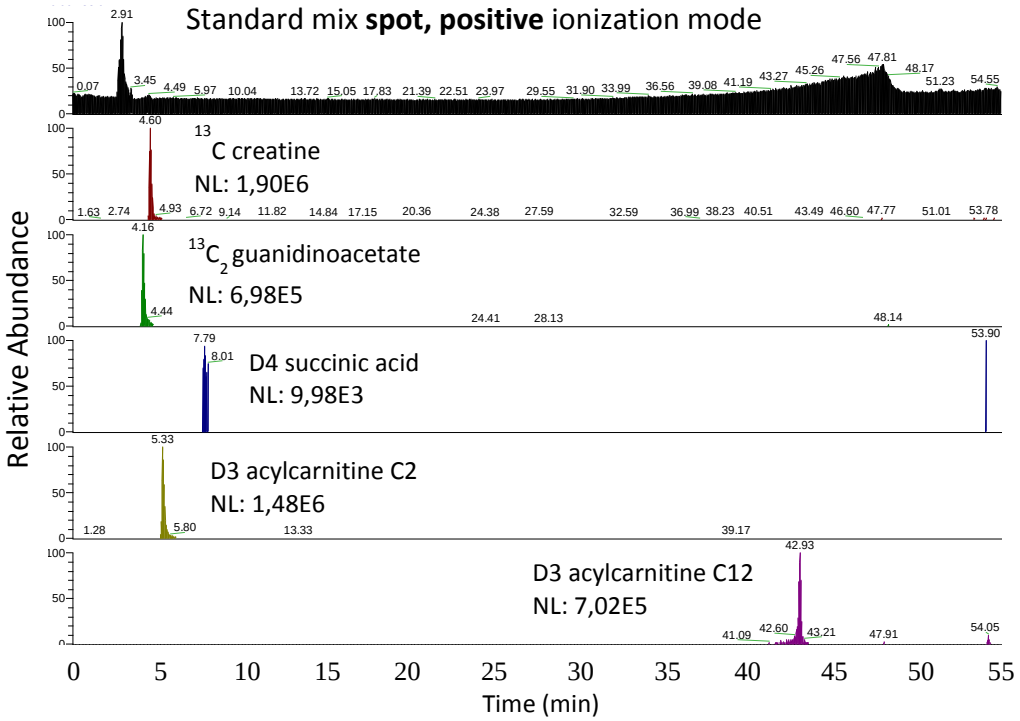

Figure 49 Total ion chromatogram and extracted ion chromatogram of each detected compound in standard mix spot, positive ionization mode, with a concentration of 10 µmol/L of each compound. Measured intensity (NL, normalized intensity level) of each peak is listed. Analysis was performed using the initial settings except for AGC target value, which was set to 1 000 000 ion counts.

6.4 Initial testing of electrospray voltage

Initial testing of electrospray voltage was done to investigate the effect of high electrospray voltage on peak intensity. The dilution analyses (see section **4.2**) (with spray voltage set to 3.5 kV) were compared to analyses performed with 7 kV (no other parameters were changed, see **Table 5** for settings). Results are shown in **Figures 50-52**. Tables showing the average intensity of each compound in each sample are shown in **Appendix** section 6.1, Tables 14-19.

For the standard mix sample, the measured intensity for all compounds was higher using 3.5 kV than 7 kV, for both ionization modes, see **Figure 50**.

