Thesis for the Master's degree in chemistry

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Global metabolomics of urine profiles relevant to Inborn Errors of Metabolism: Liquid chromatography positive electrospray ionization tandem mass spectrometry analysis

60 study points

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“Joy is the serious business of Heaven”

C. S. Lewis (1898 -1963)

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Anja Østeby

Abstract

A robust method for global metabolomics in urine using liquid chromatography (LC) with electrospray ionization (ESI) in positive ionization, tandem mass spectrometry (MS/MS) and quadrupole time-of-flight (Q-TOF) was developed. In addition, this method was applicable for negative ionization detection as performed by Siv M. Løvoll [1].

By using an ACE C18 pentaflourphenyl (PFP) (250 x 2.1 mm, 3 um) column and separation over an hour long gradient, approximately 6000 molecular features were detected in positive mode, in a single batch of urine. Urine sample preparation was performed by using 0.22 µm cellulose acetate spin filters before injecting 8.0 µL onto the column. The method showed promising performance characteristics making it a strong candidate for future urinary metabolome analysis.

The usefulness of the method was demonstrated by showing significant clustering of human urinary metabolites in 15 subjects after eating a standardized diet for 24 hours. These novel results indicate a surprisingly fast diet-dependent change in the metabolome.

Using the method developed, in combination with a broad range of analytical techniques, an unknown compound in a black urine sample from a patient was also successfully identified as the metabolite, 5-aminosalicyclic acid (5-ASA). Even though this compound turned out to originate from an anti-inflammatory drug, the workflow shows strong potential as a method to identify new diagnostic biomarkers in the future.

Abbreviations

5-ASA	5-aminosalicylic acid
AA	Acetic acid
ACU	Alcaptonuria
APCI	Atmospheric pressure chemical ionization
CV	Coefficient of variance
EI	Electron ionization
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
FA	Formic acid
FT-IR	Fourier Transform Infrared Spectroscopy
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
LC	Liquid chromatography
ID	Inner diameter
IEM	Inborn errors of metabolism
m/z	Mass to charge ratio
MeOH	Methanol
MFE	Molecular feature extraction
MP	Mobile phase
MPP	Mass profiler professional
MS	Mass spectrometry
MS 1	Single mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular mass
N-Ac-5-ASA	N-Acetylated-5-aminosalicyclic acid
NBS	Newborn screening
NMR	Nuclear magnetic resonance
PFP	Pentafluorophenyl
PKU	Phenylketonuria
PVDF	Polyvinylidene fluoride
Q-TOF	Quadrupol- time of flight
RP	Reversed phase
SP	Stationary phase
TIC	Total ion chromatogram
t_R	Retention time
UV	Ultra violet
XRD	X-Ray Diffraction

Table of content

1 Introduction

Inborn errors of metabolism (IEM) constitute a large group of genetic diseases that involve change in the complex biochemical and metabolic pathways of living cells [2]. The individual IEM are relatively rare, but are collectively common. If not diagnosed early, morbidity and mortality of newborns may result from the disorder. Symptoms are often diffuse and diagnostic methods are difficult. Newborn screening (NBS) is performed for specific IEM to allow early diagnosis and treatment. Starting more than 40 years ago, every Norwegian newborn has been screened for 2 diseases, phenylketonuria (PKU) and hypothyroidism. Since 2012 the screening has been expanded to include 23 different diseases of which 20 are IEM [3]. All of these disorders can be treated, some with great success.

Metabolomics involves the systematic study of all metabolites in a given organism or biological sample [4]. Metabolomics is an emerging field and an important tool for clinical research into human diseases. This development is allowed by sensitive and accurate analytical methods like mass spectrometry (MS) in tandem with chemometric software. This combination allows the identification and comparison of thousands of metabolites simultaneously. Metabolomics has therefore become a method to discover biomarkers that can be used to diagnose diseases, including IEM. **Table 1** lists some definitions commonly used in this thesis.

Table 1: Important definitions in the field of metabolomics used in this thesis

Metabolism	Involves the multitude of complex biochemical processes that occur in living cells in order to maintain essential cellular activities [2].
Metabolites	Small molecules that are products and intermediates of metabolic reactions present in living organisms [5].
Metabolome	Involves the complete set of endogenous low-molecular weight components in a biological sample such as cells, tissue, urine or plasma [6].
Metabolomics	Complete analysis of the whole metabolome under a given set of conditions [7].
Biomarker	A characteristic that can be measured in a biological sample as an indicator of normal or pathogenic processes, as well a response to therapeutic interventions [8].
Differential diagnosis	Different diseases that can all cause the clinical symptoms presented.

However, the use of metabolomics for disease diagnostics is not a recent invention. Already in 4000 BC urine was already an important marker for health and disease [9]. Obviously, there were no advanced analytical instrumentation like LC or MS to measure diagnostic metabolites, but color, smell and taste were used to examine urine. Specific characteristics of urine corresponded to certain diseases and physicians began to diagnose urine samples without prior consultation with the patient. In 1934 the Norwegian biochemist Ivar A. Følling discovered excess amounts of phenylalanine in patients with PKU. Little did he know that he had just performed the first screening for IEM and that phenylalanine would still be a biomarker for PKU almost 80 years later!

The motivation for this thesis was that the Section for Inborn Errors of Metabolism wanted to develop a method suitable for studying the urinary metabolome using a combination of LC and Q-TOF. For IEM, the identification of new diseases relies to a large extent on the identification of accumulated metabolites in body fluids of affected patients. In order to detect new biomarkers for diseases, the metabolome of the healthy state may be compared to the metabolome of a disease state.

1.1.1 Metabolism

Metabolism involves the multitude of complex biochemical processes that occur in living cells in order to maintain cellular activities [2]. These processes include essential reactions that provide the cell with adenosine triphosphate as an energy source and synthesis of molecules that are required for the structure and function of the cell. Every reaction is a component of specific metabolic pathways that depend on enzymes that metabolize substrates to other molecules. The consequences of interference in a normal enzymatic or metabolic pathway vary in degree depending upon the position in the pathway. An IEM often causes a change in a specific metabolic pathway. Today, one of the main goals of biochemistry and cell biology is to identify all of the components of the entire intracellular metabolism; the intracellular metabolome [10].

1.1.2 Metabolites and biomarkes

Metabolites are small molecules such as amino acids, organic acids and sugars that are present in living organisms [10]. These substances are constantly involved in a range of essential processes in cells and most of them are products and intermediates of metabolic reactions. Depending on the metabolic pathway and reactions, specific metabolites are involved. Measurement of metabolite concentrations in body fluids is very useful because it reflects the intracellular processes of the organism. Therefore, the intracellular metabolites can reveal the regulation in an organism due to genetic modifications and environmental variations. The metabolites present in cells are in exchange with the cells environment, thus, the metabolic profile found in body fluids can reflect the intracellular metabolism and the transport system for metabolites in and out of the cells. This is the fundamental basis for the use of body fluid metabolite analysis as a diagnostic tool in IEM. Metabolites that are used in clinical research and diagnosis of human diseases are referred to as biomarkers. These biomarkers give a unique signature that can be used for diagnoses by comparing metabolic differences in the healthy and the diseased states [11]. For instance, identification of high levels of the biomarker phenylalanine, can indicate PKU [12].

1.1.3 Metabolome

The term “metabolome” includes the complete set of endogenous, low molecular mass (MW) metabolites in an organism or cell [13]. It is necessary to gain knowledge of the metabolome in addition to the genome and proteome in order to comprehend the cellular mechanism. However, the complexity and the large number of metabolites have made it challenging to measure the metabolome systematically and efficiently. Metabolome profiling is an approach to measure and analyze factors in biosamples such as urine or blood. Urine can provide biological information since deficiency of an enzyme can for instance be discovered by accumulation of the enzyme’s substrate in a patient sample. Therefore, a profile comparison between two samples that shows a clear difference between peaks indicates differences in metabolism.

1.2 Metabolomics

Metabolomics is the complete analysis of the whole metabolome in a given organism or biological sample (3) and can be divided into targeted and global metabolomics respectively. Targeted metabolomics is the quantitative analysis of specific metabolites that are associated with a specific pathway, enzyme or metabolite class [14]. Depending on the analyte of interest, considerations involving the sample preparation and the analytical method must be taken. Diagnostics for IEM, is based on targeted specific metabolites in which MS/MS analysis with multiple reaction monitoring, neutral loss, precursor ion scan or product ion scan are frequently used. However, even though targeted metabolomics is undoubtedly useful in many clinical applications, significant information may be missed.

Metabolomics has become an important tool in clinical research of human diseases like IEM that provides a “snapshot” of the physiology of the specific cell of organ [12] and therefore allows a holistic investigation of the metabolome. Increasing research in this field can partly be explained by more sensitive and accurate analytical methods like MS, in addition to the application of chemometric software. Study of the metabolome under a given set of conditions can provide significant information about metabolic pathways that are not yet fully understood. Global metabolomics has therefore become an approach to discover

biomarkers that can be used to diagnose diseases, and global metabolomics experimental approaches will be further discussed in 1.6.

To sum up, laboratory diagnostics of IEM today are based on targeted metabolomics. New IEM are identified as new diagnostic metabolites are described. Global metabolomics is used to better understand the biochemical consequences of known IEM and to identify new diagnostic metabolites.

1.2.1 Inborn errors of metabolism

The term IEM refers to inherited defects in metabolism and was first described by Sir Archibald Garrod in his address to the Royal College of Physicians in 1908 [15]. For more than 40 years, and still today, GC-MS has been the key instrumentation used to identify urinary organic acids which are essential for routine diagnosis of IEM. However, LC in combination with MS/MS has become increasingly important for quantitative metabolite screening in clinical diagnostics, due to improvements of sensitivity and specificity, when profiling classes of metabolites [14].

IEM are a large group of genetic diseases, and today more than 1000 different conditions have been described [16]. Generally, IEM are classified into three different groups based on the pathophysiology: Group 1: Disorders involving complex molecules, Group 2: Disorders that give rise to intoxication and Group 3: Disorders involving energy metabolism [17]. Individually, these conditions are rare, but collectively they are common. The conditions are complicated, and symptoms can vary from mild to severe among the affected children. However, some IEM do not show any symptoms in childhood but appear later in life. The rates of morbidity and mortality are high and early diagnosis and treatment are essential. A majority of these diseases are caused by genetic mutations which affect specific enzymes that catalyze specific reactions. The cells in our body continually metabolize a large number of different substances. An IEM results in the of absence of a normal working enzyme and therefore affects the complex biochemical and metabolic pathways in living cells [2]. Hence an IEM always results in an alteration of the metabolome.

In a healthy state, enzymes are working properly and thereby producing metabolites normally. The LC chromatogram illustrated in **Figure 1** shows a theoretical separation of compounds in a normal metabolome profile.

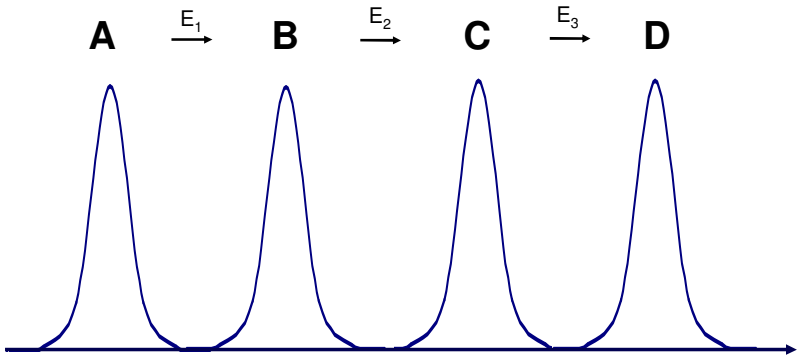

Figure 1: The chromatogram shows a theoretical separation of the compounds in a healthy state and illustrates metabolism involving conversion of metabolites A, B and C with specific enzymes E1, E2 and E3.

A gene mutation resulting in a defective enzyme in a metabolic pathway may result in an inability to metabolize one or several substances. The LC chromatogram illustrated in **Figure 2** shows a theoretic separation of the compounds where a specific metabolite G accumulates as a result of deficient enzyme E6. The resulting clinical picture and treatment strategy depends on a number of factors including the nature of the metabolite that builds up, the lack of metabolite after the block and whether the IEM affects an anabolic or katabolic pathway.

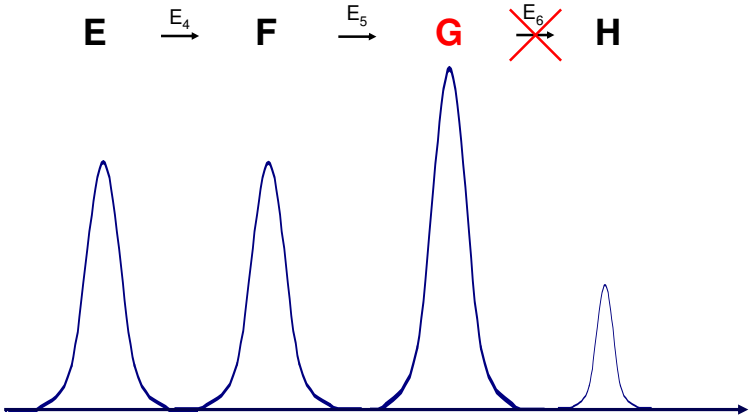

Figure 2: The chromatogram shows a theoretical separation of the compounds in a sample from a patient with IEM and illustrates metabolism involving conversion of metabolites E, F and G with specific enzymes E4, E5 and E6. An IEM may result from a defect or absence of a specific enzyme (E6) leading to accumulation of metabolite G and lower concentration of H.

Alternatively, a defective enzyme may result in pathological metabolites that are normally not present in a healthy state. The LC chromatogram illustrated in **Figure 3** shows a theoretical separation of the compounds including the pathological metabolites X and Y that accumulate because K is not metabolized to L.

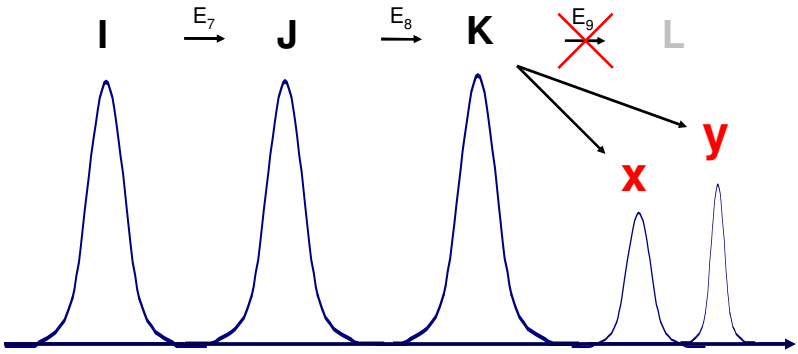

Figure 3: The chromatogram shows a theoretical separation of the compounds in a sample from a patient with IEM and illustrates metabolism involving conversion of metabolites I, J and K with specific enzymes E_7, E_8 and E_9. An IEM may result from a defect or absence of a specific enzyme (E_9) leading to accumulation of the pathological metabolites X or Y, and lower concentration of L.

In diagnostics of an IEM at the clinical level, characteristics like appearance or smell may suggest a particular disease. Diagnostics of IEM are seldom provided from the clinical picture alone; clinical symptoms are diverse. Many IEM are differential diagnosis to non-IEM diseases. Laboratory analysis of biological fluids that reflect the body's metabolism has therefore become an important tool for diagnosis of IEM. In a diagnostic lab, IEM can be diagnosed at three levels: the gene level, the gene products level and the metabolite level [16]. A general scheme for laboratory diagnosis of IEM is given in **Figure 4.**

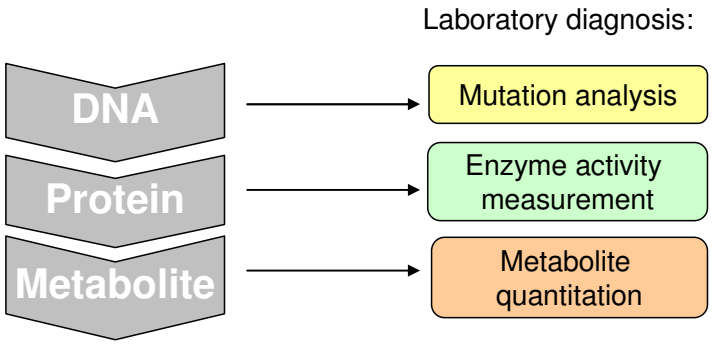

Figure 4: Laboratory diagnosis of an IEM is performed at three different levels: the gene level, the gene product level and the metabolite level.

Diagnostics at the gene level is performed by mutation analysis. Commonly full blood and mutation analyses are performed in leukocytes. The enzyme activity can be measured. Diagnostic enzymology involves measurement of changes in activity of specific enzymes in disease to study pathological changes in body fluids, tissues and fibroblasts. Understanding how cells and intact organisms regulate the overall metabolism is critical to distinguish functionally regulated enzymes from abnormalities in order to comprehend the molecular basis of disease.

Biochemical routine analysis provides important information at the metabolite level. Further examination may be performed to detect specific pathological metabolites when an IEM is suspected. These analyses are performed in urine, plasma or cerebrospinal fluid. The analyses are time-consuming, expensive, difficult to interpret, and the pre-analytical aspect of sample treatment is important. A suspected IEM based on clinical information can be strengthened or weakened after analysis of specific metabolites since the IEM metabolome is changed significantly compared to a healthy metabolome. Normally, laboratory diagnostics at the metabolite level is sufficient to start treatment of the disease. Follow up analysis is often performed, either by enzyme activity measurement or mutation analysis.

1.2.2 Nomenclature of IEM

The nomenclature of IEM is closely related to the metabolomics of the specific disease. An IEM ending with -uria or -emia means elevated levels of specific diagnostic metabolites in urine or blood, respectively. The PKU disease is caused when the defective enzyme phenylalanine hydroxylase is not able to convert phenylalanine into tyrosine that normally is further catabolised to fumarate, which is an intermediate in the Krebs Cycle [18]. Phenylketonuria therefore means high levels of phenylketones in urine. Another example is Metylmalonic aciduria; a branched-chain organic aciduria caused by a deficiency of methylmalonyl-CoA mutase leading to elevated levels of methylmalonic acids in urine [17]. More examples of IEM caused by deficient enzymes resulting in specific accumulated metabolites are provided in **Table 2.**

Table 2: Examples of IEM in which accumulation of a specific metabolite results in different symptoms.

IEM	Deficient enzyme	Accumulated metabolite	Main clinical findings
Phenylketonuria	Phenylalanine hydroxylase	Phenylalanine	Progressive mental retardation
Methylmalonic aciduria	Methylmalonyl-CoA mutase	Methylmalonyl-CoA	Metabolic acidosis
Tyrosynemia type II	Tyrosine amino transferase	Succinylacetone	Inflammation of cornea
Medium-chain-dehydrogenase (MCAD) deficiency	Medium-chain-dehydrogenase (β-Oxidation defect)	Acylcarnitines; C6, C8, C10, C10:1	Hypoglycemia Sudden infant death syndrome
Maple syrup urine disease (MSUD)	Brached chain oxo/keto acid dehydrogenase	Leucine Isoleucine Valine	Characteristic maple syrup odor Developmental delay

1.2.3 Metabolomics in biofluids

Biofluids are biological fluids including tears, sweat, breast milk or sperm. In diagnostics plasma and urine have traditionally been used as they often reflect the healthy or diseased state of an individual and can easily be collected. Ever since 4000 BC smell, taste and color of urine have been important diagnostic markers of disease [9]. Abnormal urine color might be explained by medications or specific foods, but can also be caused by a pathological condition [19], see examples in **Table 3.**

Table 3: Abnormal urine color can be resulting from certain medical conditions, medications and foods.

Abnormal urine color	Medical condition	Medication	Food
Red	Sickle cell anemia	Ibuprofen	Blackberries
Blue or green	Blue diaper syndrome	Methylene blue	Herbicides
Brown	Hemolytic anemia	Aceptaminophen overdose	Fava beans
White	Proteinuria	-	-
Black	Alcaptonuria	Sorbitol	-

On the molecular level, plasma and urine provide complementary information about the metabolic state of an organism [20]. Plasma gives an instantaneous snapshot of metabolites from when the sample was taken, whereas urine represents an average picture of polar metabolites that are excreted in inconsistent amounts according to the homeostatic control. Concentration, solubility and stability are important considerations of the analyte while the complexity of the matrix is equally important. Generally, the concentration of the analyte in plasma is low and the amount of sample available is limited. In urine the concentration of both analyte and interfering compounds is often high. Sample preparation for lab diagnostics should be selective and reproducible, and the analytical method of detection should be specific.

In order to achieve a holistic knowledge of human biology an ultimate starting point for investigation of metabolome variation is to quantify all metabolites in a biofluid [21]. Detected deviation in the metabolome could be caused by biological variation, but possibly also experimental variation. Sample collection, storage, freeze-thaw cycles and inter-individual biological variation due to physiological and environmental factors should therefore be taken into account. Sample preparation in global metabolomics for unbiased detection of a large diversity of metabolites in body fluids should be unselective, simple and fast, with a minimal number of steps, reproducible and potentially including a metabolism-quenching step [22]. Sampling time (random vs. timed, vs. 24-h), urine volume correction, dilution and pH adjustments should also be considered.