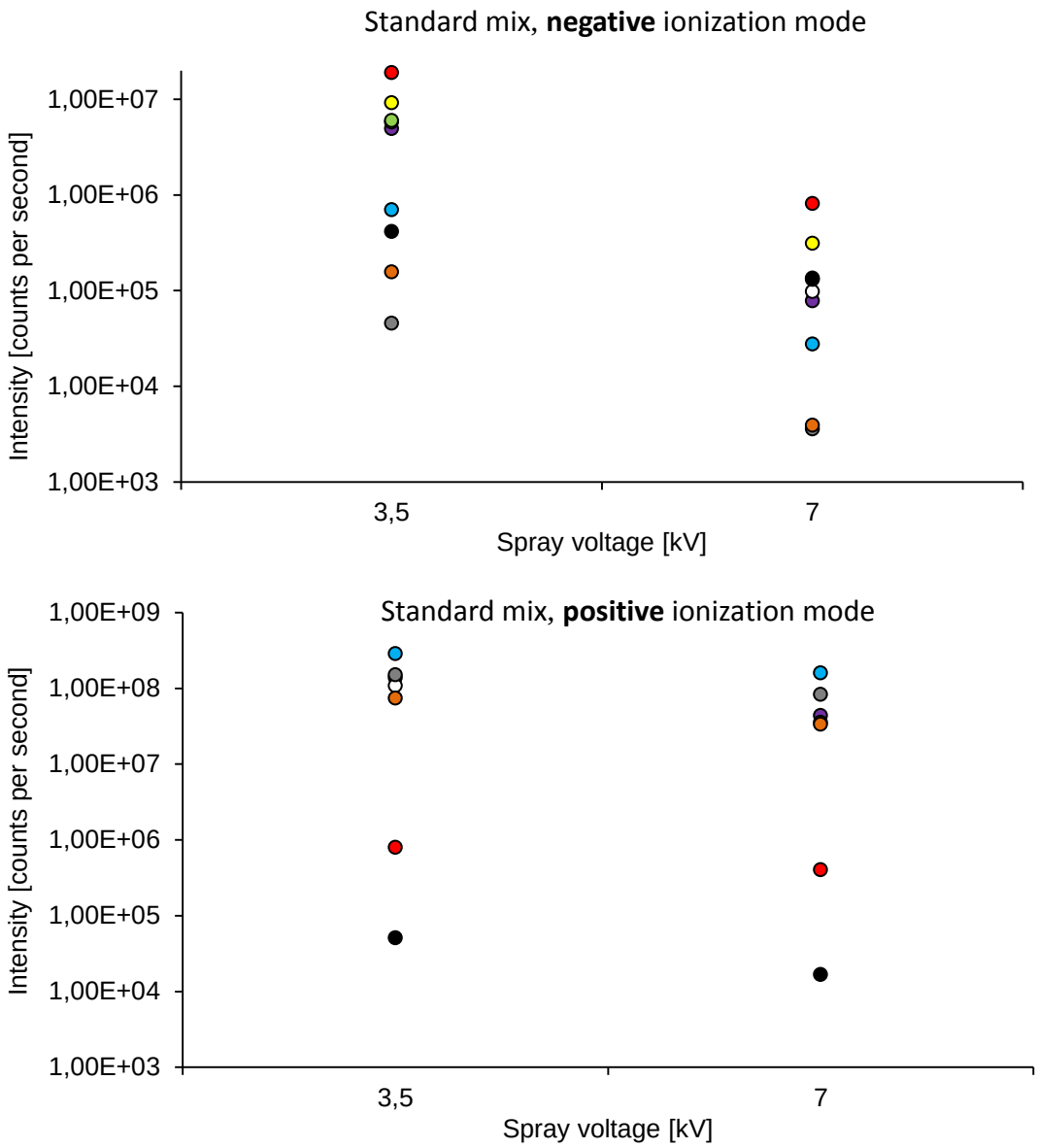

Figure 50 Measured intensity of all compounds in standard mix, negative (top) and positive (bottom) ionization mode, for electrospray voltage values 3.5 and 7 kV. Intensity shown is the average intensity measured from three injections. The color code for each compound is shown in **Table 6**.

For the standard mix spot sample, all standard mix compounds that were detected with both spray voltages had higher signal intensity with 3.5 kV. Additionally, more compounds were detected with 3.5 kV, see **Figure 51**.

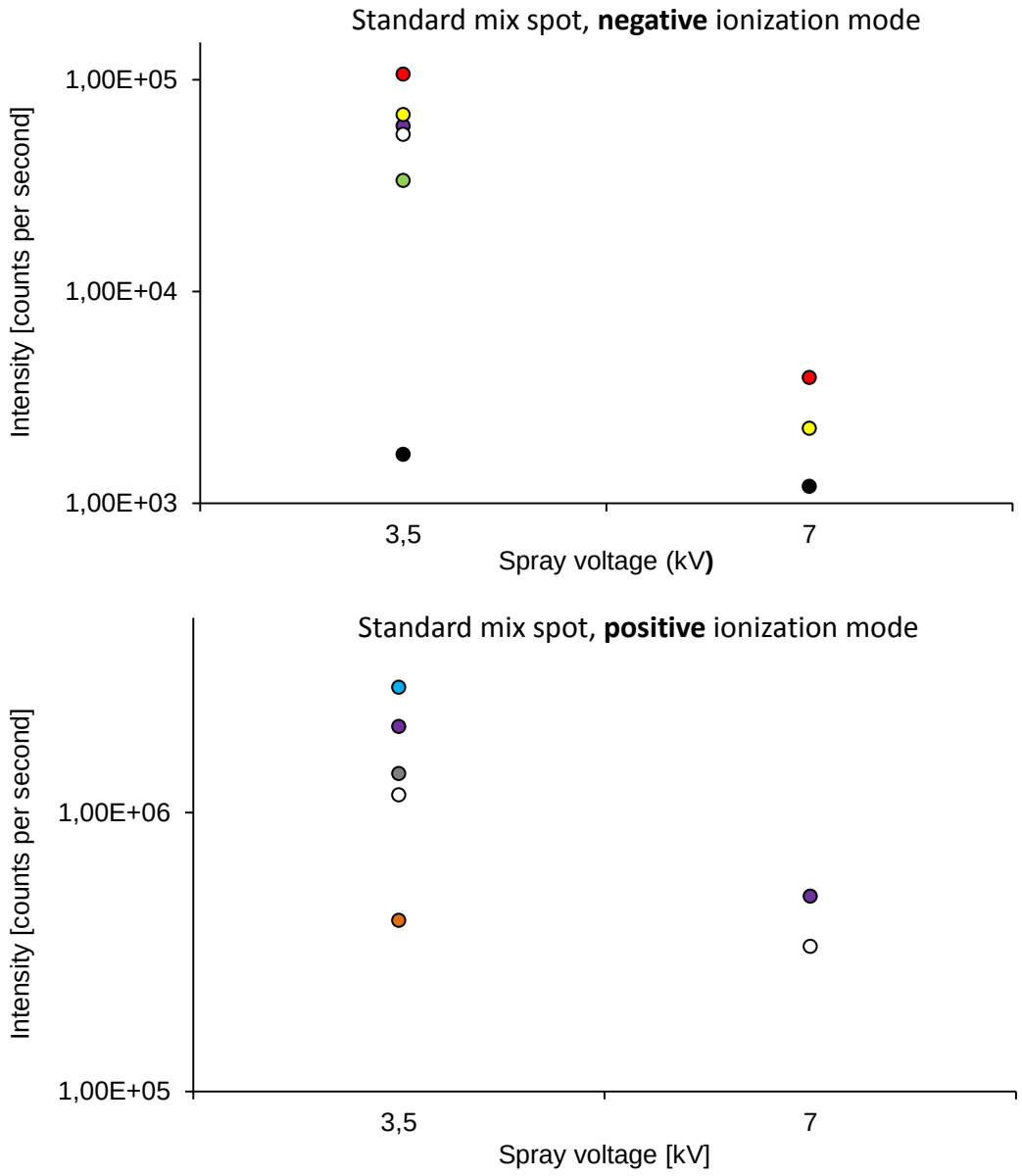

Figure 51 Measured intensity of all compounds in standard mix spot, negative (top) and positive (bottom) ionization mode, for electrospray voltage values 3.5 and 7 kV. Intensity shown is the average intensity measured from three injections. The color code for each compound is shown in **Table 6**.