The concentration of compounds in a urine sample is dependent on the urine volume. Creatinine is a breakdown product in the muscle which is excreted over a 24 hour period and is therefore often used as a standard correcting for dilution of the sample. In order to make up for different urine concentration in patients, diagnostic laboratory normalize urine samples by using creatinine as a reference for quantitation of diagnostic metabolites by measuring the metabolite/creatinine ratio in a sample. In addition, there are several approaches regarding normalization for different analytical methods. Commonly, standardized dilution (e.g. 1+9) or dilution according to concentration of creatinine is performed. Even though dilution of urine possibly results in decrease in ion suppression, urine samples are at times preferred undiluted if the analyte of interest is present in low concentration.

1.2.4 Dietary effect of the metabolome

Metabolomics in biofluids is highly dependent on intake of different foods and drinks. Lloyd et. al. propose a MS method for identification of urinary metabolites after consumption of specific foods, and thereby illustrates an example of a strategy for identifying effects of dietary exposure using MS and metabolomics [23]. In this study, a systematic experiment for investigation of the dietary effect on the urinary metabolome in a healthy population is examined.

1.3 Laboratory diagnosis of IEM in Norway

Laboratory diagnosis of IEM is performed in the targeted approach based on measurement of specific diagnostic metabolites. Already in 1934, when the Norwegian biochemist Professor Asbjørn Følling examined two children with severe developmental delays, urine odor and decreased skin pigmentation, the first screening was performed [24]. Følling discovered excess amounts of phenylketone that is a by-product of phenylalanine. The observations indicated that the children were suffering from a disease known today as PKU. A screening assay became available in 1963, when Guthrie et. al. developed a simple test that could be used on all infants, to identify those affected with PKU [25]. A few drops of blood from the 2-3 year old child spotted on an absorbent filter paper rapidly show elevated amounts of phenylalanine. If the test was positive, treatment was accomplished and mental retardation avoided. Thereby, the worldwide NBS program was established. Today more accurate and rapid methods, such as MS/MS, LC-MS and gas chromatography mass spectrometry (GC-MS) have replaced the early screening for amino acids, and the NBS samples are collected 2-3 days after birth. Laboratory diagnosis of IEM in Norway is performed at two different laboratories, one doing NBS and the other carrying out the biochemical diagnostics.

1.3.1 Newborn screening for IEM

NBS procedures are performed with the intention of revealing genetic disorders. The Norwegian Directorate of Health suggests specific newborn screening criteria [26] according to World Health Organization (WHO) principles and decides which of the IEM should be included in the screening. There is a balance regarding what analysis should be included in the screening. On the one hand the NBS should promote better health and life quality for the children affected by IEM. On the other hand, protection of newborns and parents from unnecessary concerns about disease should be avoided, since expansion of screening leads to an increase in false positive results. Costs per analysis, advanced analysis equipment and the requirement of a competent staff are also important considerations. In 2012 the list of diseases included in NBS in Norway was dramatically expanded from 2 to 23 different inborn diseases, of which 21 are IEM. The aim of newborn screening is therefore to detect inborn diseases at an early stage in order to introduce preventive measures and to begin necessary treatment.

NBS criteria are listed below:

1. There is a serious disease
2. There is an effective treatment against the most serious symptoms.
3. The effect of treatment increases when performed at an early stage.
4. The disease can not be detected at birth without a specific screening.
5. There is a satisfactory test for the specific disease with high specificity and sensitivity and low rate false positive results.
6. The cost of screening shall be lower than the cost of treating someone with a fully developed disease.

1.3.2 Biochemical diagnosis of IEM

The Section of Inborn Errors of Metabolism at the Dept. of Medical Biochemistry at Oslo University Hospital, Rikshospitalet is the National Resource Centre for laboratory diagnosis of IEM in Norway. Biochemical diagnosis of IEM is performed on newborns with positive screening results in any of the 21 diseases. In addition, samples and clinical information from patients with symptoms indicating an IEM are sent to MBK and specific analysis is performed based on this information. The large diversity of important diagnostic analysis is illustrated in **Table 4**.

Table 4: Laboratory diagnosis of IEM performed at MBK, Rikshospitalet by detection of specific analytes in different body fluids illustrates the broad range of analytical techniques utilized.

Analyte	Method / technique	Matrix
Amino acids quantitative	Amino acid analyzator	Urine Plasma
Acylcarnitines	LC-MS/MS	Plasma Serum
Amino acids	Paper chromatography	Urine
Carnitine, total and free	LC-MS/MS	Urine
Carbohydrate-deficient transferrin	Capillary electrophoresis (CE)	Serum
Creatine and guanidinoacetate	LC-MS/MS	Urine Plasma
Cysteine in leukocytes	Cysteine binding assay	Blood
7-dehydrocholesterol	GC-MS	Serum
Metylmalonic acid	LC-MS/MS	Serum
Mucopolysaccharides	Thin layer chromatography	Urine
Organic acids	GC-MS	Urine
Orotic acid	MS/MS	Urine
Oxalic acid	MS/MS	Urine
Purines and Pyrimidines	LC-MS	Urine
Sugars	Sugar paper chromatography	Urine
Very long chain fatty acid and phytanic acid	GC-MS	Serum
Enzyme activity	Mutation analysis	Erythrocytes Fibroblasts Leukocytes
Creatinine, glucose, urine acid, glycerol, nitrate, pH, ketones, leukocytes, nitroprussid	Standard clinical chemistry Urine stix	Urine

Class specific analysis refers to the measurement of classes of components that produces a common set of fragments. A well known example is the analysis of acylcarnitines by MS/MS. Since a complex mixture can contain thousands of molecules and different molecules may share the same MW, a secondary separation system like LC or GC is often used. MS/MS enables control of the formation of molecular and fragment ions. Particular compound classes can be measured specifically, since certain classes share common fragment classes with fragment ions. Details on the currently used method for acylcarnitine analysis can be found in appendix (chapter 8.2). Class specific analysis is of great importance in laboratory diagnostics as it provides reliable quantitative data on important diagnostic markers. However, this targeted approach only provides information about the analytes of interest and will not reveal potential unknown metabolites.

1.4 Reversed phase liquid chromatography

LC is a separation technique where compounds migrate through a column with different velocities due to different equilibriums between a solid stationary phase (SP) and the liquid mobile phase (MP). Depending on the analyte of interest a broad range of column SP chemistries can be selected and C18 is the most commonly used. The MP in reversed phase (RP) is polar and consists of an aqueous and organic solvent with a pH adjustment additive, e.g. a buffer. Reversed phase chromatography is the most frequently used separation principle where the SP is nonpolar and eluent strength increases with less polar solvent [27].

1.4.1 Column dimension

Down-scaling the inner diameter (ID) of the column will lead to an increased signal-to-noise (S/N) ratio with concentration sensitive detectors, such as ESI MS, since dilution of the analyte(s) will decrease [28]. Other benefits compared to larger ID columns are lower consumption of MP that is friendlier towards the environment and the economy. In addition, small-bore columns do not require flow splitting before the mass spectrometer. The disadvantages of miniaturization are critical dead volumes, risk of clogging and a less rugged system. In this study narrow bore columns (ID 2 mm) were used.

1.5 Mass spectrometry

The principle of MS is; to generate ions in gas phase, to separate these ions by their mass to charge ratio (*m/z*) and to detect the ions qualitatively and quantitatively according to their *m/z* and abundances [29]. The mass spectrometer consisting of an ion source, a mass analyzer and a detector provides both qualitative and quantitative information about the composition of both organic and inorganic compounds in complex samples. Information about isotope ratios provides information about characteristic isotopic pattern like Cl and Br, which may be used to identify unknown compounds. A *mass spectrum* is a representation of signal intensity versus *m/z* ratio versus intensity in a sample that has successfully been transferred into ions in gas phase. The advantages of MS are identification and quantification of a broad range of MW analytes, detection levels from ng/mL to pg/mL and structural information [14]. However, the disadvantage of using MS is the inability to separate isobaric or isomeric compounds. Using ESI MS, possible ion suppression is also a well known weakness.

1.5.1 Ionization techniques

There are several ionization techniques including the most commonly used ionization techniques ESI, electron ionization (EI), atmospheric pressure chemical ionisation (APCI) and matrix assisted laser desorption ionisation. Today, the LC-MS technology is based on ionization techniques developed by Fenn et al. in the 1980s [30]. Among the atmospheric pressure ionization techniques, ESI has a higher dynamic range (polarity and mass range) than APCI and is widely used in applications for proteomics, drugs and metabolites. In ESI the ionization technique is based on formation of gas phase of ions from the ions in liquid between the LC capillary and the MS inlet. This ionization process can be divided into three major steps, a) production of charged droplets at the capillary tip, b) evaporation of charged droplets results in higher density of charges in the droplet, c) production of gas phase ions [31]. The ionization process is illustrated in **Figure 5.**

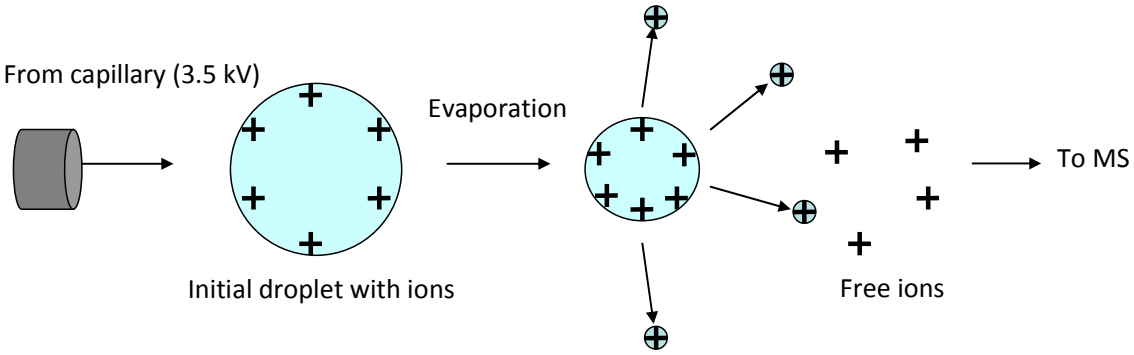

Figure 5: Ions in gas phase are formed in the electrospray interface for MS. Charged droplets produced at the capillary tip is evaporated bringing the charges closer together until free ions are released.

ESI is convenient for coupling to LC and has become a powerful tool for analyzing ionic and ionisable components. This ”soft” ionization technique, driven by an electric potential, results in less fragmentation than EI, where molecular ions and some fragments can be produced. For macromolecules multiple charges are quite common. Ion suppression is one of the most important challenges in ESI when there is competition for charge between co-eluting compounds. Organic modifiers like methanol (MeOH) and acetonitrile (ACN) are often used, while salts with high ionic strength should be avoided as they are likely to form adducts, which can lead to a decrease in selectivity and sensitivity, and contamination of the ion source might also occur. ESI is concentration sensitive and small ID columns are advantageous for increased response. Degree of ionization, surface activity, hydrophobisity, adduct-ion stability and solvation energy also determine the response of different compounds.

The choice of ionization mode, positive $[M+H]^+$ or negative $[M-H]^-$, depends on the analyte(s) of interest. ESI in positive mode can effectively ionise a broad range of medium polar and polar molecules and is therefore the most commonly used mode in LC-MS [32]. Generally, most molecules more easily stabilize positive ions than negative ions. Detection of negative ions is obtained with less chemical background noise and less adducts formation. For these reasons negative ionisation generally provides higher selectivity than positive ionisation. In order to increase detection of different metabolites, and thereby provide a more holistic insight into the metabolome, APCI has been shown to provide a significant increase in the number of additional metabolites revealed compared to ESI [33]. Because of

the large diversity in molecules, one methodology cannot cover all types of molecules and the different ionization techniques are therefore complementary.

1.5.2 Tandem mass spectrometry

The charged compounds in gas phase can be separated by different mass separators, like the quadrupole, time-of-flight (TOF), ion trap or magnetic sector, and a combination of these. In the following section, the instrument set up relevant to this master thesis will be described. The principle of the quadrupole mass filter is detection of ions with specific mass by varying the electric field. Typically, this mass separator consists of four parallel metal rods carrying both a constant voltage and a radio frequency oscillation [27]. Only ions with a particular *m/z* ratio reach stable oscillations that allow them to pass through the separator and to be detected, while unstable ions collide with the rods before reaching the detector. Because of its low cost, the quadrupole is one of the most common mass analysers. The TOF mass analyzer principle is separation based on accelerating all ions to identical kinetic energy. The ions reach a velocity which depends on their mass. Ions with different mass and the same energy will be separated, as light ions will reach a higher velocity than heavy ions and will therefore reach the detector first. In this study a mass separator of the type quadrupole time-of-flight (Q-TOF) was used and a schematic illustration is provided in **Figure 6.**

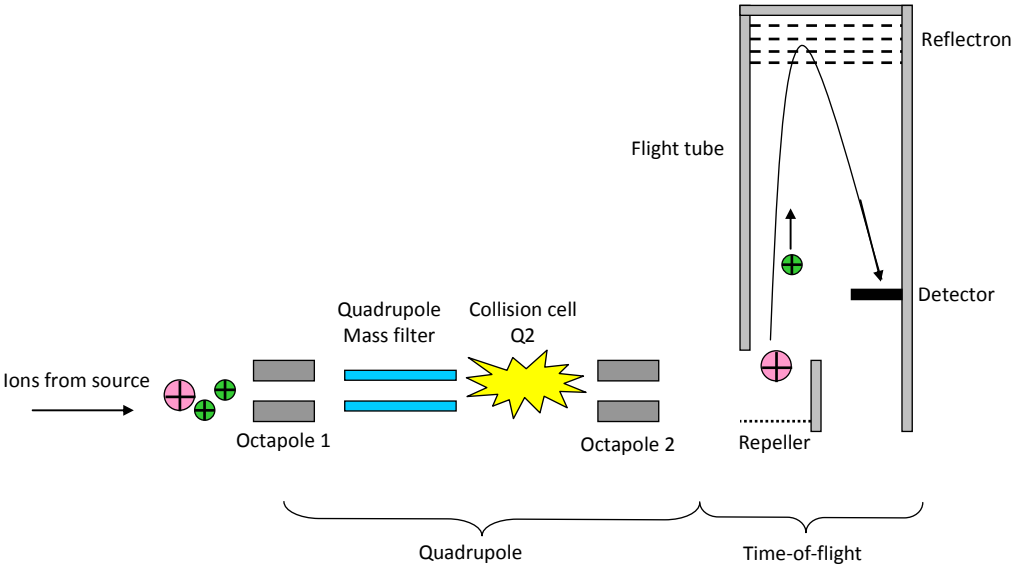

Figure 6: A schematic illustration of a MS/MS Q-TOF mass separator used in this study, consisting of a quadrupole , a hexapole collision cell and a TOF to produce spectrum.

1.5.3 Data processing

Molecular feature extraction (MFE) is one of the data mining algorithms available in the software MassHunter (Agilent Technologies). MFE, which is the method of choice for untargeted discovery workflows that operate on single mass spectrometry (MS1) data to find unique compounds, includes the following: 1. Raw data appears as a 3-dimensional array between retention time (t_R), *m/z* and abundance. 2. Background noise is removed. 3. Search for features with common elution profiler including adducts, isotope clusters, dimers and charge states. Method parameters can be adjusted according to the desirable data. The "extraction peak filters" is an adjustable parameter that depends on the chemical noise level in the spectrum. The compound filter is a threshold for absolute height (in counts) set according to the chromatogram level. Different ion species can be included or excluded depending on the probability of these ions being present in the system. In this study, MFE was used for method development and method evaluation, and to study the intra- and inter-individual biological variation in metabolome affected by diet.

Mass profiler professional (MPP) is a LC-MS statistical analysis software provided by Agilent Technologies for compound identification and biochemical pathway analysis. Principal component analysis (PCA) is based on capturing variance in a data set using vectors of the first three principal components [34]. Samples within one experimental condition should be more similar than those from different conditions, and is therefore expected to group closer together. In this study, PCA was performed to examine how the metabolome in healthy volunteers alters with different diet intake.

1.6 Global metabolomics

Global metabolomics, also referred to as untargeted metabolomics, is measurement of a large number of metabolites in an unbiased approach [14]. In other words, untargeted metabolomics is the study of the metabolome with the aim to quantify all the metabolites detected in one sample. Unlike targeted metabolomics, where specific metabolites are analyzed, untargeted metabolomics is used to capture as much information possible about all metabolites in the sample. Since untargeted metabolomics involves the study of all metabolites in one sample, it is important to evaluate sample collection, sample preparation,

chromatographic procedure and data processing [35] reduce sample pretreatment to a minimum, in order to avoid exclusion of metabolites.

A typical metabolomics experimental set-up for biomarker discovery of an IEM is shown in **Figure 7.** This approach can be used to discover now biomarkers for known diseases, or to find new IEM by identification of diagnostic metabolites in samples from patients suspected to have an IEM, but where no diagnostic metabolite is identified using the routine methods. The samples are processed depending on the chosen technology for separation, for instance LC-MS, GC-MS or Nuclear Magnetic Resonance (NMR). Data from patients and a healthy control group is compared to identify differences. The definition of a normal metabolome must rely on the measurement of the global metabolome in an adequate healthy control group. The importance of obtaining normal data must be emphasized, because the metabolome will vary between healthy individuals. Identification of an "abnormal peak" might be either a "normal" metabolite in an abnormal concentration (G in **Figure 2**), or a pathological metabolite (X and Y in **Figure 3**).

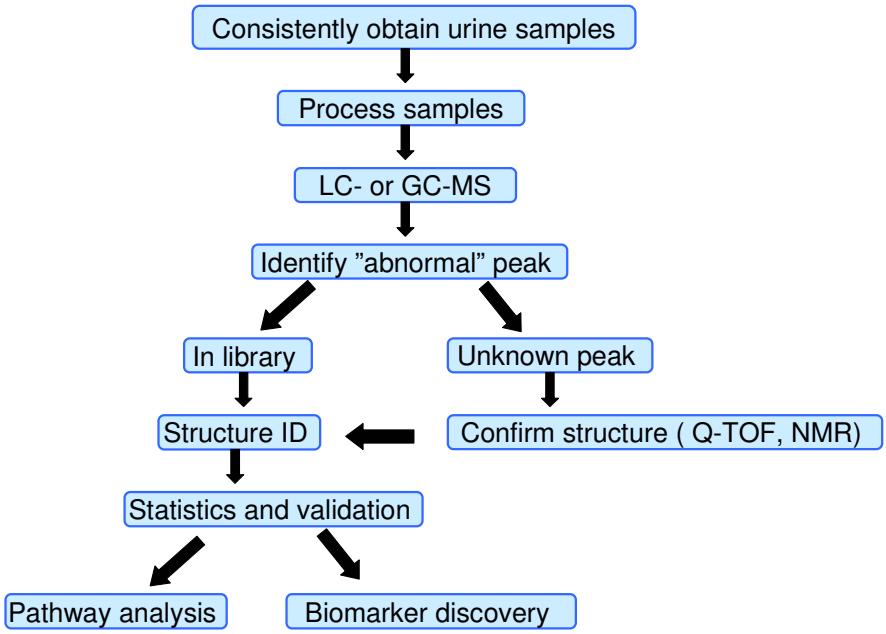

Figure 7: A typical global metabolomics experimental setup for potential biomarker discovery, adapted from [11].

For global metabolomics, method development should be examined to find the most optimal conditions for a complete analytical system, including analytical columns, MP etc. Kloos et. al. tested different column chemistries for fast urinary metabolic profiling by LC TOF MS [36]**.** Performance of RP LC vs. hydrophilic interaction liquid chromatography (HILIC) materials were examined in combination in a non-targeted manner. For the RP columns, different MP conditions were examined and the number of detected molecular features when eluting with MeOH compared to ACN minimal. Kloos et. al. recommend the use of HILIC for urinary metabolomic profiling with high throughput. The PFP column gave the best results of three different RP columns with regards to the number of molecular features. Furthermore, this column was chosen as it provides π-π-interactions in addition to classic C18-RPLC interactions with analytes. Roux et. al. conclude that a minimum of two different complementary chromatographic methods should be used for urinary metabolomics in order to discriminate between isomers [37]. LC separation was performed by a C18 (150 x 2.1 mm, 1.9 µm) column combined with LC separation by a pentaflourphenylpropyl (PFPP) (250 x 2.1 mm, 5.0 µm) column, to separate a broad range of different chemical families such as acylcarnitines, organic acids and amino acids.

1.7 Aim of study

The primary goal of this study was to develop a robust and reproducible LC Q-TOF MS method with positive ionization, useful for untargeted urine metabolomics. The method developed should also be applicable to negative ionization which was studied by Siv M. Løvoll [1]. The method will be validated and used to study the normal metabolome for intra- and inter- individual biological variation. During the study, a black urine sample from a patient, suitable for investigation and identification at Section for Inborn Errors of Metabolism, was used as a spotlight project. Several methods were utilized in order to identify the compound responsible for the black color.

2 Experimental

2.1 Materials and methods

Chemicals

Type I water was obtained from Milli-Q ultrapure water purification system (Millipore, Billerica, MA, US) was used to prepare standards and MP in combination with high purity MeOH and ACN was obtained from Merck (MeOH, Dramstadt, Germany), high purity acetic acid (AA) and formic acid (FA) obtained from Sigma (Fluka, Sigma Aldrich, Dramstadt, Germany). Ammonium acetate was obtained from Riedel-DeHaen (Seelze, Germany) to investigate the unknown compound in black urine, potassiumhydroxide, 5-aminosalicyclic acid (5-ASA) and homogentisic acid obtained from Sigma were used.

A standard mixture called magic mix was prepared including the following chemicals: 3-hydroxybutyrate, L-allo-isoleucine, parahydroxyfenylactate, creatinine and succinylacetone were obtained from Sigma. Hippuric acid, citric acid and deoxyadenosine was obtained from Merck. The origin of hexanoylglycine is unknown.