In the standard mix + blood spot sample, more compounds were detected with 3.5 kV in negative mode. Most compounds had higher signal intensity with 3.5 kV in both ionization modes, except for vancomycin in negative mode and D3 acylcarnitine C16 in positive mode, see **Figure 52**.

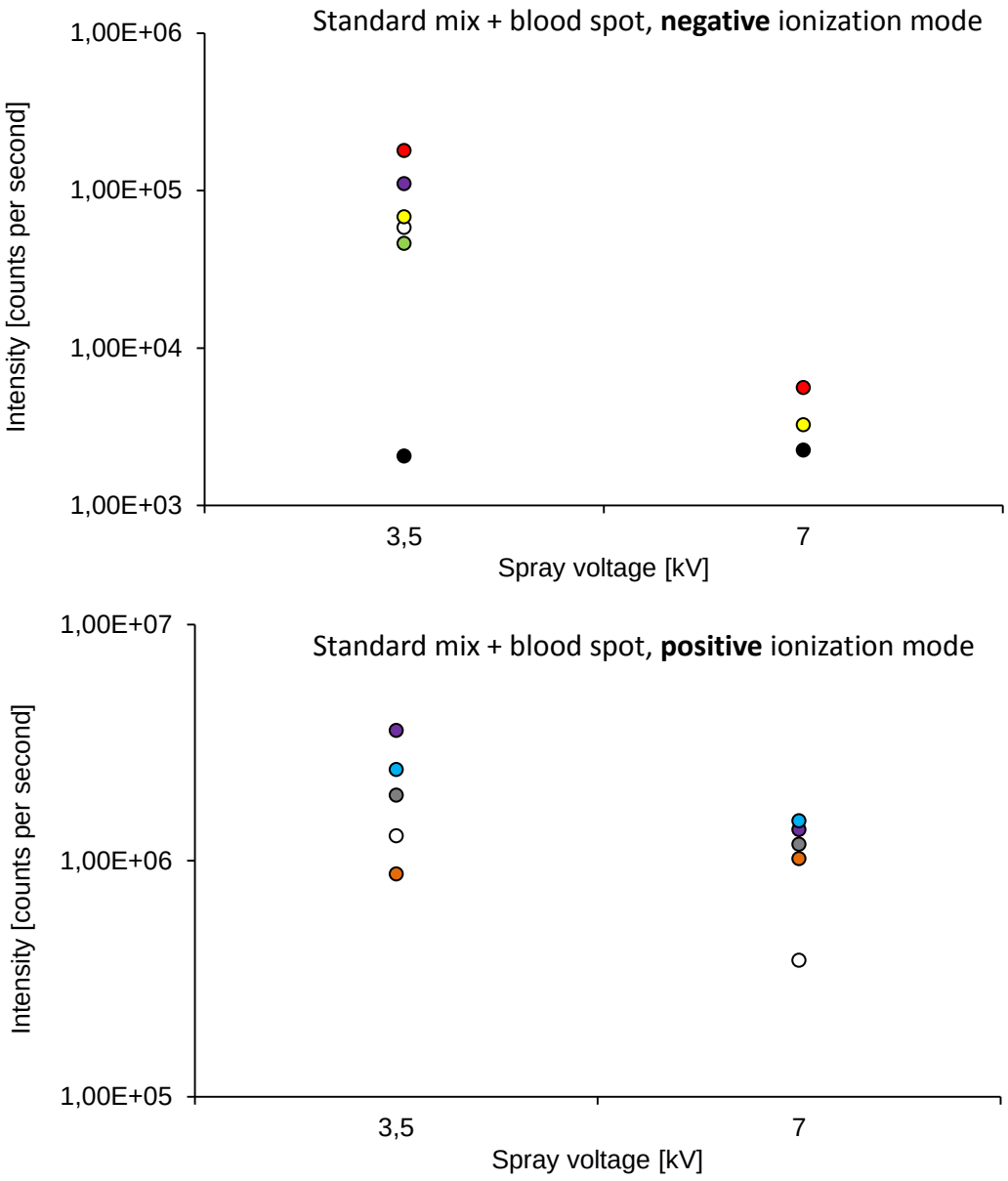

Figure 52 Measured intensity of all compounds in standard mix + blood spot, negative (top) and positive (bottom) ionization mode, for electrospray voltage values 3.5 and 7 kV. Intensity shown is the average intensity measured from three injections. The color code for each compound is shown in **Table 6**.

In summary, results from the initial electrospray voltage experiments indicated that spray voltage set to 3.5 kV was a better choice than 7 kV.

6.5 Carry-over

Some carry-over was observed for acylcarnitines D3 C12 and D3 C16. There were also some detection problems with these compounds, indicating stability issues. The effect of carry-over was considered not to influence the results of the parameter testing. Nevertheless, evaluation of carry-over will be part of the method validation.

Carry-over in diagnostic samples can be a major problem because of the risk of false positives, i.e. diagnosing a patient because the previous run included a sample from a patient with a disease. If a compound is known to always be present at the same level in the sample/instrument/column or other places, it is not necessarily a problem – the contribution can be subtracted from the analysis result. A more serious case is if carry-over suddenly occurs from a compound used as a biomarker.

Carry-over can be caused by compounds accumulating different places – such as on the column, injection needle, other autosampler parts, solvents, pipettes, punchers etc [33]. It can be difficult to locate the source of the carry-over. One way to do so is to gradually investigate the different parts used in the sample preparation and the analysis, i.e. no injection of sample, only mobile phase, to see if the carry-over is caused by compounds present in the mobile phases, or injection of sample with and without the analytical column to investigate if the column is the carry-over source. This can be tedious and difficult. To check for carry-over, it is important to analyze blank samples regularly, i.e. direct injection of a water sample (through the column but not prepared as the other samples, for example).

6.6 Poster presented at «Det 16. norske seminar i massespektrometri», Hafjell 2017

Oslo University Hospital

OPTIMIZATION OF MASS SPECTROMETRIC PARAMETERS FOR UNTARGETED METABOLOMICS OF DRIED BLOOD SPOTS

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Background

Metabolism is the set of chemical processes in the cells of living organisms involving the breaking down and building of complex compounds and molecules. All the small molecular compounds that participate or are the end products in the metabolism constitute the metabolome. Metabolome analysis results in a metabolic profile unique for each individual and each sample material from a single individual. In Norway, laboratory diagnostics of Inborn Errors of Metabolism (IEMs) is done at The Department of Medical Biochemistry at Oslo University Hospital, Rikshospitalet. An IEM is an inherited disease characterized by deficient ability to break down or synthesize one or several specific compounds. About 1500 different IEMs are described. Most IEMs are caused by a mutation in the DNA leading to a dysfunctional protein, typically an enzyme. This malfunction results in deviations in the metabolome, and these deviations are the basis of the diagnostics. [1] Metabolomics consists of separating, identifying and quantifying low-molecular weight metabolites (typically <1,000 Da [2]) in samples, providing information about the physiological state of an organism [3]. Untargeted metabolomics involves analysing a sample with the purpose of detecting as many metabolites as possible, thereby maximizing the coverage of the metabolome. Orbitrap mass spectrometry coupled to liquid chromatography (LC-MS) is a powerful tool for untargeted metabolomics, due to the high resolution and sensitivity of this technique. Metabolomics is anticipated to play an important role in the future diagnostics of IEMs.

1.Hagve, T.-A., *Klinisk Biokjemi og Fysiologi*. 4 ed. 2011.
2. Qi, Y., et al., *Global Metabolic Profiling Using Ultra-Performance Liquid Chromatography/Quadrupole Time-of-Flight Mass Spectrometry*, in *Mass Spectrometry in Metabolomics: Methods and Protocols (Methods in Molecular Biology)*, D. Raftery, Editor. 2014, Humana Press. p. 15-27.
3. Roessner, U. and J. Bowne, *What is metabolomics all about?* BioTechniques Special Issue, 2009. **46**(5): p.363-365.