Artificial urine was prepared including the following chemicals: potassiumsulfate and dipotassiumhydrogenphosphate obtained from Merck, ureic acid and potassiumdihydrogen phosphate was obtained from VWR (West Chester, PE, US), potassiumchloride was obtained from Chemi-Teknik AS (Oslo, Norway) and magnesium sulfate was obtained from Sigma.

MS reference ion solution and tuning mix were obtained from Agilent Technologies (St. Clara, CA, USA)

2.2 Samples

2.2.1 Control and standards

Batch urine was prepared as a long term quality control by collecting urine from healthy volunteers and was pooled in order to increase the complexity of the urine. Furthermore the sample was aliquoted and stored at -70 °C, until the day of analysis.

The magic mix was prepared based on compounds with a broad spectrum of physio-chemical properties reflecting the diversity of metabolites found in urine. A selection of 9 different endogenous compounds of interest in lab diagnosis of IEM was prepared in water and artificial urine respectively, concentration of creatinine 5.0 mmol/L and the remaining 0.5 mmol/L. Artificial urine dissolved in water used for evaluation of matrix effects was prepared using a mixture of different salts, made according to a recipe provided by Oslo University Hospital, see appendix (chapter 8.3).

2.2.2 Urine collection and preparation

Urine samples used for investigation of the metabolome were collected by taking midstream of the first morning urine from healthy volunteers and were stored at -70°C until the day of analysis. For RPLC separation urine samples were thawed and filtered through a 0.22 µm cellulose acetate spin filter (Agilent Technologies) at 14800 rpm, at 4 °C for 5 min.

2.2.3 Columns

The various RP columns examined were Pursuit XR's Diphenyl and Polaris Ether obtained from Matriks AS (Oslo, Norway), Kinetex C18 (Phenomenex), Atlantis C18 T3 obtained from Waters, C18 H2o (Fortis) obtained from Holger Teknologi AS, C18 PFP both 150 and 250 mm ACE Technologies (Advanced Chromatography Technologies, Aberdeen, Scotland) used with C18 PFP guard cartridge obtained from TeknoLab AS (Kolbotn, Norway), see **Table 5.** Additionally, a zwitterionic (ZIC) HILIC (Syncrosis 150 x 2.1 mm, 5 µm. 100 Å) (Thermo Scientific) was examined. All columns except C18 PFP were examined using an online 0.5 µm prefilter (Phenomenex).

Table 5: Analytical RP columns examined during method development

Column	Column length (mm)	Column diameter (mm)	Particle Size (µm)	Pore Size (Å)	Surface area (m^2/g)	Carbon load (%)
Pursuit XR's C18 Diphenyl	250	2.0	3	100	440	14.6
Polaris C 18 Ether	250	2.0	3	180	200	12.1
Kinetex C18	100	2.1	2.6	100	200	12.0
Atlantis T3 C18 Polar endcapped	150	2.1	3	100	330	14.0
Fortis H2o C18 Polar endcapped	150	2.1	3	120	380	18.0
ACE C18 PFP	150	2.1	3	100	300	14.3
ACE C18PFP	250	2.1	3	100	300	14.3

2.3 Liquid chromatography and mass spectrometry

The LC-MS analyses were performed using an Agilent 1200 series LC (Agilent Technologies) coupled to a tandem MS Q-TOF 6520 series (Agilent Technologies) fitted with an ESI source operating in positive mode. The LC-pump was operated at a flow rate of150 µL/min. The MP consisted of H_2O and MeOH both containing 0.1 % of AA, and gradient separation was performed according to **Table 6.**

Table 6: Gradient elution A: H_2O + 0.1 % AA and B: MeOH + 0.1% AA

Time (min)	% A: 100 % H_2O + 0.1 % AA	% B: 100 % MeOH+ 0.1 % AA
0	98	2
10	98	2
50	0	100
55	0	100
55.1	98	2
65	98	2

The separations were performed on an ACE PFP C18 (250 x 2.1mm, 3 µm) column particles equipped with a C18 PFP precolumn (lenght 1.0 cm). The LC system was equipped with an 8 µL loop and 8.0 µL was injected. The software used was MassHunter version B.04.00 (Agilent Technologies). Before every analysis the MS was calibrated using a calibration solution provided by the manufacturer and the mass accuracy was within the range of 2 ppm. Reference masses obtained from manufacturer were introduced to the MS during all analysis and the raw data are automatically calibrated in MassHunter Workstation Software Version B.04.00 (Agilent Technologies). All analyses were performed at ambient temperatures.

Ionization was performed in positive mode with capillary- and skimmer voltage set on 3500 V and 65 V respectively. Nebulizer gas and drying gas was set at 30 psig and 5 L/min respectively. MS 1 was employed at low dynamic range *m/z* 100-1700.

2.4 Data processing

In this study the MFE parameters were set based on one batch urine, and within the manufacturers' recommendations according to **Table 7.**

Table 7: MFE parameters for data processing for determining the total number of molecular features detected

Parameter	Setting
Peak filters	≥ 500 counts
Compound filters (absolute height)	≥ 5000 counts
Allowed ion species	$[H^+]$, $[Na^+]$ and $[NH_4^+]$

Data from urine samples from investigation of diet effect were processed in MPP MassHunter version B.02.01 (Agilent Technologies)

3 Results and discussion

3.1 Method development

A robust method for untargeted metabolomics in healthy human urine using LC Q-TOF MS with ESI in positive mode was to be developed. Generally, positive ionization is the most commonly used ionization technique while negative ionization is often preferred for metabolites that are cannot easily be ionized in positive mode [38]. Since the optimal conditions for the two ionization modes not often are the same, the method developed must potentially compromise, since this thesis is one half of a study where both positive and negative is included. Selection of LC-column, gradient, MP and sample preparation will be evaluated in this study. The setup giving the highest number of molecular features for the batch urine will be the method of choice.

3.1.1 Selection of RP column

To develop a sensitive and rugged RP LC tandem MS/MS method for untargeted metabolomics in human urine, several different analytical columns were examined. By comparing the number of different separated compounds in batch urine, the column separating the highest number was chosen.

LC separation was performed on 7 different RP analytical columns with different properties, see **Table 5.** In addition one HILIC column with separation conditions was examined. The choice of column chemistries and diameter were done in collaboration with the application specialists for clinical application at the different column manufacturer.
Batch urine was used as a real biological matrix to monitor the total number of different molecular features separated by each column, and thereby compare column performance. Injection volume used was 5.0 µL and the gradient used is shown in **Table 8.**

Table 8: Gradient elution A: H_2O + 0.1 % FA and B: MeOH + 0.1% FA

Time (min)	% A: 100 % H_2O + 0.1 % FA	% B: 100 % MeOH + 0.1 % FA
0	98	2
10	98	2
40	50	50
50	10	90
54	98	2
64	98	2

A Magic mix of 9 known endogenous compounds, see **Figure 8**, dissolved in water was injected onto the 7 different RP columns to compare the separation capability of the columns. The compounds of the magic mix are well known is the diagnostic laboratory work of IEM. The biological source of each compound in addition to diagnostic function or correlation to a specific IEM disorder is listed in **Table 9.**

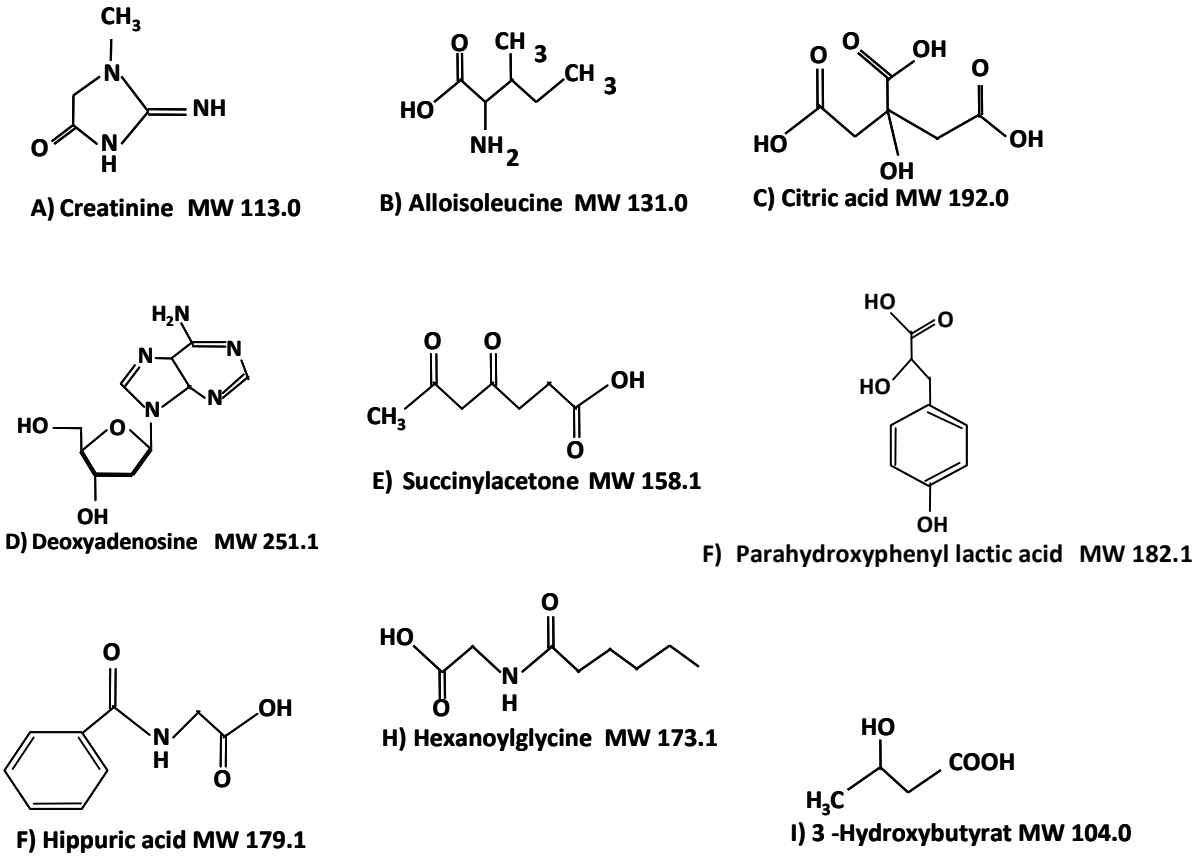

Figure 8: Structure of the 9 compounds in the Magic mix.

Table 9: The compounds of the Magic mix listed with biological source, in addition to biological function or correlation to s specific IEM disorder

Compound	Source	Function / IEM disorder
A) Creatinine	Endogenous breakdown product from muscle	Reflects urine dilution Normal constituent of urine
B) Alloisoleucine	Exogenous, branched chained amino acid from diet (protein)	Branched chain organic aciduria Eg. MSUD
C) Citric acid	Endogenous (tricarboxylic acid cycle) and exogenous	Metabolic acidosis. Relevant for lab diagnosis of crystal growth inhibitor.
D) Deoxyadenosine	DNA nucleoside A	Purine pyrimidine metabolism deficiency
E) Succinylacetone	Endogenous, breakdown product of tyrosine metabolism	Patognomonisk Tyrosynemia type I
F) Parahydroxyphenyl lactic acid	Endogenous breakdown product from tyrosine metabolism	Disorder of tyrosine metabolism, Eg. Tyrosynemia
G) Hippuric acid	Endogenous. Concentration increase with consumption of phenolic compounds	Organic aciduria Normal constituent of urine
H) Hexanoylglycine	Endogenous. Metabolite for fatty acid metabolism	Fatty acid oxidation disorder Eg. MCAD
I) 3-hydroxybutyric acid	Ketone body synthesized in liver	Branched chain organic aciduria

The separation of compounds in the Magic mix with compound A-H was examined for 7 different RP columns, see **Figure 9**. Each compound were separated by all 7 columns in identical order, except for citric acid (compound C) eluting both prior to and after alloisoleucine (compound B). The largest difference in elution time was approximately 15 minutes between Kinetex C18 and Pursuit XR's Diphenyl.

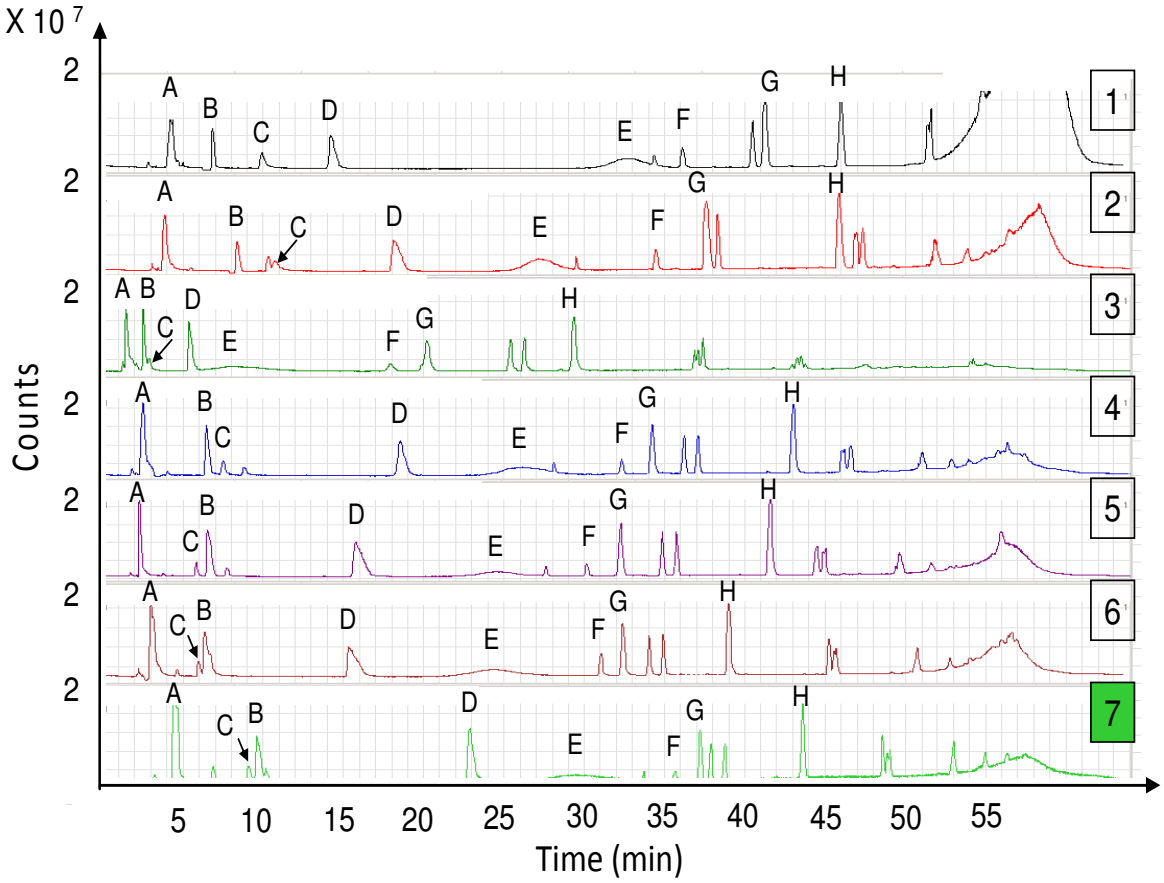

Figure 9: Total ion chromatograms (TIC) of compounds in Magic mix separated on 7 RP columns with gradient in Table 8. 5.0 uL were injected. Detection was carried out in positive mode with mass range *m/z* 50-2800. The compounds injected were: A. Creatinine (5 mmol/L); B. Alloisoleucine (0.5 mmol/L) ; C. Citric acid (0.5mmol/L); D. Deoxyadenosine (0.5 mmol/L); E. Succinylacetone (0.5 mmol/L) ;F. Parahydroxyphenyl lactic acid (0.5 mmol/L); G. Hippuric acid (0.5 mmol/L) and H. Hexanoylglycine (0.5 mmol/L), see Figure 9. The columns examines were: 1: Pursuit XR's Diphenyl; 2. Polaris C18 Ether; 3. Kinetex C18; 4. Atlantis T3 C18; 5. Fortis H2o C18; 6. ACE C18 PFP (150 mm) and 7. ACE C18 PFP (250 mm), see Table 5.

Succinylacetone (compound E) was separated with all columns, although in a very broad peak in **Figure 10.**

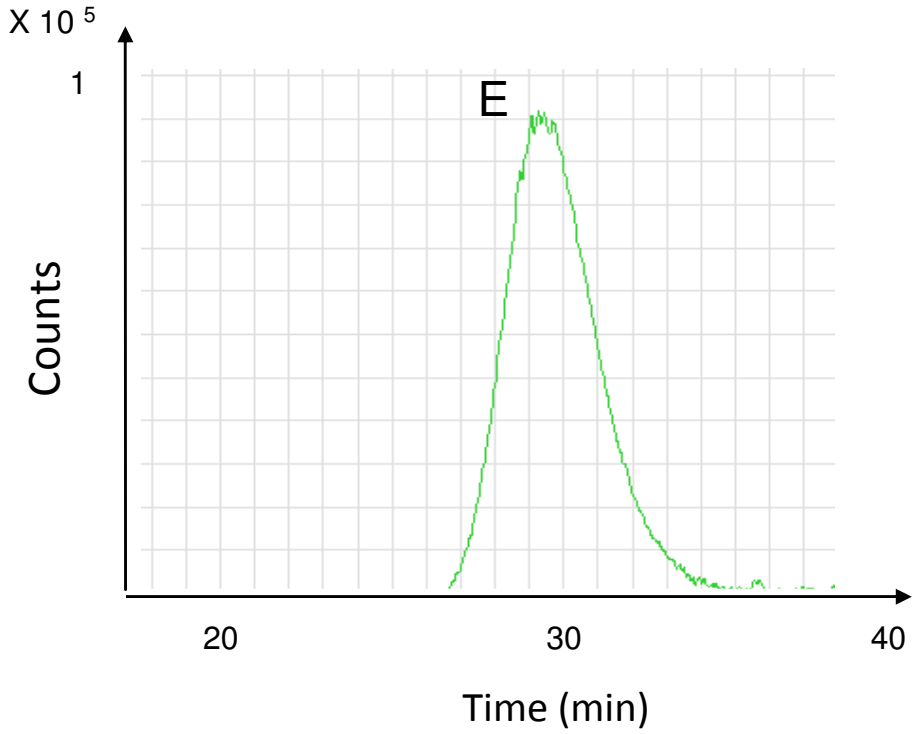

Figure 10: Chromatogram (EIC) of succinylacetone (E)(0.5 mmol/L) separated on an ACE (250 x 2.1 mm, 3 µm) column with the gradient described in Table 8. 5.0 µL was injected. Detection was carried out in positive mode with m/z 50-2800.

All columns were examined using the same chromatographic conditions to detect the highest number of molecular features in the batch urine. The ACE C18 PFP (250 x 2.1 mm, 3 µm) column (marked green in Figure 10) separated the most different molecular features in batch urine, see **Figure 11.**

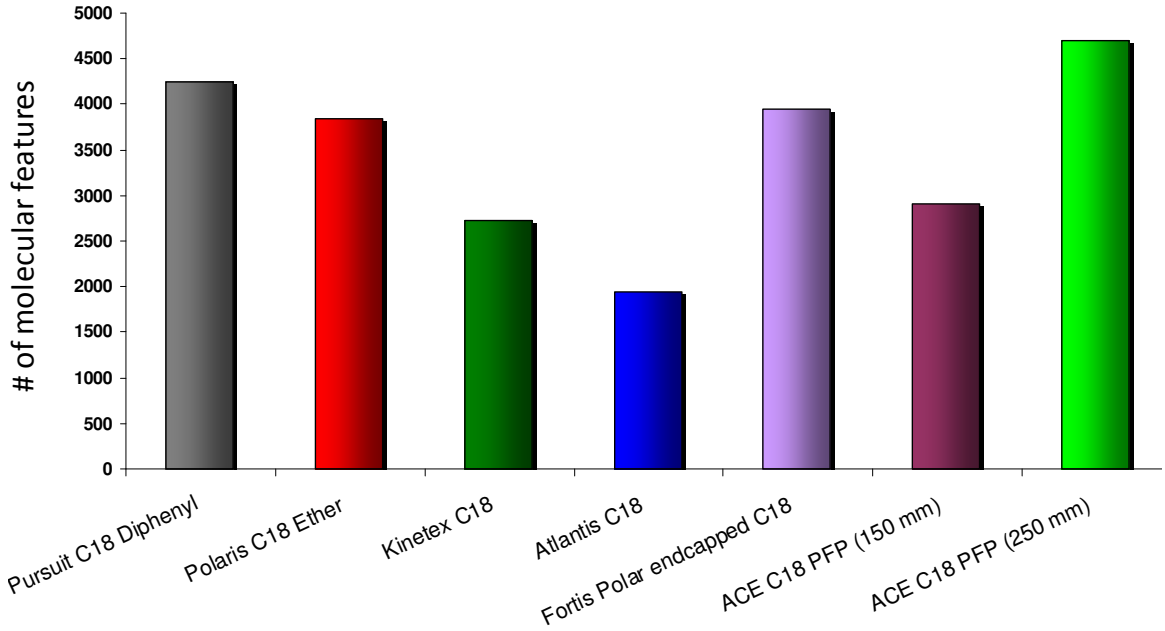

Figure 11: The diagram provides the total numbers of molecular features detected in batch urine by 7 different RP columns, see Table 5. Data were collected in the mass range *m/z* 50-2800. MFE filter was used according to parameters in Table 7.

The ACE C18 PFP (250 x 2.1 mm, 3 µm) column provided the highest number in both positive and negative ionization [1] and was therefore chosen for further metabolomics studies in urine. Since the Fortis polar endcapped C18 (150 mm) column detected a higher number of molecular features than ACE C18 PFP (150 x 2.1 mm, 3 um) column suggests that a longer Fortis column also could have been examines. In addition, all columns were tested with identical chromatographic conditions and it could be argued that each column should be optimized separately to provide best possible separation. However, our aim was to create an LC-MS method providing the highest number of detectable molecular features of urinary compounds and conclusion will be drawn based on the experimental approach. Further optimization with the selected column was performed during method development.

3.1.1.1 Evaluation of HILIC

HILIC, which is suitable for separation of polar compounds, has good potential for metabolomics in urine. Zang et. al. [39] evaluated RP, aqueous normal phase and HILIC with Orbitrap MS for metabolomic studies of human urine. According to this study ZIC-HILIC was able to detect most unique *m/z* features (33 %) in positive ionization and RP detected the fewest (6%). HILIC provided the best separation of isomers and best repeatability in their study.

In the present study a ZIC-HILIC Syncrosis (150 x 2.1 mm, 5 µm) column was examined based on method used by Zhang et. al. [39] for metabolomics. The gradient shown in **Table 10** was used and the resulting chromatogram is shown in **Figure 12.**

Table 10: Gradient elution A: H_2O + 0.1 % FA and B: ACN + 0.1 % FA

Time (min)	% A: 100 % H_2O + 0.1 % FA	% B: 100 % ACN + 0.1 % FA
0	20	80
30	80	20
35	95	5
45	20	80

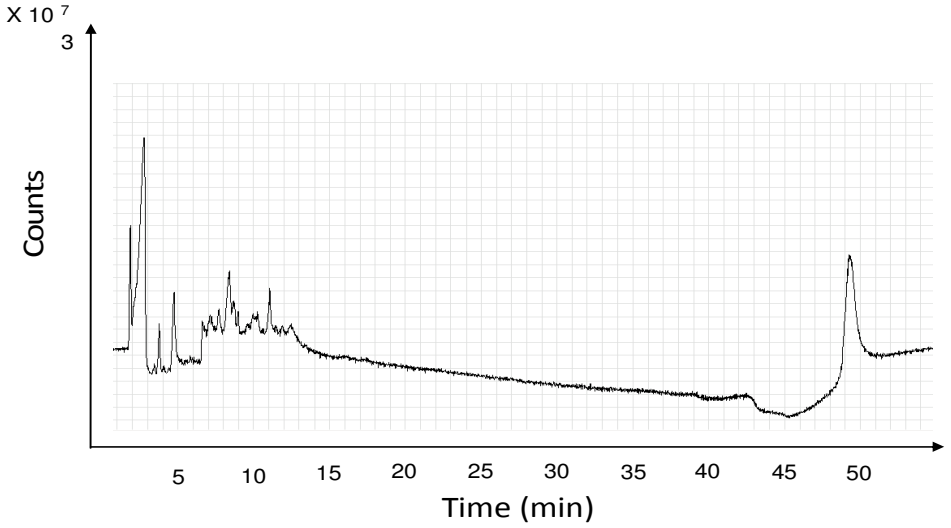

Figure 12: Chromatogram (TIC) of batch urine separated on a ZIC-HILIC Syncrosis (150 x 2.1 mm, 5 µm) column with the gradient shown in Table 10. Detection was carried out in positive mode in mass range m/z 50-2800. The batch urine was diluted (5x) with ACN and 5.0 µL was injected.

The reason for the poor chromatography using the ZIC-HILIC column could be many. First, there might have been water in the needle seat. The requirements of the system to dilute the urine sample in organic solvent to prevent “plug elution” is not ideal in this study, as compounds present in low concentration may not been detected after dilution (5x). Because of the poor chromatography obtained, further studies were not carried out with the HILIC column.

3.1.2 Mobile phase

Organic solvents used in MP in ESI MS/MS may be associated with ion suppression and adduct formation. Depending on the analyte of interest MP, additives and pH among others can be optimized in order to increase the formation of ions in the solution phase, and thereby improve sensitivity of detection [40]. In this study MeOH was examined in combination with different additives for detection of polar metabolites in urine, since ACN has weaker elution strength in RP which is not optimal for polar compounds separated on RP.

MeOH in combination with 0.1 % FA and 0.1 % AA resulted in 3801 and 5445 molecular features respectively, and AA was therefore chosen for further studies. Ammonium acetate (10 mM) was also tested but did not result match the number of molecular features detected with AA.

3.1.3 Gradient profile evaluation

In RP chromatography retention of analytes of interest in addition to the complexity of the sample, influence the optimal gradient profile since polar compounds will elute earlier than non-polar compounds. In study four gradient profiles were investigated to possibly increase the number of molecular features, see **Figure 13.** All of the examined profiles were approximately one hour long to possibly increase separation.

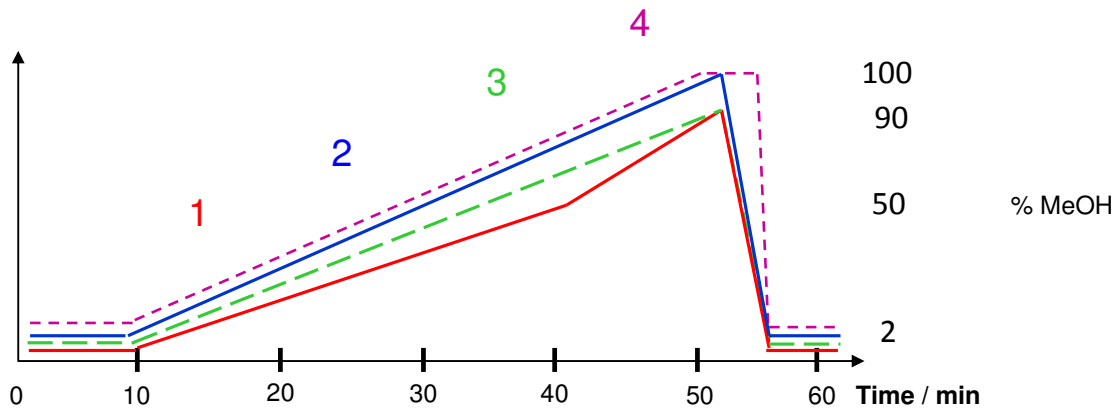

Figure 13: The figure shows four gradient profiles tested. 5.0 µL of batch urine was injected in triplicate and separated on an ACE C18 PFP (250 x 2.1 mm, 3 µm) column.

MFE was performed according to **Table 7** in time intervals based on the slope of gradient no 1. The mean value of number of molecular features detected after injection of batch urine in triplicate for gradient profile 1-4 are provided in **Table 12.**

Table 12: The number of molecular features detected (CV %) in batch urine by using four the gradient 1-4, see Figure 13.

Interval (min)	Gradient profiles 1-4			
	1	2	3	4
0-10	1352 **(0.4%)**	1306 **(1.3%)**	1293 **(1.4%)**	1406 **(0.3%)**
10-40	2341 **(1.0%)**	2342 **(2.9%)**	2779 **(1.1%)**	3545 **(0.7%)**
40-50	1150 **(2.2%)**	1159 **(1.7%)**	1520 **(0.4%)**	1584 **(4.5%)**
50-54	325 **(2.6%)**	747 **(3.2%)**	365 **(1.6%)**	391 **(5.1%)**
54-65	487 **(4.8%)**	1014 **(4.4%)**	320 **(23.9%)**	751 **(7.4%)**
Total	5655	6568	6277	7678

From the number of molecular features it was concluded that profile number four was most suitable for further the aim of this study. The performance of the column was evaluated to gain a better understanding of how the analytes spread throughout the gradient. TIC of batch urine with gradient profile no 4 is shown see **Figure 14.**

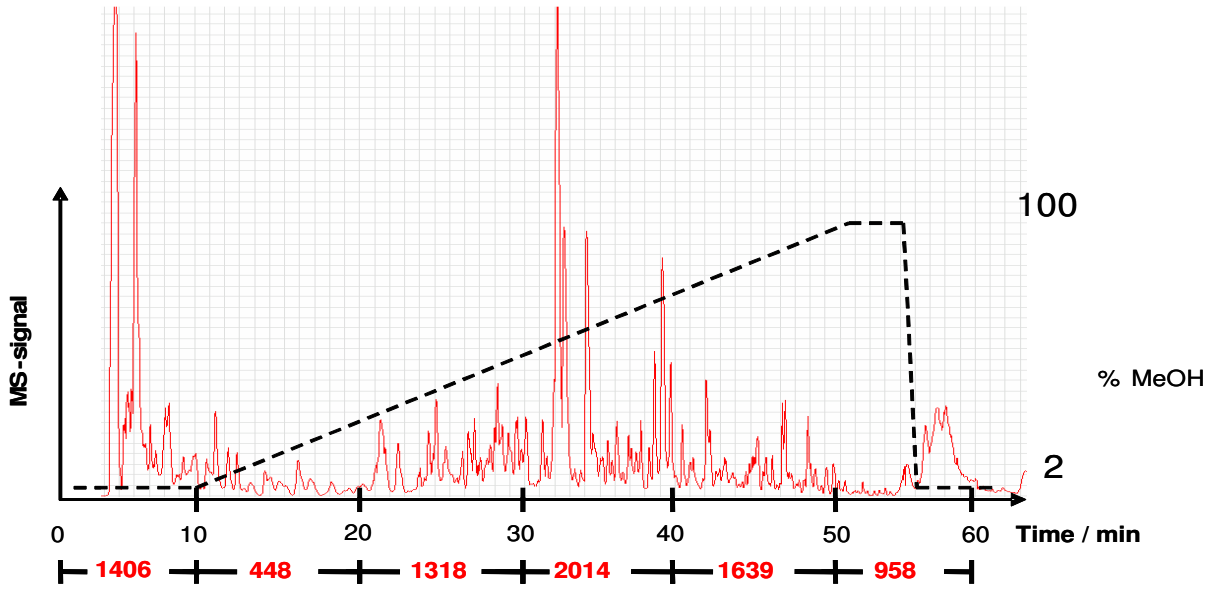

Figure 14: Chromatogram (TIC) of batch urine separated on a C18 PFP (250 x 2.1 mm, 3 µm) column with the gradient described in Table 6. Detection was carried out in positive mode and 5.0 µL was injected. The data were collected in the mass range *m/z* 100-1700. The dotted line represents the used gradient. MFE was used according to Table 7.

The high number of molecular features found throughout the gradient shown in the TIC above demonstrates the usefulness of utilizing an hour long gradient.

3.1.4 Sample preparation

Ideally, no sample preparation should be performed in metabolomics because of the possibility of handling the samples differently and eventual loss of analytes. In addition, shorter time consumption is always preferable. However, advanced analytical technologies require a minimum of sample preparation for instrument maintenance. In this study, different sample preparation filters were tested to evaluate if the filter added compounds to the sample. Polyvinylidene flouride (PVDF) 0.22 µm syringe filter (Millipore) was tested, but eliminated due to the unpractical filtration technique. Further, two different spin filters, suggested by manufacturers useful for metabolomics is urine, were tested: Cellulose acetate 0.22 µm spin filter (Agilent Technologies) and PVDF 0.22 µm spin filter (Millipore).

From the results (not shown), it was observed a small increase of approximately 50 features, between filtrated- and un-filtrated H_2O, and the increase was practically identical. For global metabolomic studies, it is not ideal to use filers that add compounds to the filter. However, if the identical features are added to each sample, there might not be a difficulty after all. Nevertheless, this emphasizes the importance of sufficient evaluation prior to a study.
For the purposes of this work, Cellulose acetate 0.22 µm spin filter (Agilent Technologies) was the filter of choice mainly due to economic considerations.

3.2 Effect of different injection volume

In diagnostics injection volumes is dependent on the concentration of the analyte of interest and the availability of sample. However, in global metabolomics detecting as many molecular features as possible it is not obvious what injection volume would provide most compounds. One advantage of urine analyses is the availability of large sample volumes. The developed method uses 8.0 µL, but effect of smaller injection volumes was tested to investigate possible decrease in ion suppression. Three different injection volumes tested were 0.1 µL, 5.0 µL and 8.0 µL and the number detected features were compared. The TICs are provided in **Figure 15.**

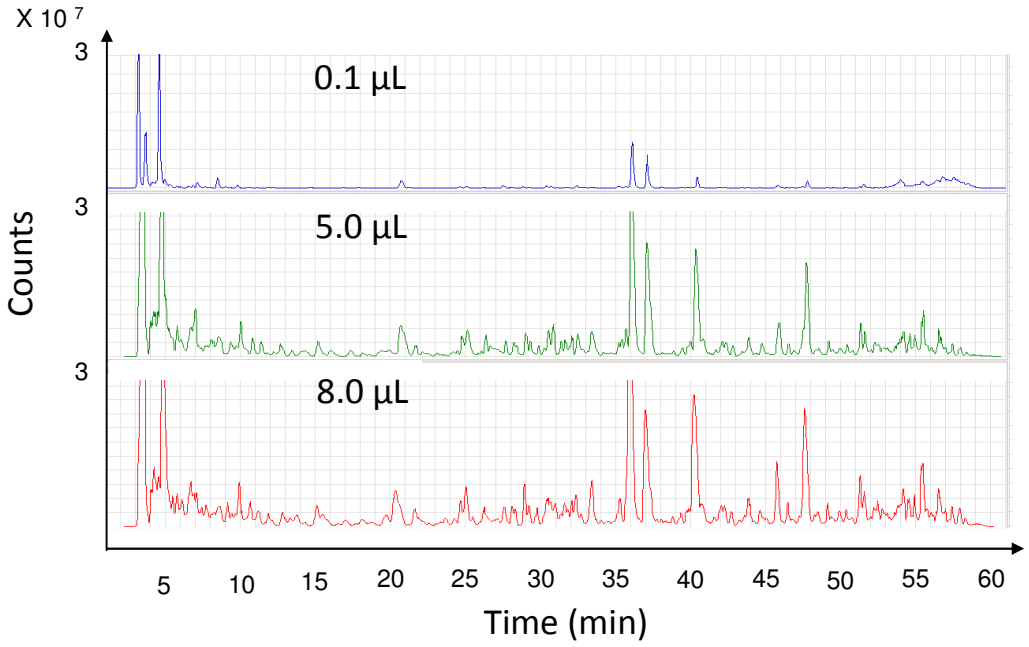

Figure 15: Chromatogram (TIC) of batch urine separated on ACE C18 PFP (250 x 2.1 mm, 3 µm) column for examination of varying the injecting volume. 0.1 µL, 5.0 µL and 8.0 µL were injected onto the column and detection by Q-TOF was performed in positive mode. Used gradient is provided in Table 8. The data were collected in the mass range m/z 100-1700.

The highest number of molecular features was found in the largest injection volume, which indicates an up concentration of compounds present in low concentration. Therefore, increasing of injection volume does not seem to become a larger challenge when it comes to ion suppression, which can be due to the long gradient time of approximately one hour. Based on the number of molecular features 8.0 µL will be used further in this study.

3.3 Studies of possible matrix effects

Ion suppression is one of the main challenges in ESI MS, particularly regarding complex samples. Dilution of urine sample could potentially result in less degree of ion suppression. In this study, investigation of possible ion suppression was performed by diluting urine samples based on concentration of creatinine. The number of molecular features extracted for four different urine samples collected from one healthy volunteer and the respective diluted urines is shown in **Table 15.**

Table 15: Urine sample 1 with creatinine concentration of 20 mmol/L was diluted to 13 mmol/L and 7 mmol/L and 0.6 mmol/L respectively. Urine sample 2 with creatinine concentration of 13 mmol/L was diluted to 7 mmol/L and 0.6 mmol/L respectively. Urine sample 3 with creatinine concentration of 20 mmol/L was diluted to 13 mmol/L and 7 mmol/L and 0.6 mmol/L respectively. Urine sample 4 with creatinine concentration of 0.6 mmol/L was not diluted. Injection volume was 5.0 µL and gradient used is shown in Table 8. The data were collected in the mass range m/z 100-1700 and the MFE was performed according to Table 7.

Sample	Dilution factor (DF)	Creatinine concentration (mmol/L)	# molecular features
1	-	20.3	7538
1	1.5	13.1	6519
1	2.9	6.9	5529
1	33.8	0.6	1886
2	-	13.1	6514
2	1.9	6.9	5558
2	21.8	0.6	1713
3	-	6.9	5763
3	11.5	0.6	1933
4	-	0.6	2282

Dilution resulted in a decrease in total number of molecular features in sample 1-3, which suggests that ion suppression might not be significant for this method. The numbers of molecular features detected in different samples with matching creatinine concentration are quite similar. However, since these samples are not collected identically, the total number only gives a certain idea.

3.3.1 Effect of urine dilution on molecular features

Compound with *m/z* 103.03 eluting at 5 minutes was selected randomly and extracted from urine sample nr 1, with creatinine concentration of 20, 13 and 7 mmol/L respectively, see **Figure 16.** Peak are and height are shown in **Table 16.** The compound could not be detected in the urine diluted to creatinine concentration of 0.6 (mmol/L).

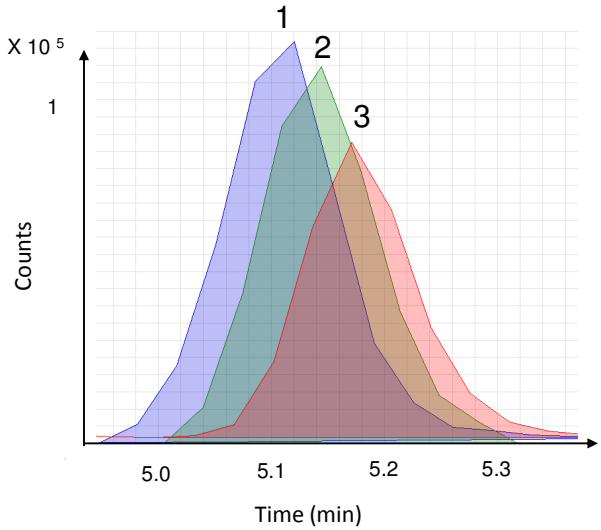

Figure 16: Chromatogram (EIC) of compound *m/z* 103.03 in the identical urine sample diluted to different concentration of creatinine; 1 (20 mmol/L), 2 (13 mmol/L) and 3 (7 mmol/L) separated on a ACE C18 PFP (250 x 2.1 mm, 3 µm) column with the gradient described in Table 8. Detection was carried out in positive mode and 5.0 µL was injected. The data were collected in the mass range m/z 100-1700.

Table 16: t_R, peak area and height of compound *m/z* 103.0 extracted from sample 1 (see Table 15) with creatinine concentration of 20, 13 and 6 (mmol/L).

Sample	Creatinine (mmol/L)	t_R of *m/z* 103.03	Area
1	20	5.12	901646
1	13	5.14	824600
1	6	5.17	612261

Although, the compound *m/z* 103.03 was detected in the urine sample with various dilution grades, the MFE filter setting determines whether the compound will be counted for a molecular feature. The effect of adjusting MFE filter settings will be discussed in the following section.

MFE filter settings may be adjusted based on the analytes of interested and intensity of background noise in the chromatogram and spectrum. Increasing the peak filter setting and compound filter will eliminate more peaks coming from the noise. However, increasing the peak filter can also potentially eliminate actual compounds.

In this study, the MFE filter used have been according to Setting A in **Table 17.** Using these settings, the compound *m/z* 103.03 could only be detected using MFE in the samples with creatinine concentration of 20 and 13 mmol/L. In contrast, the same compound could not be detected in the sample with creatinine concentration of 6 mmol/L, until the filters were lowered according to Setting B.

Table 17: Evaluated MFE parameters including peak filter, compound filter and allowed ion species.

Parameter	Setting A	Setting B
Peak filters	≥ 500 counts	≥ 100 counts
Compound filters (absolute height)	≥ 5000 counts	≥ 1000 counts
Allowed ion species	$[H^+]$, $[Na^+]$ and $[NH_4^+]$	$[H^+]$, $[Na^+]$ and $[NH_4^+]$

This example illustrates how the number of molecular features is highly dependent on the MFE parameters. The parameters used in this study, setting A, are set according to the noise ration from one injection. Further evaluation of these setting could be performed.

Matrix effect is a well-known drawback in LC-MS that may lead to either ion suppression or ion enhancement of analytes, due to co-eluting residual matrix components. Artificial urine is a matrix of salts that is used for preparing calibration standards for methods used in diagnostics where ion suppression appears.