Objective

The objective was to develop a robust and sensitive Q Exactive Orbitrap mass spectrometer (MS) method for untargeted metabolomics of dried blood spots (DBS) using electrospray ionization (ESI). This was achieved by optimizing MS parameters including ESI spray voltage and needle position, resolution, and automatic gain control (AGC) target. The MS method will be merged with a liquid chromatography method being developed by master student Camilla Elene Arnesen, see poster "Optimization of liquid chromatographic parameters for untargeted metabolomics of dried blood spots".

Materials and methods

A standard mix covering a wide polarity range representing the blood metabolome was used throughout this work. The standard mix was an aqueous solution consisting of the following non-endogenous compounds: isotopically labelled acylcarnitines (C2, C12 and C16), creatine, guanidinoacetate, glycolic acid, succinic acid, glucose, and non-labelled vancomycin (a drug absent in blood if not taken by the patient). The use of isotopically labelled compounds and a drug ensures that the only contribution to the signal intensity in the mass spectra is from the added compounds, independent of levels of endogenous compounds. Three sample types were used: standard mix injected with no prior sample preparation, standard mix spotted onto filter paper, and standard mix + whole blood (50:50 v/v) spotted onto filter paper.

Sample preparation of DBS was done as follows: To one punch of the DBS,100 µL of 80 % aqueous methanol (MeOH) with 0.1 % formic acid (FA) was added and extraction performed by mixing for 45 minutes (45 °C, 700 rpm). After evaporation to dryness (N_2, 40 °C), 100 µL of starting mobile phase was added, as shown in **Table 1**. The sample solution was mixed and injected. Settings used in all experiments are shown in **Table 2**. Samples were injected three times in both positive and negative ionization mode. All experiments were performed using a C18 Diphenyl column. Mobile phase A and B consisted of Type 1 water with 0.1 % FA, and MeOH with 0.1 % FA, respectively. LC parameters were kept constant in all experiments.

To find a suitable setting for ESI spray voltage and needle position, resolution, and AGC target, all sample types were injected with a concentration of 10 µmol/L (standard mix and standard mix spot) and 5 µmol/L (standard mix + blood spot) of each compound. Results for the latter are shown in **Figures 1-8**.

Table 1: Gradient program used in all experiments. B is the organic phase (MeOH). Equilibration was performed for 10 minutes after each analysis.

Time (min)	% B
0	5
10	5
50	100
55	100
55.1	5

Table 2: Settings used in all experiments shown.

Parameter	Setting
Scan ranges (m/z)	50-750, 750-1700
Scan type	Full MS
Capillary temperature (°C)	250
Aux gas heater temperature (°C)	300
Injection volume (µL)	2
Flow rate (µL/min)	150
Column temperature	Room temperature

Results and discussion

Several spray voltage values were tested: 1, 2, 3.5, 4, 5, 6 and 7 kV, see **Figures 1-2**. Overall, a spray voltage of 3.5 kV was considered to be the best choice, as this gave a high intensity for all compounds, and, with some exceptions, the highest number of detectable standard mix compounds. Each point in all figures shown represent the average intensity measured from three injections.

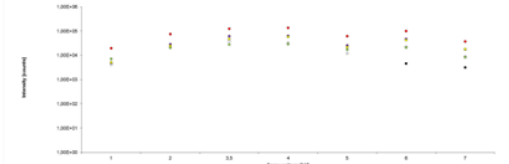

Figure 1: Average intensity of each compound in standard mix + blood spot for all spray voltage values tested, negative ionization mode.

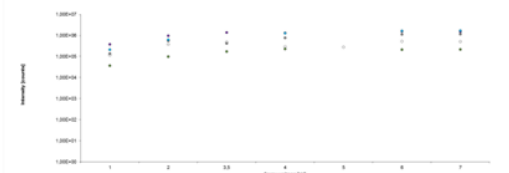

Figure 2: Average intensity of each compound in standard mix + blood spot for all spray voltage values tested, positive ionization mode.