In this study, the degree of matrix effects was evaluated for the developed method by comparing signals from compounds from the Magic mix standards spiked (1:1) in batch urine, artificial urine and H_2O respectively, and peak area of selected compounds are compared in **Figure 17.**

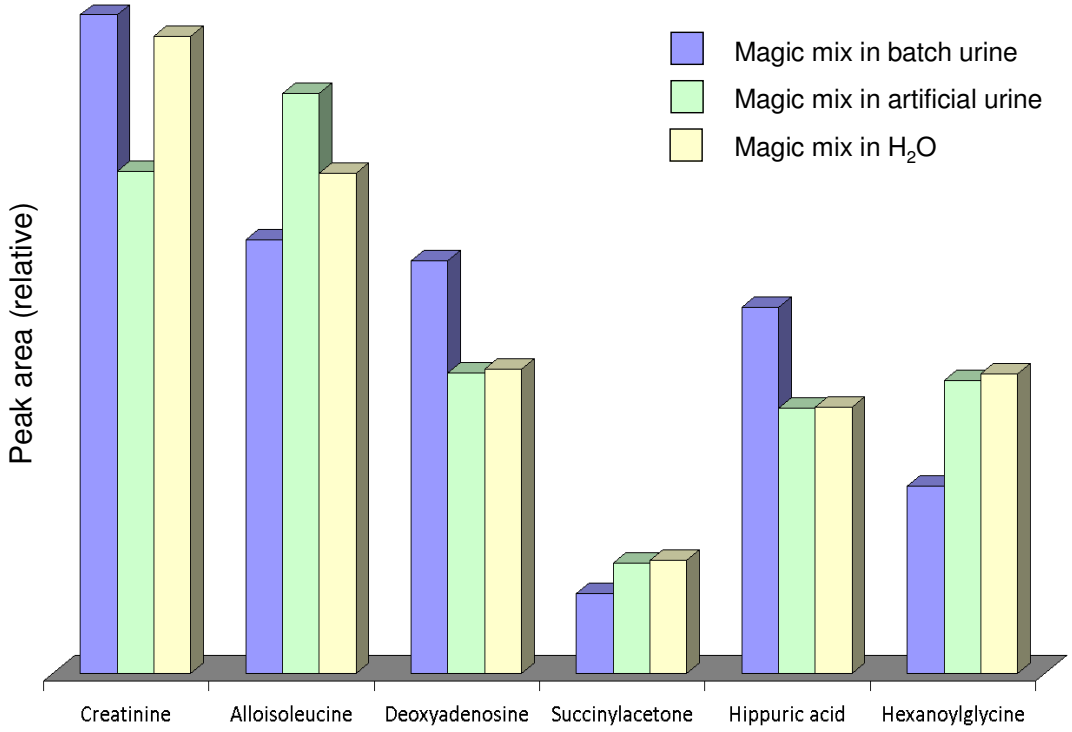

Figure 17: Evaluation of matrix effects by comparing the peak area of compounds in the Magic mix spiked in batch urine, artificial urine and H_2O respectively.

The endogenous compounds creatinine, deoxyadenosine and hippuric were found at highest levels in the batch urine as expected. Similar signals of deoxyadenosine, succinylacetone, hippuric acid and hexanoylglycine in the artificial urine and H_2O, indicates a small degree of matrix effects. For creatinine, it was observed lower signals in artificial urine than in water. No obvious explanation for higher levels of alloisoleucine found in artificial urine rather than in H_20 was found.

3.3.2 Sample stability

In laboratory diagnostics it is well known that some compounds have limited stability. The preanalytical factors can be crucial to obtain reliable results. Secondly the stability will influence the allowable length of the analysis series, and thereby what time limit should be set between the start of the analysis until the last sample is injected. Stability of the batch urine was examined by analysing samples after 0-5 days of storage in room temperature. From the TICs (results are not shown), no significant changes between the chromatograms were found. The number of molecular features were detected with MFE is shown in **Table 18.**

Table 18: The number of molecular features detected in batch urine stored in room temperature for 0-5 days. The samples were separated on ACE C18 PFP (250 x 2.1mm, 3 µm) column and detection was performed in positive mode. Injection volume was 5.0 µL and the used gradient is provided in Table 6. The data were collected in mass range *m/z* 100-1700.

Days stored in room temperature	# molecular features
0	5527
1	5227
2	6331
3	5941
4	6601
5	6631

The results show that the number of molecular features increased with increased storage time at room temperature, which indicates compound degradation and eventually oxidation. The number of molecular features was fairly stable after one day at room temperature. Nevertheless, after 2 days in room temperature, the number of compounds increased significantly and will therefore be the limit of analysis series time.

3.4 Evaluation of developed method

Repeatability of analytical methods is a measure of precision within a series. Normally, the expected coefficient of variance (CV) should not exceed 5-10 %. For global metabolomics in particular, both repeatability and reproducibility should be evaluated throughout the run. In this study repeatability of the developed LC Q-TOF MS method was evaluated with regard to t_R and signal intensity precisions within one series. Batch urine was injected 10 times in one series according to developed method provided in the Experimental section. The results are shown as overlaying TICs in **Figure 18**.

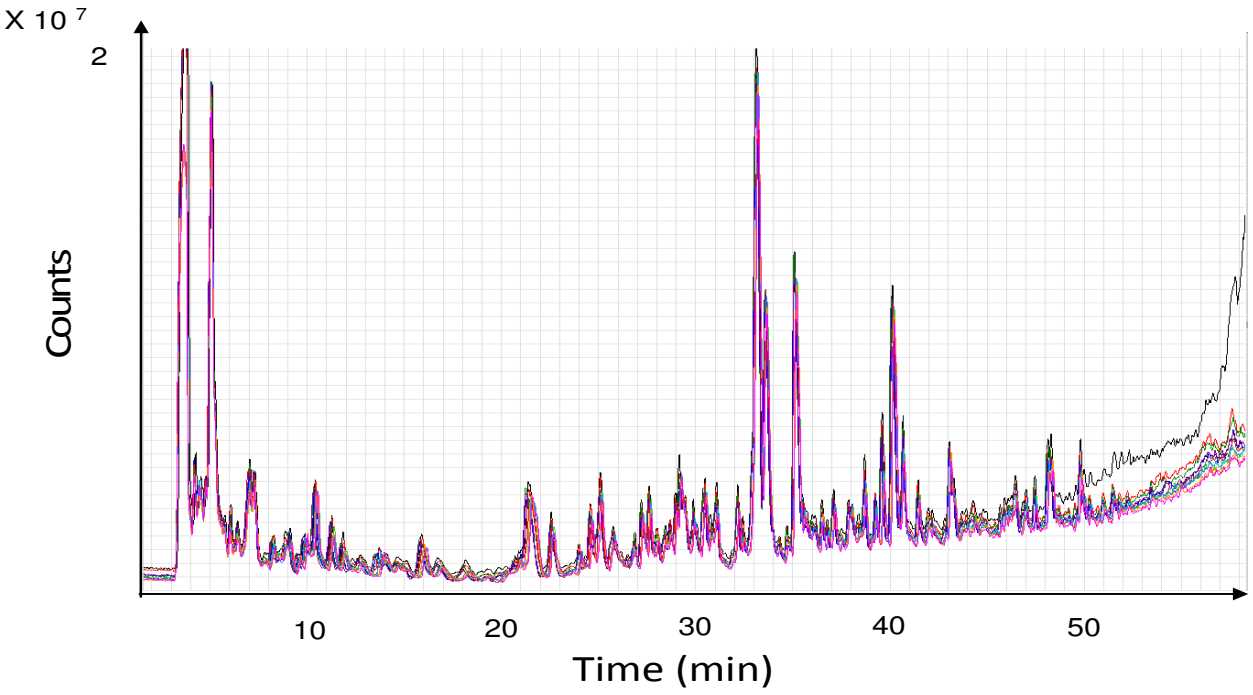

Figure 18: Chromatograms (TICs) shows 10 injections of batch urine injected in one series. The injection volume was 8.0 µL and separation was performed on an ACE C18 PFP (250 x 2.1 mm, 3 µm) column with the gradient described in Table 6. Detection was carried out in positive mode and data were collected in the mass range m/z 100-1700.

Five unidentified compounds were randomly selected throughout the gradient and the respective t_R, peak area, height and CV % is provided in **Table 19.**

Table 19: Mean value of t_R, peak area and peak height, calculated for five randomly selected compounds in batch urine after 10 injections in series for evaluation of the repeatability of the method, see Figure 19.

Compound	t_R mean (range) CV %	Peak area mean (range) CV %	Peak height mean (range) CV %
I	5.1 (5.0 - 5.1) 0.4 %	3.5 (3.3 - 3.7) 10^8 4.5 %	1.8 (1.7 - 1.8) 10^7 1.0 %
2	10.4 (10.3 - 10.5) 0.7 %	6.2 (5.3 - 6.7) 10^6 6.9 %	6.0 (5.5 - 6.3)10^5 4.2 %
3	25.1 (25.0 - 25.2) 0. 2 %	3.5 (2.2 - 4.6) 10^7 18.5 %	3.1 (2.5-4.4) 10^6 18.8 %
4	33.1 (33.1 - 33.2) 0.15 %	31 (2.9 - 3.3)10^8 4.6 %	1.8 (1.7 - 1.8) 10^7 2.4 %
5	49.8 (4.8 - 4.9) 0.1 %	1.2 (1.2 - 1.4)10^7 5.4 %	1.6 (1.6 - 2.0) 10^6 20.9 %

The CV % for t_R, peak area and peak height range was calculated to 0.4-0.7 %, 4.5-18.5 % and 1.0- 20.9 % respectively. The peak area of compound 3 was significant higher mean value and the reason for this is not known. A slight trend was observed in which peak area for several of the selected compounds was either all low or all high in the same sample.

Reproducibility of analytical methods is a measure of precision between series, and is essential in diagnostic laboratories. In this study reproducibility of the developed LC Q-TOF MS method was evaluated by injecting batch urine aliquots from freezer, 10 times over a period of three weeks. The overlaying TICs are shown in **Figure 19.**

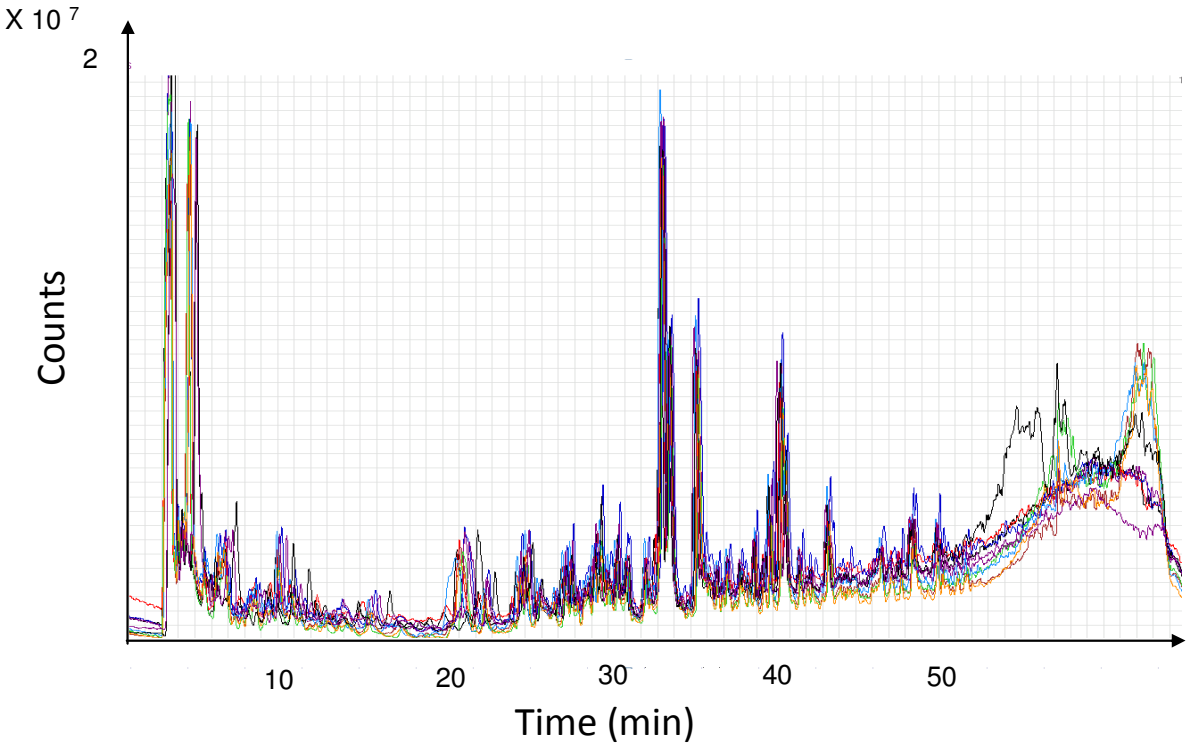

Figure 19: Chromatograms (TICs) show 10 injections of batch urine between different series. Injection volume was 8.0 µL and separation was performed on an ACE C18 PFP (250 x 2.1 mm, 3 µm) column with the gradient described in Table 6. Detection was carried out in positive mode and data were collected in the mass range m/z 100-1700.

Five unidentified compounds were randomly selected throughout the gradient and the respective t_R, peak area, height and CV % is provided in **Table 20.**

Table 20: Mean value of t_R, peak area and peak height, calculated for five selected compounds in batch urine after 10 injections in series for evaluation of the repeatability of the method, see Figure 20.

Compound	t_R mean (range) CV %	Peak area mean (range) CV %	Peak height mean (range) CV %
6	4.7 (4.4 - 4.7) 4.6 %	3.1 (2.7 - 3.5) x 10^8 9.8 %	1.8 (1.7 - 1.8) x 10^7 1.9 %
7	11.2 (10.9 - 11.6) 1.9 %	3.07 (2.7 - 3.5) x 10^8 16.5 %	8.4 (6.7 - 9.8) x 10^5 14.1 %
8	25.1 (24.9 - 25.4) 0.7 %	3.6 (2.8- 5.5) x 10^7 25 %	2.9 (2.2 – 4.0) x 10^6 22.0 %
9	35.4 (35.2-35.5) 0.3 %	2.7 (2.2 - 3.3) x 10^8 14.9 %	1.4 (1.4 - 1.7) x 10^7 32.2 %
10	39.8 (39.6-40.04) 0.3 %	3.6 (2.5 – 4.9) x 10^7 28.8 %	3.8 (2.6 - 5.1) 10^6 21.8 %

The CV % for t_R, peak area and peak height range was calculated to 0.3-4.6 %, 9.8-28.8 % and 1.9- 32.2 % respectively. A weak tendency of the peak area and peak height increasing throughout the run was observed for these particular compounds.

3.5 Intra- and inter- individual biological variation of the normal urinary metabolome

The term “intra-individual biological variation” describes continual changes over a short or long period, within one individual as a result of diet, activity, hormones etc., while “inter-individual biological variation” describes differences in the urinary metabolome due to diverse lifestyle, age and genetic differences. Both terms need to be considered in metabolomic studies. In this study, two different experiments were set up in order to obtain a better understanding about the inter- and intra- individual biologic variation of the normal urinary metabolome.

3.5.1 Investigation of the intra- individual biologic variation

The intra-individual biological variation was investigated by analyzing samples from healthy volunteers with individual diet of own choice. It was emphasized that the volunteers should eat normally. A group of 15 healthy volunteers (10 women and 5 men) participated. One urine sample was collected over a period of 10 work days, collecting a total of 10 urine samples per volunteer. Morning midstream urine was collected after 5:00 am in specific tubes and frozen at -20 ° C at home and at -70° C after arrival at the hospital and until analysis. Diet, including all food and drinks through the whole day prior to the day of urine collection in addition to eventual work out lessons was noted in a scheme delivered out prior to the experiment. Sex, age, weight, height, eventual nutrition subsidizations or medications and eventual vegetarian diet was also noted in the scheme. An example of scheme is provided in appendix (chapter 8.4). Due to lack of time, the samples were collected, but are not yet analyzed. **Figure 20** shows a PCA-score constructed in MPP shows a urinary profile for 3 healthy volunteers with different diets over 10 days.

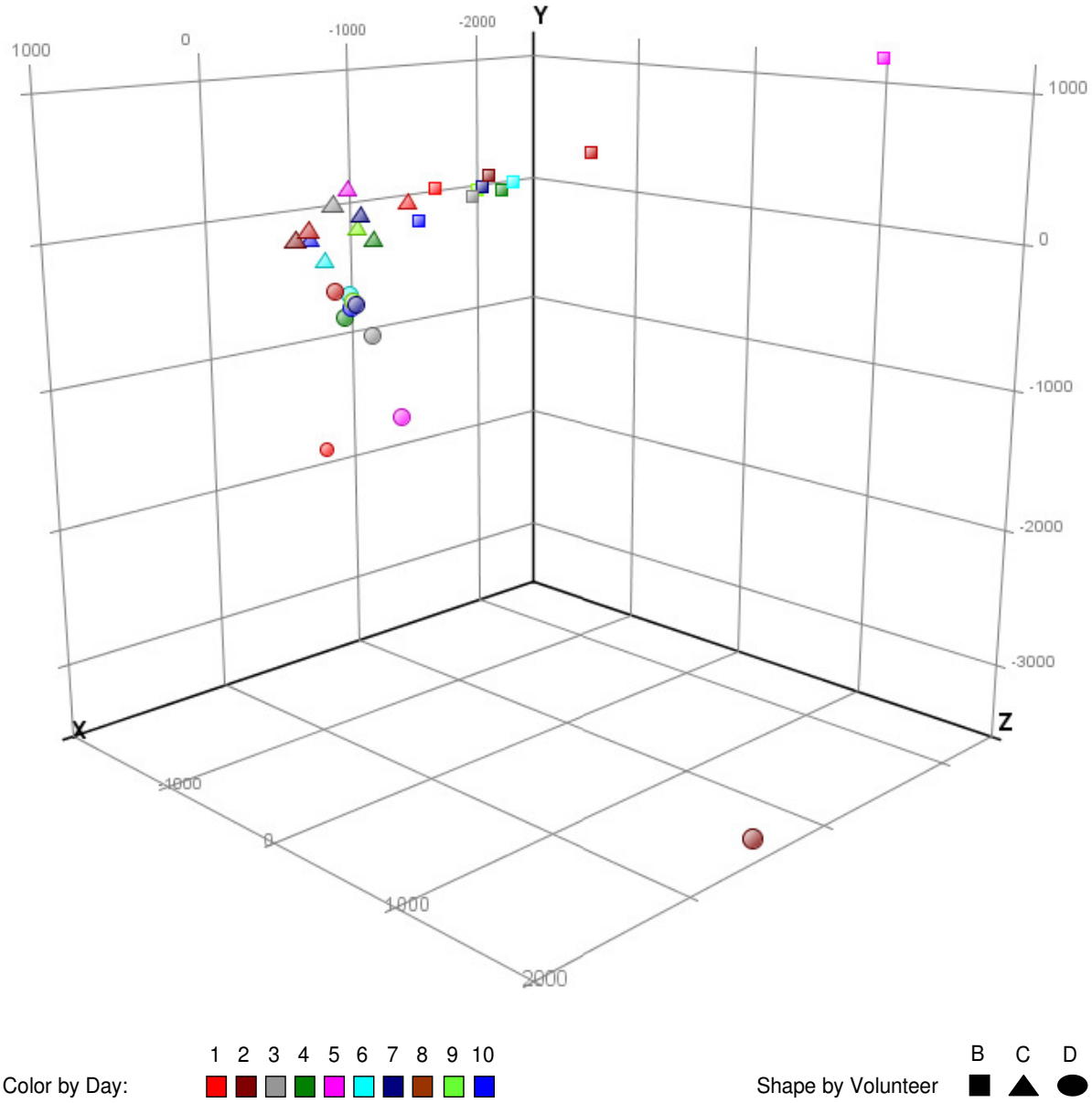

Figure 20: The PCA-score constructed is based on the molecular features shows a profile for 3 healthy volunteers with different diets over 10 days.

The PCA-scores shows a clear tendency of clustering of each subjects' metabolome over a 10 days period. This could reflect limited variation in each person's diet, or genetically driven differences between individuals in how the metabolism is regulated. From the information about the diet intake from each volunteer, no particular trend could be observed.

3.5.2 Investigation of the inter-individual biologic variation

The inter-individual biological variation was investigated by analyzing urine samples from healthy volunteers with identical diet. A group of 14 healthy volunteers (10 women and 4 men) participated over 3 days. On the first day the volunteers ate and drank according to own preferences and they were instructed to report every meal. Next day, a morning midstream urine sample was collected. During the second day, all volunteers were instructed to eat and drink identical food that was provided. The menu which included breakfast, lunch, dinner and supper was developed with emphasis on simple food and small degree of variation, with the aim of recruiting a sufficient number of participants for the trial. General information about the experiment, instructions for the procedure given to the participating volunteers is provided in appendix (chapter 8.5), in addition to the menu set for this day. On the third day, a second morning midstream urine sample was collected. All urine samples were frozen at -20 ° C at home and at -70° C after arrival at the hospital and until analysis.

Principle component analysis (PCA) perfomed in MPP of urine samples from 15 healthy volunteers identified with a letter (A-O), from day 1 and 2 identified with shape (square and triangle) see **Figure 21** was constructed. Day 1 represents different diet and day 2 standardized diets. Square shape day 1 and triangle day 2.

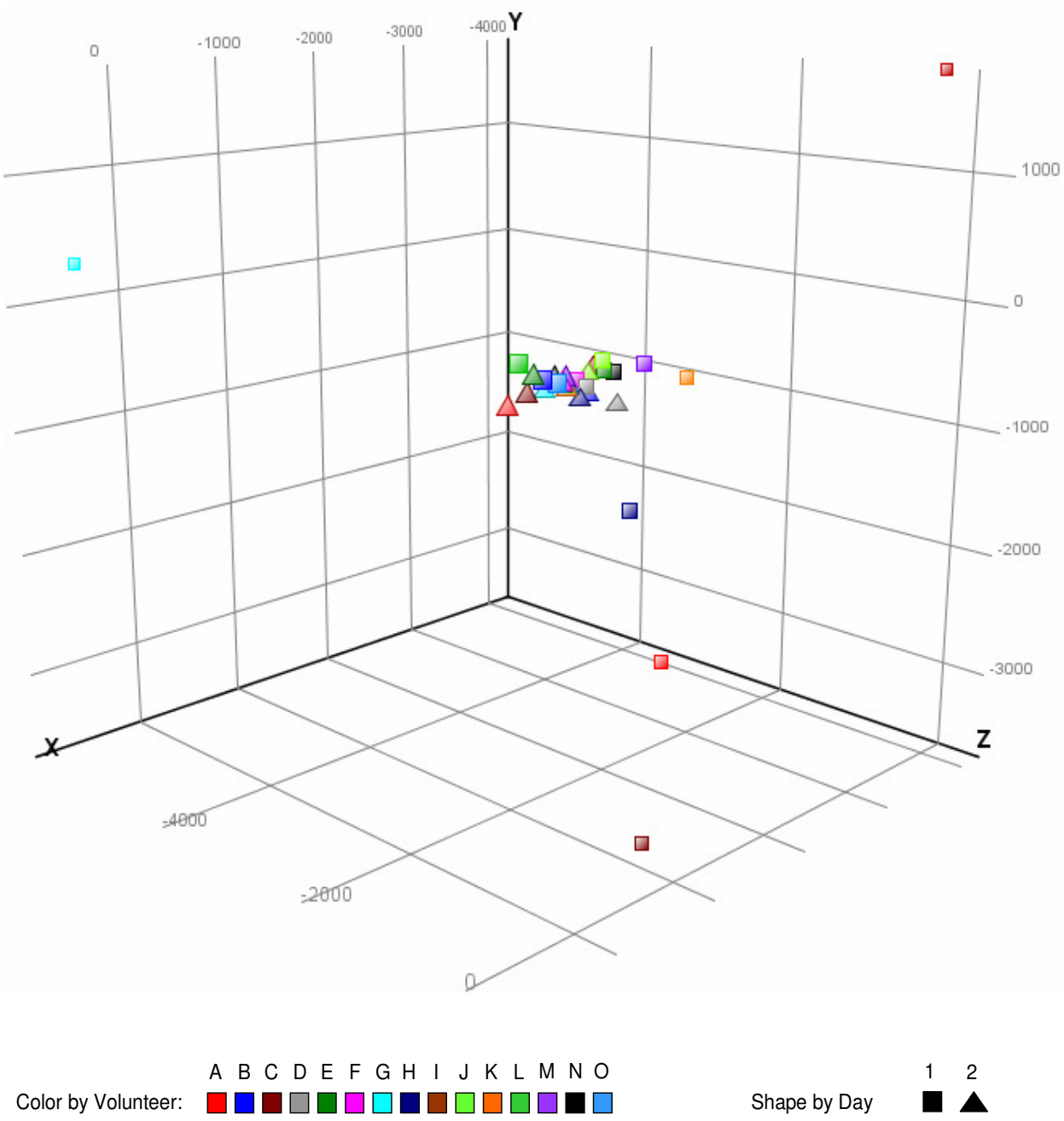

Figure 21: The PCA-score constructed is based on the molecular features shows a profile for 15 healthy volunteers with identical diet for one day.

The results indicate clustering of the points after standardized diet for 24 hours. The outliers on day one are united to a larger extent in day two, which indicates a rapid change in the metabolome as a result of diet.

The urine samples were also analyzed on ^{1}H NMR by Daniel Sachse. Results are presented in **Figure 22**. Each volunteer is identified with a letter and attached number 1-2 indicates if the sample is collected after different diet (no 1) or identical diet (no 2). As expected the samples collected after day 1, is quite scattered while the samples collected after day 2 is more grouped together. The samples A2, K2 and J2 diverge from the rest of the samples from day 2. F1 is also close to this group. According to declared information from the volunteers A, K and J, there are no obvious reasons for the diverging points, and they are not the same sex or age. Since the volunteers I and J which are identical twins are not the closest points, which was rather unexpected since the genetics plays an important role when it comes to metabolism.

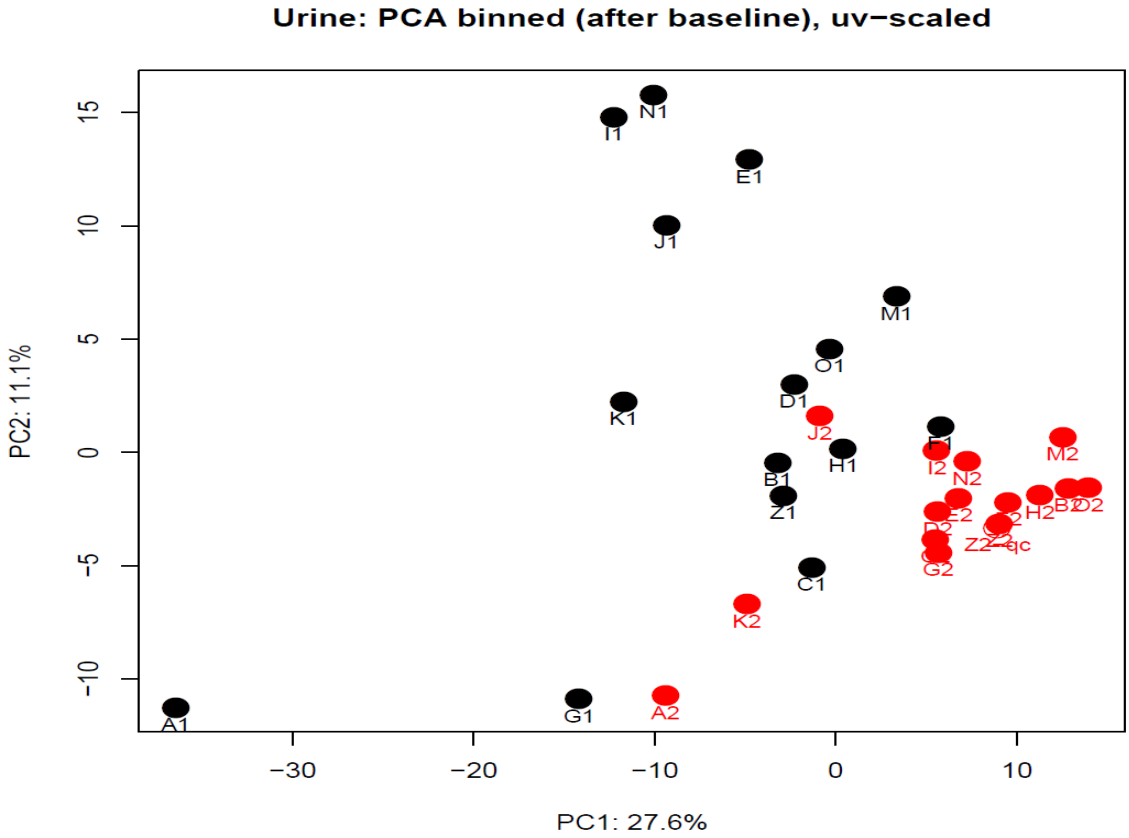

Figure 22: ^{1}H NMR PCA experiment healthy volunteers. Black dots represents the urinary metabolome from 15 healthy volunteers having diets of own choice, while the red dots represent a standardized diet.

4 Future perspectives

The developed LC Q-TOF MS method useful for both positive and negative ionisation, utilizing a global approach has shown ability to detect a large number of molecular features in urine. The samples that have already been collected during the diet experiments should be analysed and further investigation regarding biological variation should be performed. The outliers in the PCA-scores constructed should be investigated for correspondence to specific foods. Following that, the method should be used for establishing of the normal metabolome by collecting samples from a healthy population. Secondly, samples from patients suffering from a known IEM should be analysed and compared to the established normal profile to test the usefulness of the developed method. Finally, samples from the diagnostic biobank should be analysed in order to search for new diagnostic biomarkers and possibly describe new IEM. The method can also be used to investigate the biochemical consequences of, and by that identify more sensitive biomarkers for known IEM.

5 Spotlight project: Identification of unknown human urine metabolite UK460

Background – Identification of an unknown compound in a black urine sample.

A urine sample from the patient was received at the Section of Inborn Errors of Metabolism for analysis of calculi. The sample did not contain a defined not contain a defined calculi, but rather precipitate of unknown origin. Normal components of calculi are calcium, phosphor, oxalic acid, uric acid etc. Calculus is a stone formed in the body that might cause medical conditions like kidney stone or bladder stone. In addition, drug-induced stones are known, and some rare kidney stones are seen in IEM; hypoxanthine stones, 2,8-dihydroxy adenine stones and cysteine stones. No common stone component was identified in routine analysis. However, it was observed that the urine sample turned from a regular yellow sample into a dark, black color by deferral on the bench. Most samples are thrown to waste after analysis, and so was this sample. Black urine is a common indicator of alcaptonuria (ACU) which is an IEM and a new sample was received for follow up analysis. ACU is known genetic disorder, where homogentisic acid oxidase is defect and homogentisic acid accumulates as a result. The clinical picture of ACU is dark pigment spots in the skin and yellow urine turning black when exposed to air and oxidation occurs, see **Figure 23.**

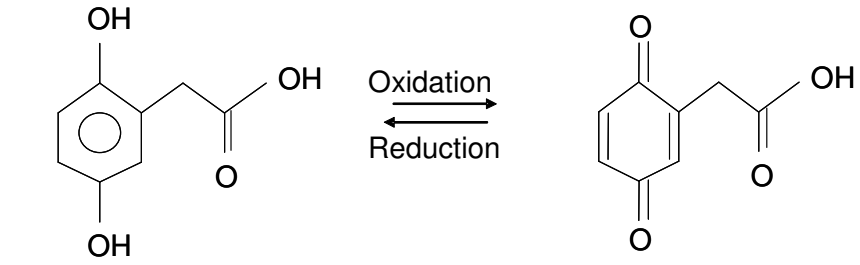

Figure 23: Homogentisic acid is the patognomonic biomarker for diagnostics of the IEM alcaptonuria. The reduced form does not contribute to color in urine, while the oxidized form result in black urine by deferral on the bench or by adding NaOH.

While waiting for arrival of the new patient sample, the usefulness of the developed LC Q-TOF MS method for identification of ACU was tested. A black urine sample from a patient already diagnosed with ACU was taken from the diagnostic biobank and analyzed. The diagnostic metabolite homogentisic acid $[M+1]^+$ = *m/z* 169 with fragmentation pattern is shown in **Figure 24.** The spectrum was identical to the spectrum obtained by analysis of homogentisic acid dissolved in water (results not shown).

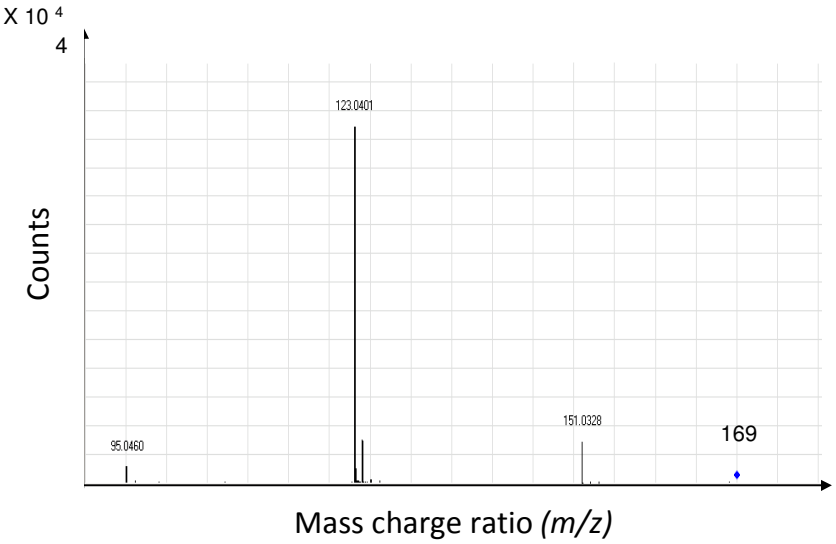

Figure 24: Mass spectrum shows the fragmentation pattern of homogentisic acid *m/z* 169 analyzed by LC Q-TOF MS in positive ionization.

5.1.1 GC-MS analysis

Sample preparation including methylation and GC-MS analysis of organic acids in urine were performed by the staff at Section for IEM according to standard operation procedure.

The new urine sample from the same patient was yellow at arrival and was methylated according to standard procedures before analysis with GC-MS. Neither homogentisic acid nor any other known biomarker was could be detected and the patient was not diagnosed with ACU. In the following, the various approaches to reveal the cause of the black color of the urine are presented. Although homogentisic acid was not found in the patient urine, a signal with great intensity was measured, but the source or structure of the compound was not known. The compound had been detected once in the past, but it was not identified and

therefore given the identity name of “Unknown 460” (UK460). The mass spectrum in **Figure 25** shows the fragmentation of UK460 by GC-MS.

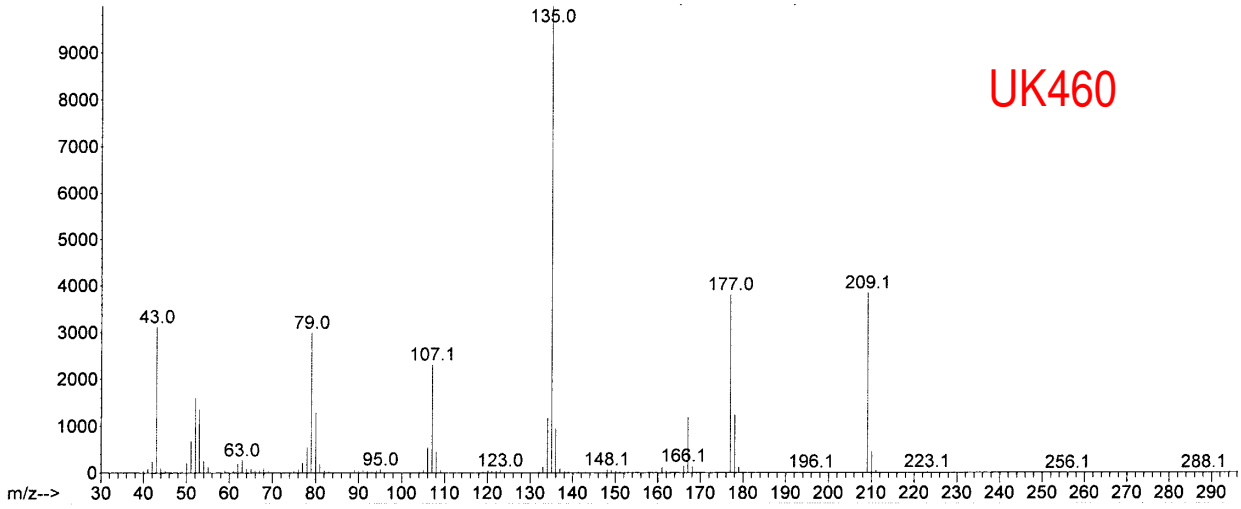

Figure 25: GC-MS mass spectrum of methylated UK460 *m/z* 209

What was particularly interesting was that two compounds with identical mass spectrum chromatographed both after 42 and 70 minutes, see **Figure 26.** The intensity of the latter peak was remarkably higher than the first. The questions arising were how UK460 chromatographing after 42 and 70 minutes, respectively, could have different t_R and if they had the same origin.

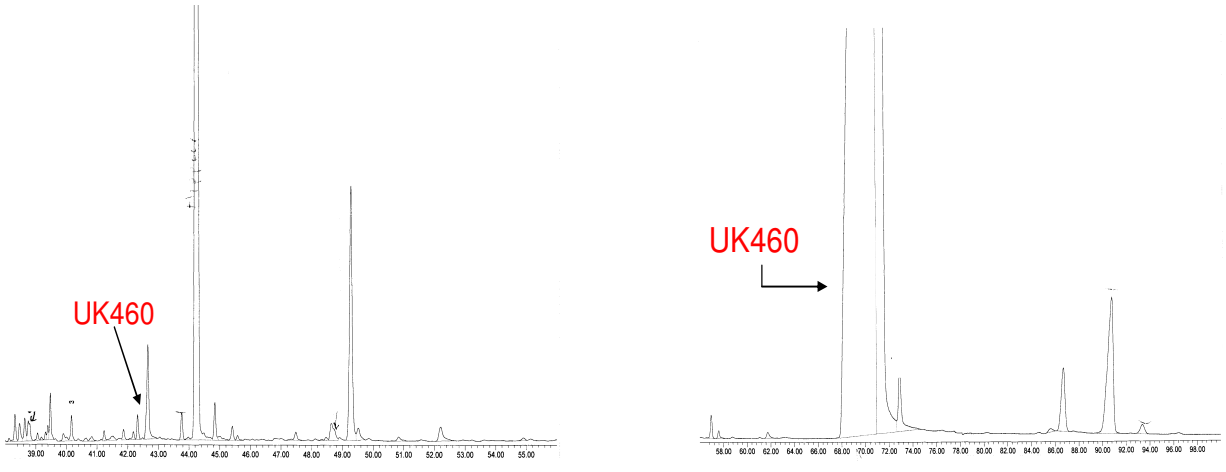

Figure 26: The two GC chromatograms show UK460 eluting both after 42 and 70 minutes.

From the GC-MS spectrum the methylated molecular ion mass was determined to be M = 209. A calculation of probable combination of the chemical elements in the structure was done under supervision by Per Ola Rønning, using Excel for low resolution GC-MS data, see appendix (chapter 8.6). The calculation was performed from the isotopic pattern and the abundance of the isotopes M+1, M+2 and M+3. The calculation was performed under the qualification of the compound having at least one nitrogen atom and two oxygen atoms since it was suspected to contain a carboxyl group. A *Solver-procedure* was performed (only for compound eluting at 70 min, due to highest intensity) to test different combination for the methylated molecular formula with best compatibility with the isotopic pattern. The number of C, H and O-atoms was calculated and the likely formula of $C_{10}H_{11}NO_4$ was suggested. Subtracting a mass of 14 leaves a formula of $C_9H_9NO_4$ with mass 195 which fits well with LC Q-TOF MS data. From the molecular formula the number of double bonds was calculated to be 6. In order to determine the structure, more information was required.

5.1.2 LC-MS/MS

The black urine patient sample was analyzed by LC Q-TOF MS with ESI. The chromatographic most abundant peak eluted at 25 min, contained *m/z* 196 and *m/z* 391, respectively, see **Figure 27.** This compound appearing as the large peak was thought to be corresponding to the unknown compound UK460 detected by GC-MS at 42 and 70 min and not found in healthy batch urine which indicates an exogenous compound. The MS/MS spectrum from the two co-eluting compounds *m/z* 391 and *m/z* 196 are shown in **Figure 28**. The fragment patterns from *m/z* 391 shows that the most abundant daughter ion is *m/z* 196.This indicated the presence of a monomeric and dimeric form of the molecule.

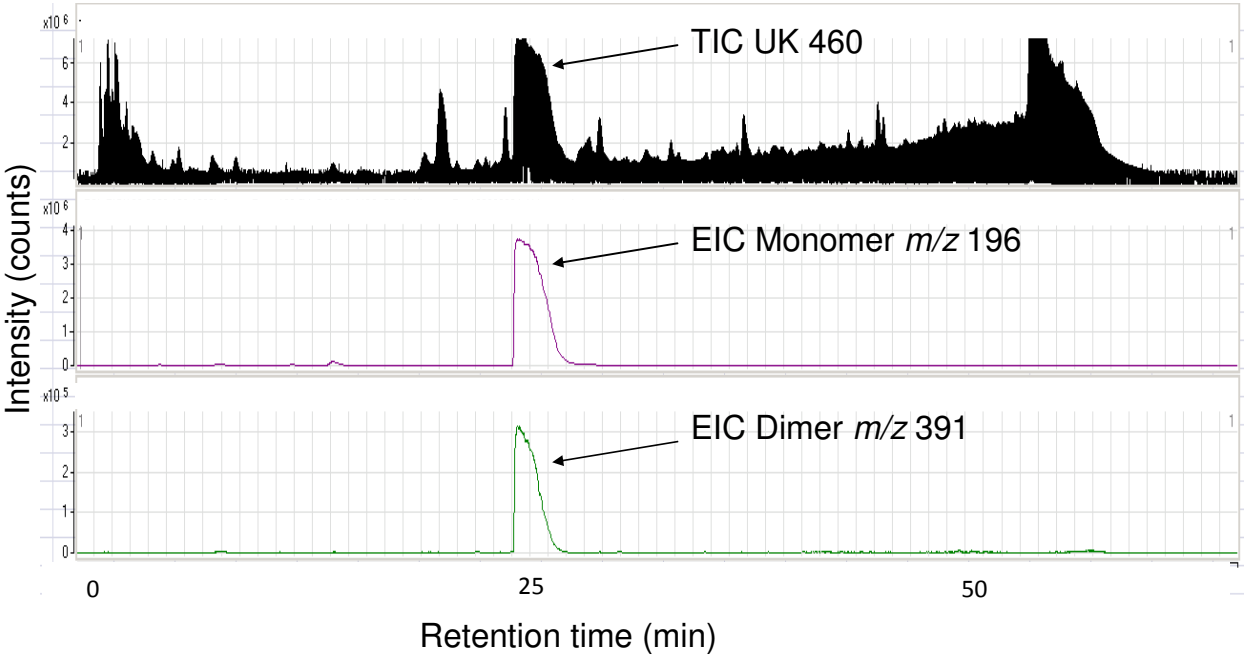

Figure 27: TIC and EIC of *m/z* 196 and *m/z* 391 of black urine analyzed by LC Q-TOF MS ESI positive ionization mode. The large peak eluting at 25 min in the patient sample was not visible in the batch urine. Separation was carried out on a Kinetex C18 (100 x 2.1 mm, 2.6 µm) column, utilizing gradient elution in Table 8.