Using a spray voltage of 3.5 kV, the available ESI needle positions (A, B, C and D) were tested, see **Figures 3-4**. Position A gives the shortest distance between the ESI needle outlet and the MS inlet, D the longest. As position C gave high intensities, as well as being one of the positions that gave the highest number of detected standard mix compounds for all sample types, this was considered to be the best choice.

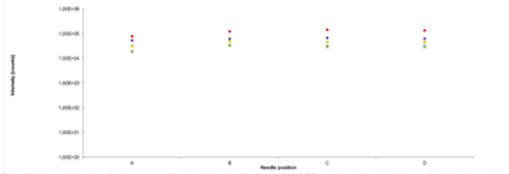

Figure 3: Average intensity of each compound in standard mix + blood spot for all ESI needle positions tested, negative ionization mode.

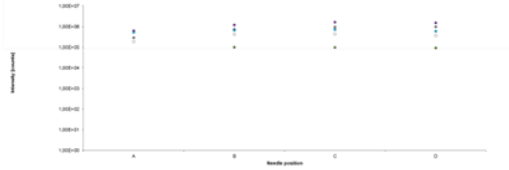

Figure 4: Average intensity of each compound in standard mix + blood spot for all ESI needle positions tested, positive ionization mode.

With a spray voltage of 3.5 kV and needle position C, the available resolution values were tested, see **Figures 5-6**. As expected, a resolution of 70 000 and 140 000 gave the highest number of detectable standard mix compounds for all sample types. To decide which was the best choice, both were used to test the available AGC target values (results for the analyses performed with a resolution of 140 000 not shown).

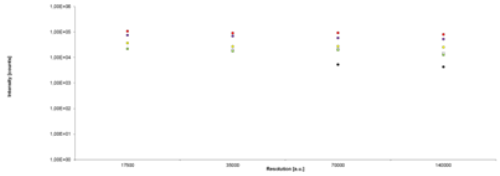

Figure 5: Average intensity of each compound in standard mix + blood spot for all resolution values tested, negative ionization mode.

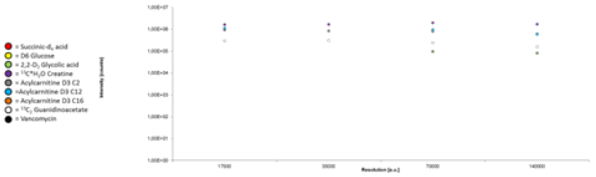

Figure 6: Average intensity of each compound in standard mix + blood spot for all resolution values tested, positive ionization mode.

All available AGC target values were tested using a spray voltage of 3.5 kV, needle position C, and resolution set to 70 000, see **Figures 7-8**. 1e6 appeared to be the best choice as this gave the highest intensities for most compounds in all sample types, and, with some exceptions, the highest number of detectable standard mix compounds.

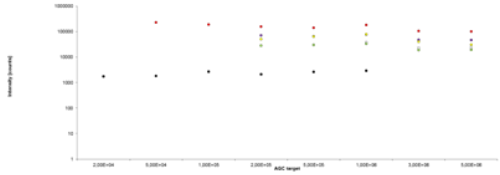

Figure 7: Average intensity of each compound in standard mix + blood spot for all AGC target values tested, negative ionization mode.

Figure 8: Average intensity of each compound in standard mix + blood spot for all AGC target values tested, positive ionization mode.

Conclusion and future work

In general, the measured intensity for the spot samples was lower than for the aqueous standard mix samples (not shown). Additionally, the number of compounds detected in the aqueous standard mix samples was higher than in spot samples. This was expected as there are several sample preparation steps where some of the compounds could be lost, e.g. extraction and evaporation. All compounds were detected in either positive or negative ionization mode in the spot samples, meaning that all these compounds are extractable from the DBS filter paper. Results of the optimization experiments indicate that the following could be suitable choices for untargeted metabolomics of DBS: spray voltage set to 3.5 kV, ESI needle position C, resolution set to 70 000 or 140 000, and AGC target set to 1e6. Other parameters will be optimized, including flow rate. The method and system appear to be robust, as there were no large variations in measured intensities for the different values of the parameters tested.

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