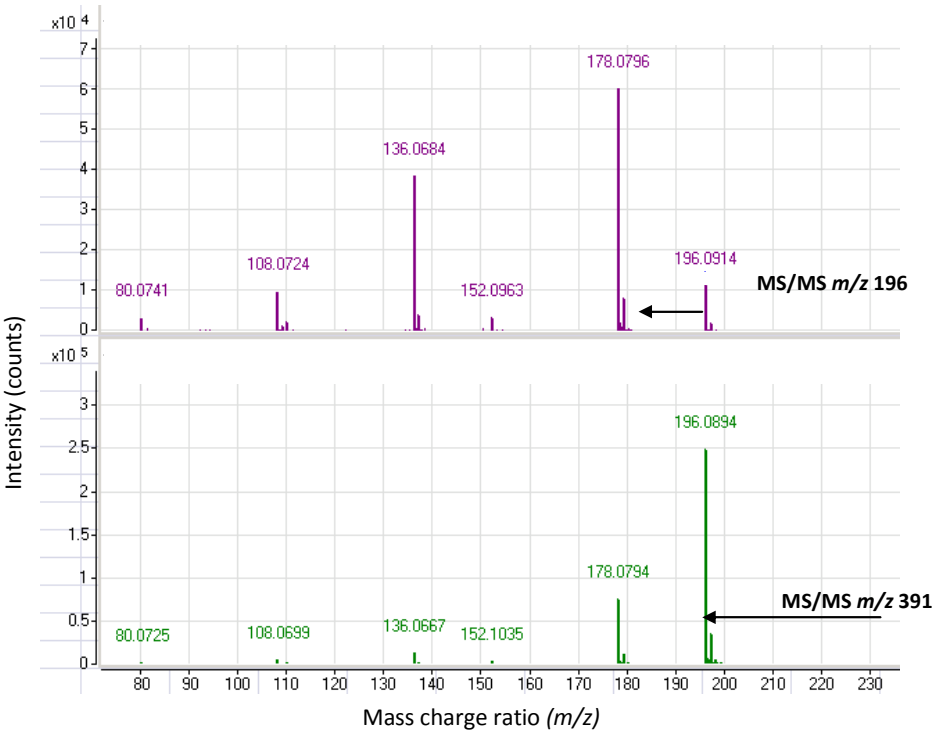

Figure 28. LC-MS/MS spectrum of black urine with identical fragmentation of the monomer ($[M+H]^+$ = *m/z* 196) and dimer ($[2M+H]^+$ = *m/z* 391)

GC-MS cannot be performed on underivatized samples. Thus, in order to be able to compare Q-TOF data with GC-MS data, the methylated patient urine sample was also analyzed by Q-TOF. By comparing the Q-TOF results obtained by analyzing the methylated and non-methylated sample, structural information of the unknown compound can be obtained. The sample was analyzed before and after exposure to air, meaning both a yellow and black edition of the same sample. Even though the black urine sample is unusual and interesting in itself, it was not certain that the unknown compound had anything to do with the color. The chromatographic most abundant peak contained two different masses of *m/z* 210 and *m/z* 419 respectively, with identical eluting at 36 min, see **Figure 29**. The retention was stronger after methylation of the compound and the split top could possibly be due to the high concentration.

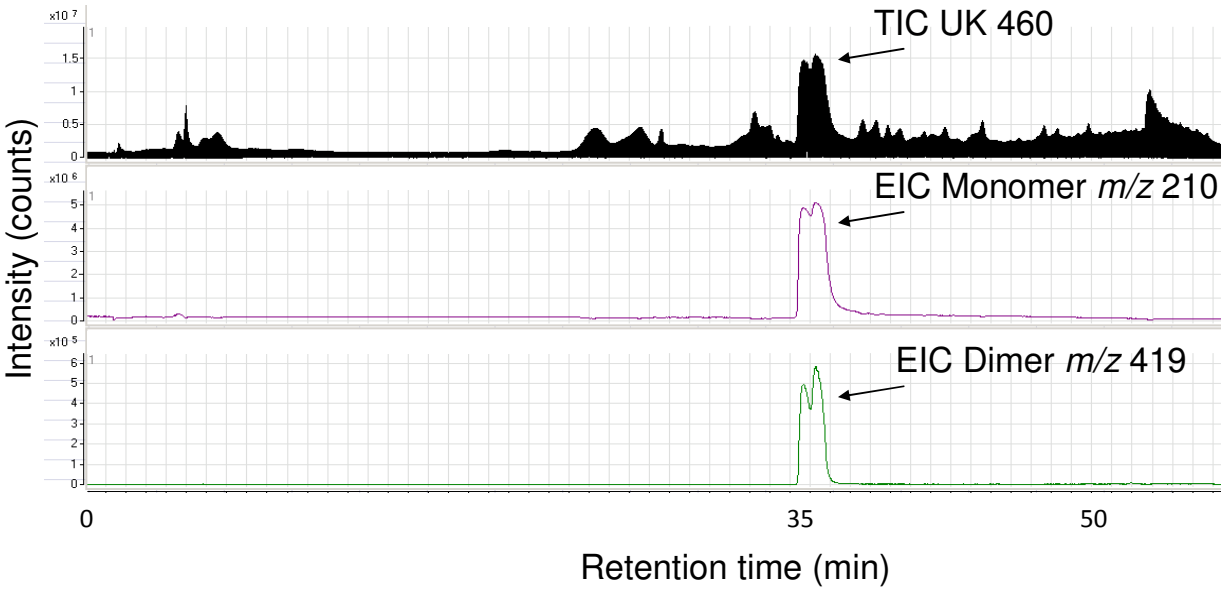

Figure 29: TIC, EIC of m/z 210 and m/z 419 of methylated black urine analyzed by LC Q-TOF MS ESI positive ionization mode. The large peak eluting at 35 min in the patient sample was not visible in the batch urine.

The MS/MS spectrum from the two co-eluting *m/z* 210 and *m/z* 419 are shown in **Figure 30**. The fragment patterns from *m/z* 419 shows that the most abundant daughter ion is *m/z* 210 and the further fragmentation patterns are identical.

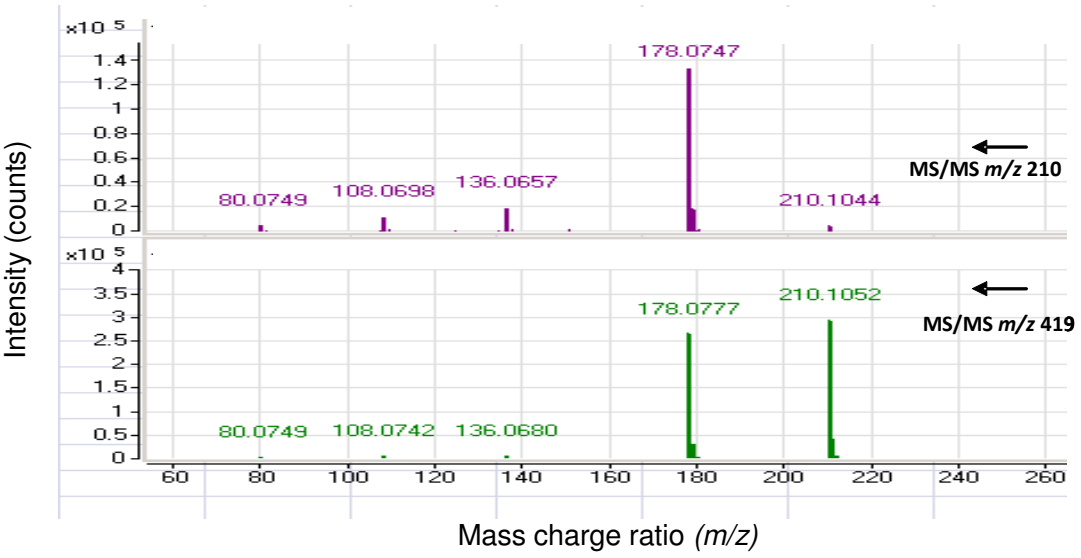

Figure 30: LC-MS/MS spectrum of methylated black urine shows identical fragmentation of the monomer ($[M+H]^+$ = *m/z* 210) and dimer ($[2M+H]^+$ = *m/z* 419)

It is interesting to note that in GC-MS, two peaks appear at different t_R with the same spectrum, while in Q-TOF two different versions of a molecule (monomer and dimer) appears at the same $t_{R,}$ but with different MW for GC-MS. Perhaps the monomer and dimmer are stable during GC and thus are chromatographically separated, but when entering the EI source they are fragmented giving identical spectrum. In contrast in LC Q-TOF, the monomer and dimer show the same chromatographic properties but are disclosed as two different molecules (monomer and dimer) in the softer ionization technique used for the Q-TOF, where the molecular ions can be detected.

Although, UK460 at this point was regarded to be a compound able to form a dimer, there is still no certainty if there is a connection between the black color observed and UK460. To investigate the origin of the black color, the sample was centrifuged and black precipitate collected. After drying, the precipitate was dissolved in water and analyzed by LC Q-TOF MS. A distinct peak eluting at 2.2 minutes was observed, referred to as “compound X” in the following (see **Figure 31**). This points to UK460 being a compound that tends to dimerisate. Total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of X *m/z* 154.08 eluting after 2.2 min is shown in **Figure 31** and fragmentation pattern of UK460 *m/z* 196, eluting after 26.1 minutes is shown in **Figure 28.** While the monomer of UK460 was found, the dimer of UK460 *m/z* 391 could not be identified in dried precipitation.

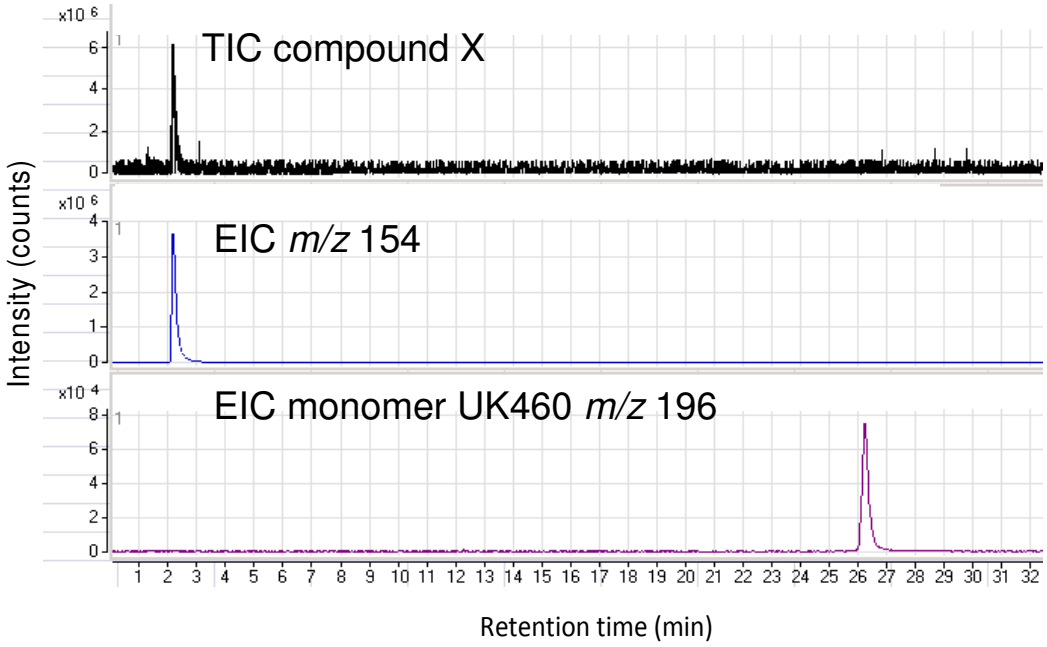

Figure 31. TIC of dried precipitation from black patient urine resolved in water and injected on LC-MS Q-TOF (black) , EIC 5-ASA $[M+H]^+$ = *m/z* 154.08 eluting after 2.2 min and fragmentation pattern of N-Acetyl-5-ASA $[M+H]^+$ = *m/z* 196 eluting after 26.1 min. The separation was performed on a Kinetex C18 (100 x 2.1, 2.6 µm) column, the injection volume was 5.0 µL and ESI positive ionization was utilized. Gradient used is shown in Table 8.

The MS/MS product ion spectrum s of dried precipitate from black patient urine are shown in **Figure 32**.

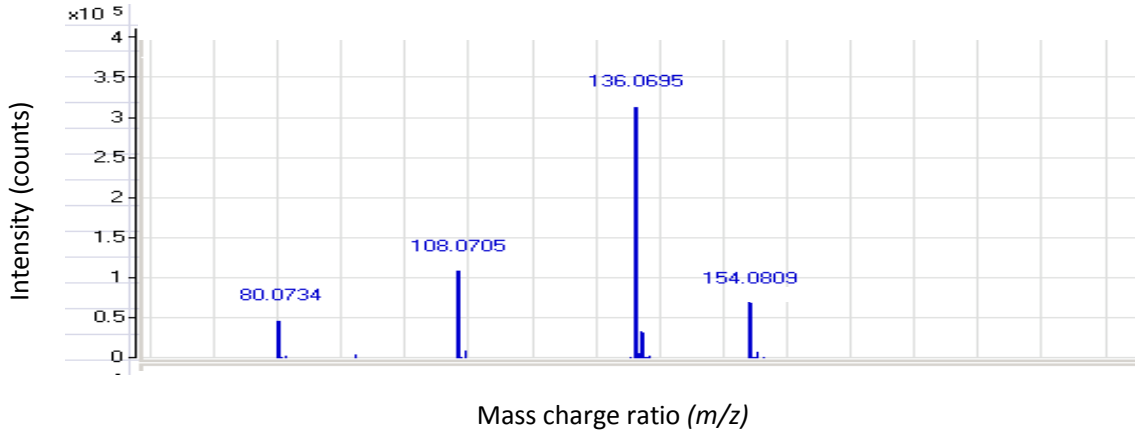

Figure 32: MS/MS product ion spectrum of dried precipitation from black patient urine, showing fragmentation pattern of component X with *m/z* 154 eluting after 2.2 min.

From a new patient a urine sample where UK460 had previously been detected by GC-MS, was analyzed by LC Q-TOF MS to compare mass spectrum to confirm that the same compound with mass of 390 was present. Chromatograms are shown **Figure 33.** (The mass spectrum are not shown)

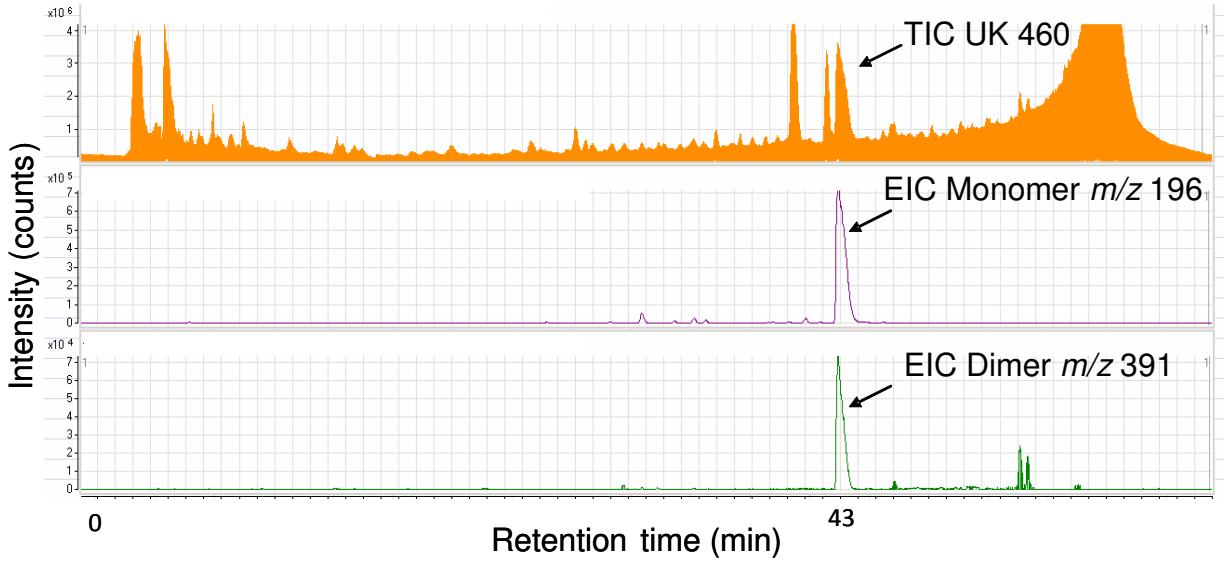

Figure 33: TIC and EIC of *m/z* 196 and *m/z* 391 of yellow patient urine sample analyzed by LC Q-TOF MS ESI positive ionization mode. The large peak eluting at 43 min in the patient sample was not visible in the batch urine. Separation was carried out on a C18 Diphenyl (250 x 2.0 mm, 3 µm) column, utilizing gradient elution in Table 8.

The difference in retention time compared to UK460 in black urine (see **Figure 27)** can be explained by separation by a longer column. Once detection of UK460 in black urine, there were first made theories regarding the correlations between UK460 and the black color. However, after detection of UK460 in normal looking yellow urine it became quite uncertain about these correlations. However, the intensities of UK460 in black urine were significantly higher than in yellow urine.

5.1.3 NMR

The unknown compound UK460 had been detected in black and yellow urine sample from two different patients. As MS was not alone able to identify the unknown compound, it was decided to analyze the samples with NMR to collect additional data for determining the structure. The sample preparation and ^{1}H NMR 600 Hz analysis were performed by Daniel Sachse at Dept. of Chemistry (UiO). More details are provided in appendix (chapter 8.7).

Three different samples were analyzed by ^{1}H NMR: black urine with UK460, yellow urine with unknown compound UK460 and batch urine without UK460 as control. 1D-specra from analysis of black patient urine with, yellow urine and batch urine are shown in **Figure 34, 35 and 36**, respectively.

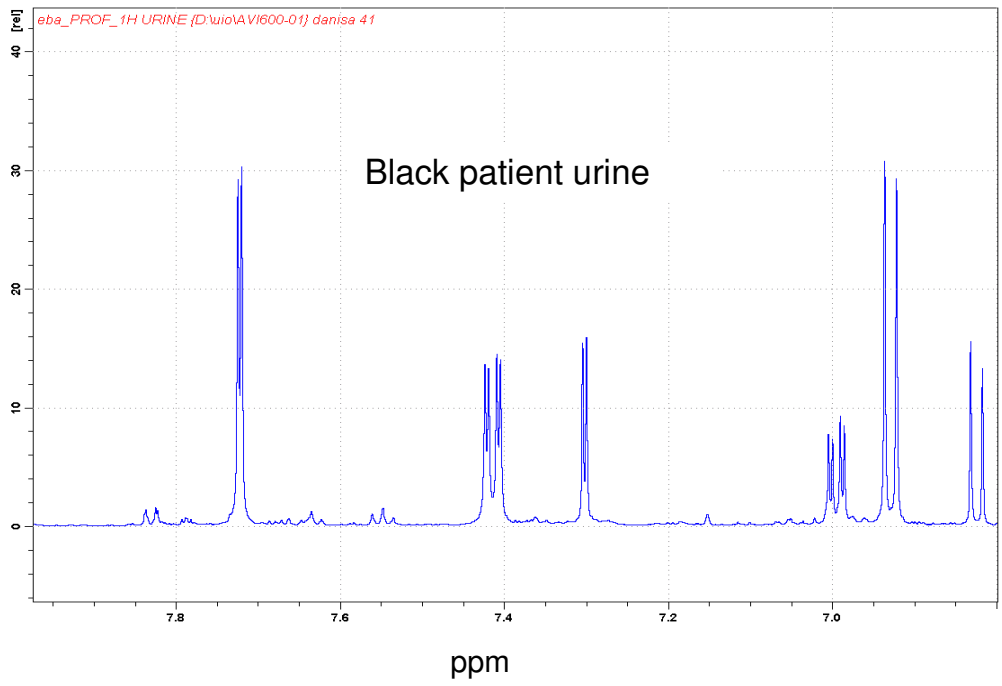

Figure 34: 1D-spectrum of black urine analyzed by ^{1}H NMR.

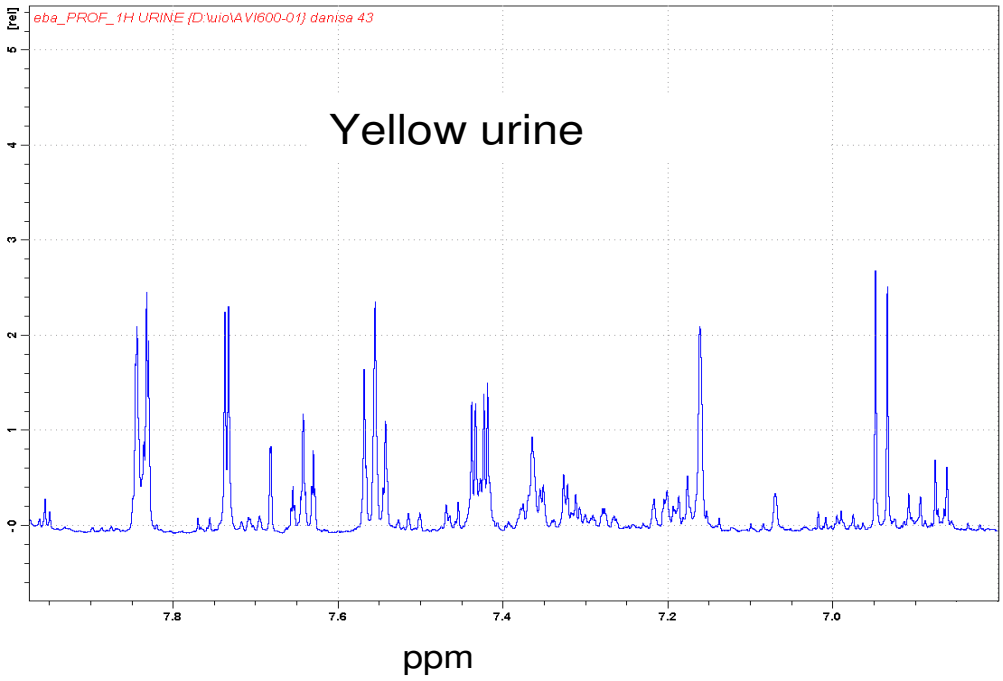

Figure 35: 1D-spectum of yellow urine analyzed by ^{1}H NMR.

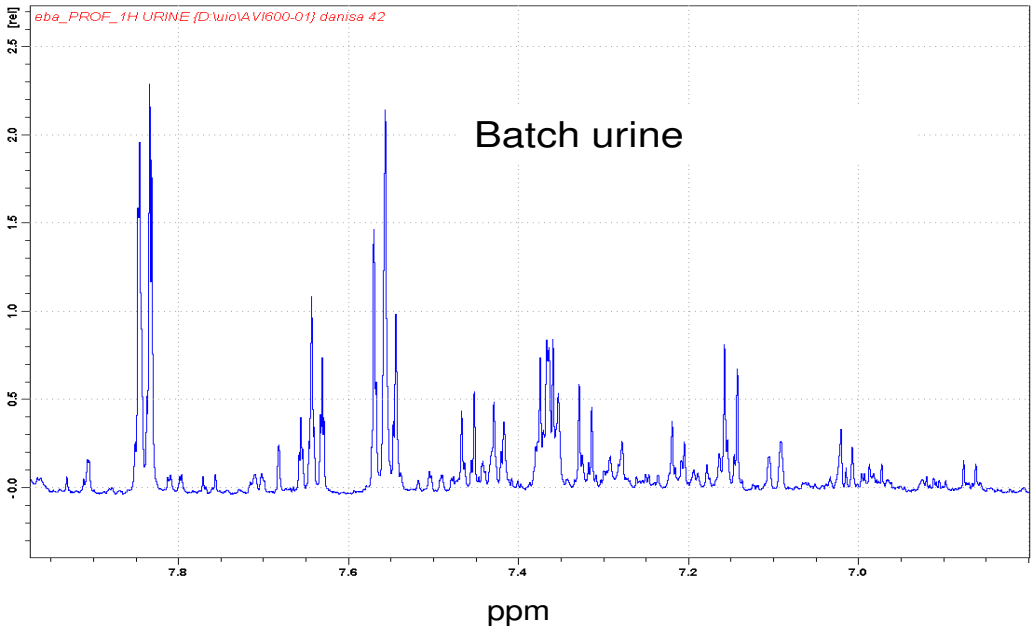

Figure 36: 1D-spectum of batch urine analyzed by ^{1}H NMR.

In black urine peaks of high intensity in the aromatic area around 7 ppm was observed. These peaks also were detected in the yellow patient urine with UK460, however in lower concentration. The spectrum of the black urine is downscaled and thus cannot be compared to the yellow urine nor be used for quantitative measures.

Based on these NMR results, it was demonstrated that UK460 was likely to contain of an aromatic structure. UK460 was not detected in batch urine which strengthens theory of this compound being exogenous. To sum up: NMR provided further information about the structure. However, UK460 was still not identified.

5.1.4 FT-IR spectroscopy

Analyses by LC, GC and NMR were not sufficient in order to determine the structure of UK460. Fourier transform infrared spectroscopy (FT-IR) of the precipitate from the black urine sample was performed on a Vertex 70 spectrometer (Bruker, Germany) under supervision by Sachin Chavan at the Dept. of Chemistry, (UiO) Catalysis group, for the purpose to identify functional chemical groups of UK460. The resulting Kubelka-Munk spectrum ranging from 4000 cm^{-1} to 360 cm^{-1} is presented in **Figure 37.**

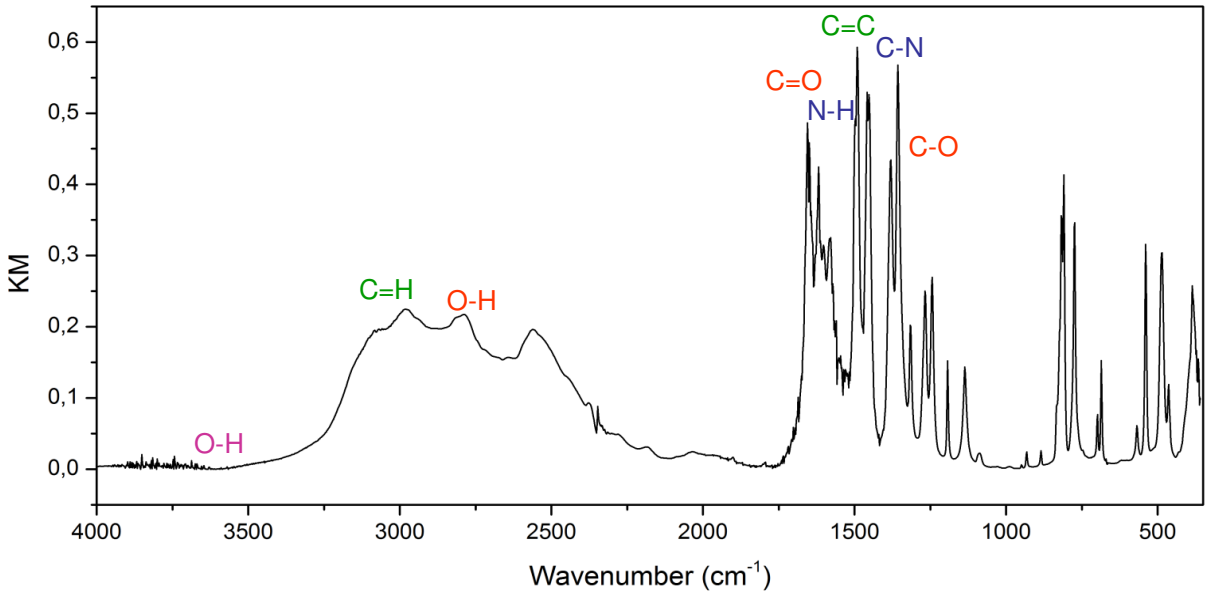

Figure 37: FT-IR Kubelka-Munk spectrum of precipitate from black urine and possible functional groups present.

An attempt to identify functional groups of the precipitate from the black urine was performed. However, identification of UK460 still appeared to be quite difficult.

5.1.5 X-ray diffraction

X-ray diffraction (XRD) of the precipitate from the black urine sample was performed on a Discover D8 Diffractometer (Bruker, Karlsruhe, Germany) by researcher Sigurd Øien at Dept. of Chemistry (UiO), Catalysis group. The purpose was to investigate potential crystalline compounds in the sample, since precipitation in urine was observed. The resulting XRD diffractogram is shown in **Figure 38.**

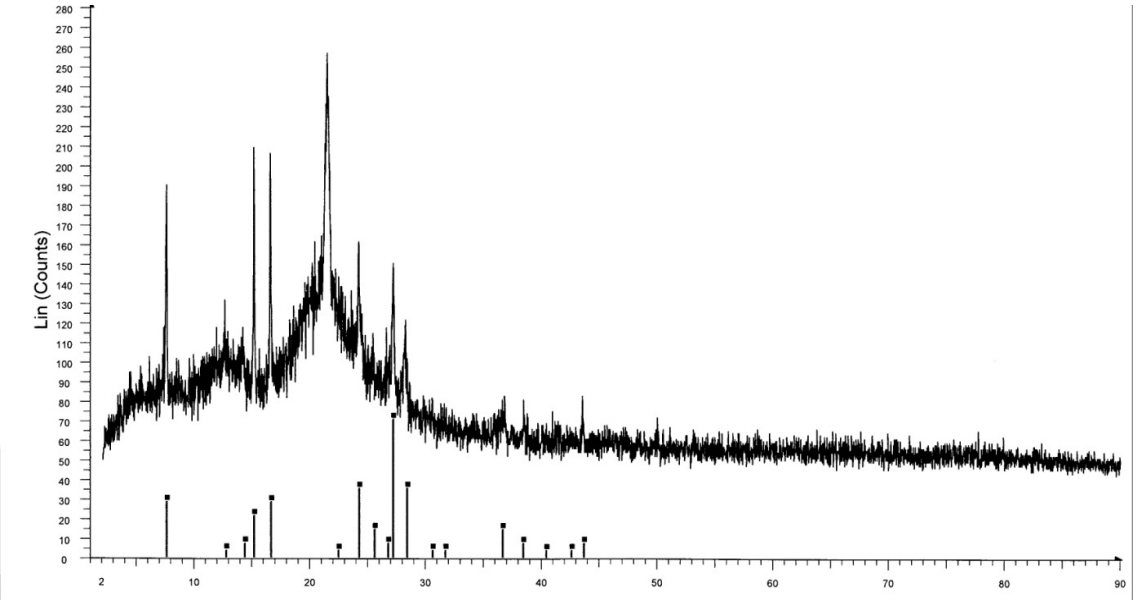

Figure 38: X-ray diffractogram of dried precipitation from the black urine sample.

The large peak at 20° is considered as noise resulting from compounds in the plastic due to sample preparation. Sharp peaks in the diffractogram appearing between 8° and 30° indicates crystalline compound (s). The diffractogram was compared to a standard database and the compound 5-ASA was confirmed present in the precipitate from the black urine sample.

Summary and concluding remarks on the compound X, main component in the precipitation, was identified as 5-ASA by XRD. Following this, the reference compound was bought and standard solution analyzed and compared to the results obtained when analyzing the patient sample.

5.1.6 Identification of black colour by LC-UV, spectroscopy and MS

Black urine was analyzed by LC-UV to determine if the compound 5-ASA was causing the unusual black color, by collecting the peak manually. UV absorbance of the black urine shown in **Figure 39,** strengthens the hypothesis that the compound in black urine eluting at 29 min does have something to do with the accumulated peak and potentially the black color.

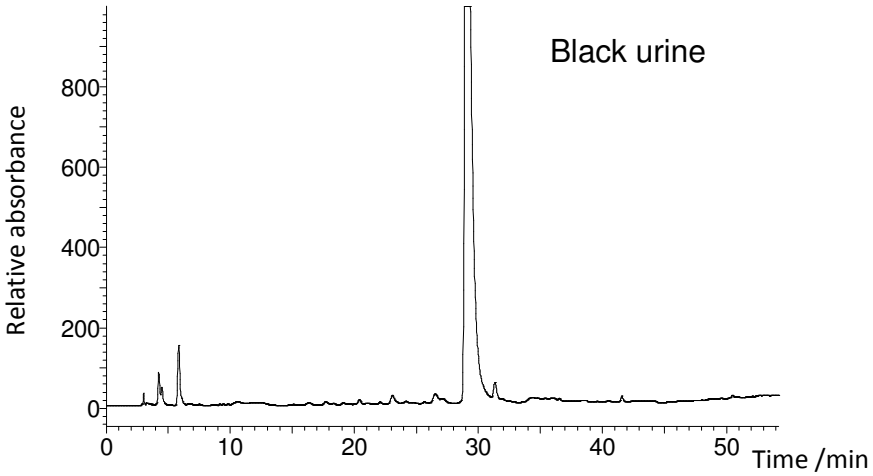

Figure 39: LC-UV chromatogram of black urine. 10 µl was injected onto aRP Eclipse XDB C18 prep 250 x 9.4 mm, 5 µm column (Agilent, USA). Gradient elution was performed according to Table 8. A Perkin Elmer HPLC system with an Agilent autosampler and 200 series pump with a flow of 3.0 ml/min. UV absorbance of black urine was measured at 250 nm by a Waters tuneable detector 486, and chromatogram generated by the software TotalChrom Navigator.

Batch urine was also analyzed using the same method as black urine, and a comparable peak could not be detected, see **Figure 40.**

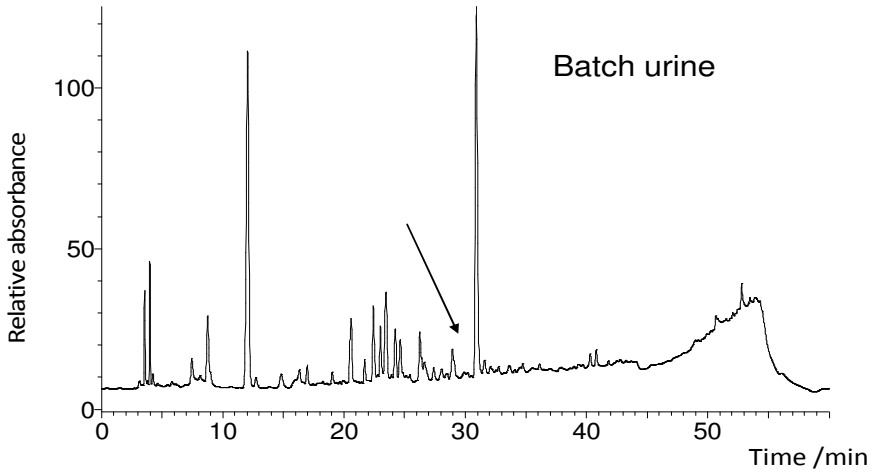

Figure 40: LC-UV chromatogram of batch urine, utilizing the identical conditions as provided in Figure 39.

The collected peak in black urine was injected directly into Esquire 3000 Plus MS ion trap (Bruker Daltonics) to identify the UV-absorbing compound present in black urine. The analysis was performed at Dept. of Chemistry (UiO) under supervision by associate professor Steven. R. Wilson. The MS spectrum in positive ionisation mode is presented in **Figure 41.** As can be seen, the spectrum contains the characteristic peak *m/z* 196. The highest peak *m/z* 214 could possibly be detection of N-Ac-5-ASA and H_2O.

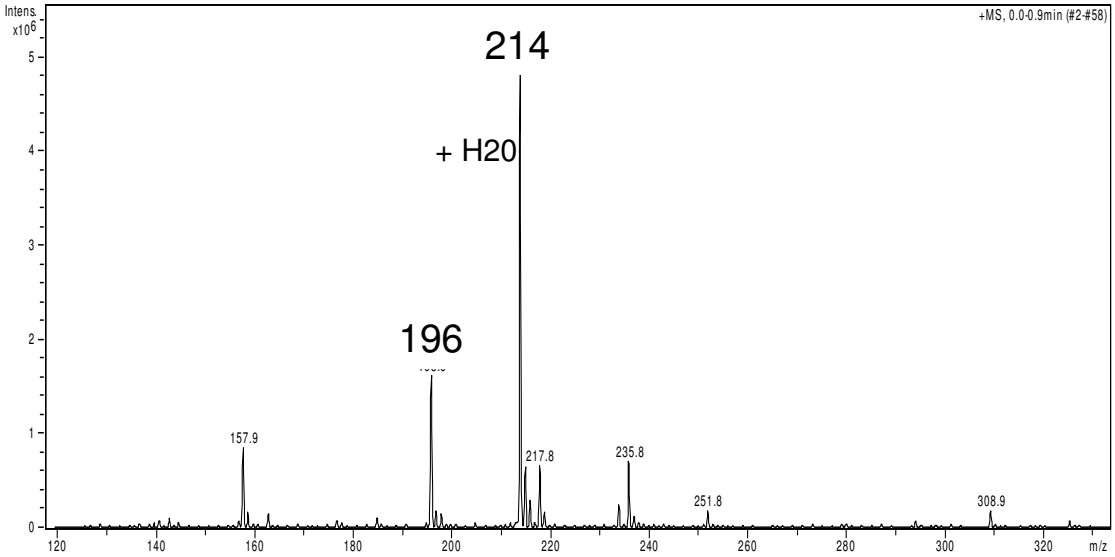

Figure 41: MS spectrum of collected peak in black urine by LC-UV analyzed on ion trap shows peak 196.

The MS/MS spectrum of this peak revealed the same fragmentation pattern (results not shown) as found for UK460, see **Figure 28**.

5.1.7 UV Spectroscopy

The metabolite N-Ac-5-ASA was collected in fractions from LC-UV, but appeared to be transparent by eye. Due to the high dilution (1:300) of the collected fraction in LC-UV the sample was analysed by UV-Visible Spectrophotometer Cary Bio 100 (Varian) to determine if N-Ac-5-ASA absorbs UV and therefore might be causing the colour of the dark urine. The analysis was performed at Dept. of Chemistry (UiO) under supervision of Steven. R. Wilson. UV detection was performed in a range between 1000-200 nm and the resulting spectrum is presented in **Figure 42.**

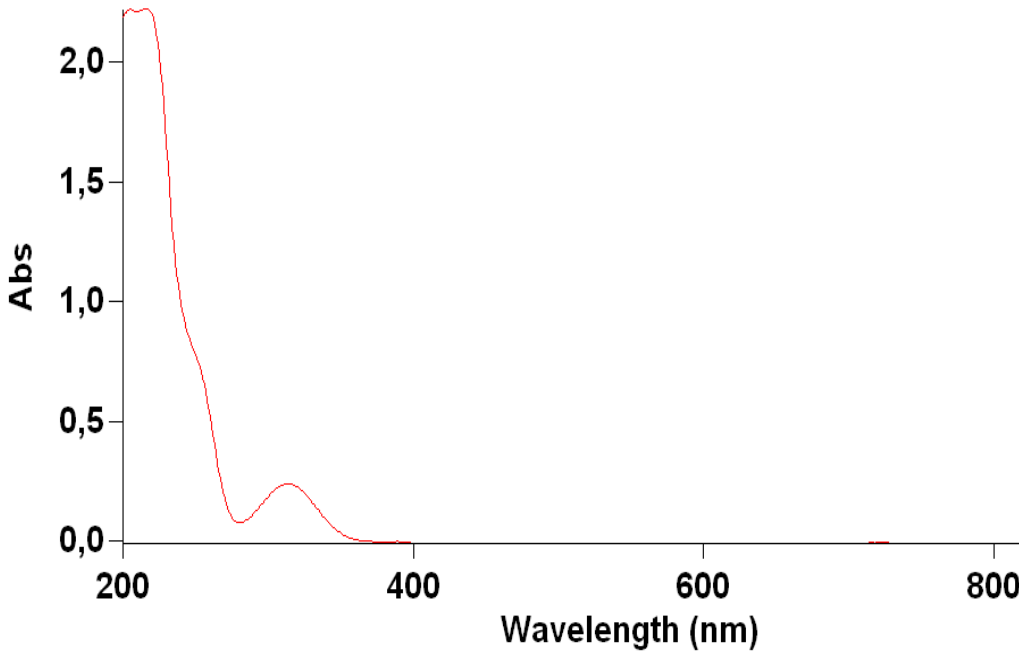

Figure 42: UV-VIS spectroscopy measurement of manually collected peak in black urine by using LC-UV, see Figure 39.

From this UV-VIS spectrum shows that the compound is strongly UV absorbent. However, the result does not immediately explain how the black urine could be a result from N-Ac-5-ASA alone. However, this sample was quite eluted and could possibly obtain further result when having N-Ac-5-ASA if present in very high concentrations.

5.2 Confirmation and concluding remarks

Compound X, the main constituent of the precipitate was identified as 5-ASA by XRD. Knowing the identity of this compound, the spectrum obtained from UK460 was investigated and it became clear that UK460 was a second metabolite with the same origin as 5-ASA.

After the compound UK460 was identified as a metabolite from an anti-inflammatory drug, Salicylazosulfapyridine, which was also confirmed by the ordering physician. Further confirmation was obtained by analyzing a urine sample from another patient that was on the drug and analyzing on LC Q-TOF MS (results are not shown).

5-ASA and N-Ac-5-ASA are known metabolites of Salicylazopyridine [41], see **Figure 43.**

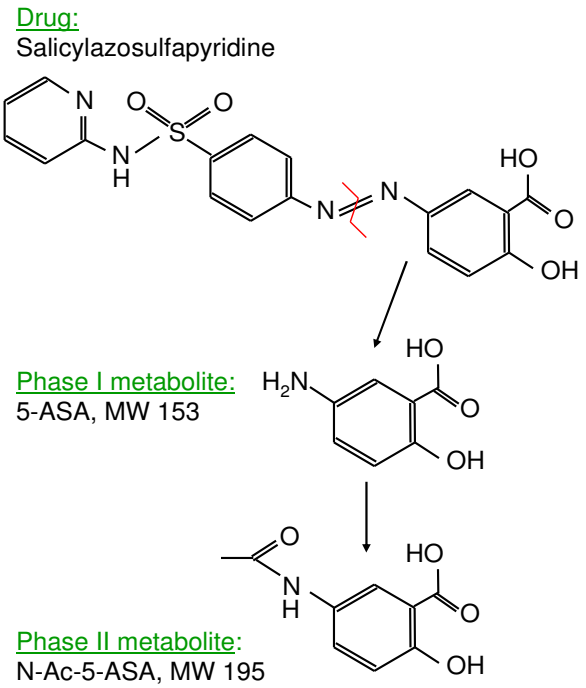

Figure 43: 5-ASA and N-Ac-5-ASA are known metabolites from the anti-inflammatoric drug Salicylazosulfapyridine.

Based on the fragmentation pattern resulted in LC-MS and GC-MS data, Per Ola Rønning has purposed a possible mechanism, shown in **Figure 44.**

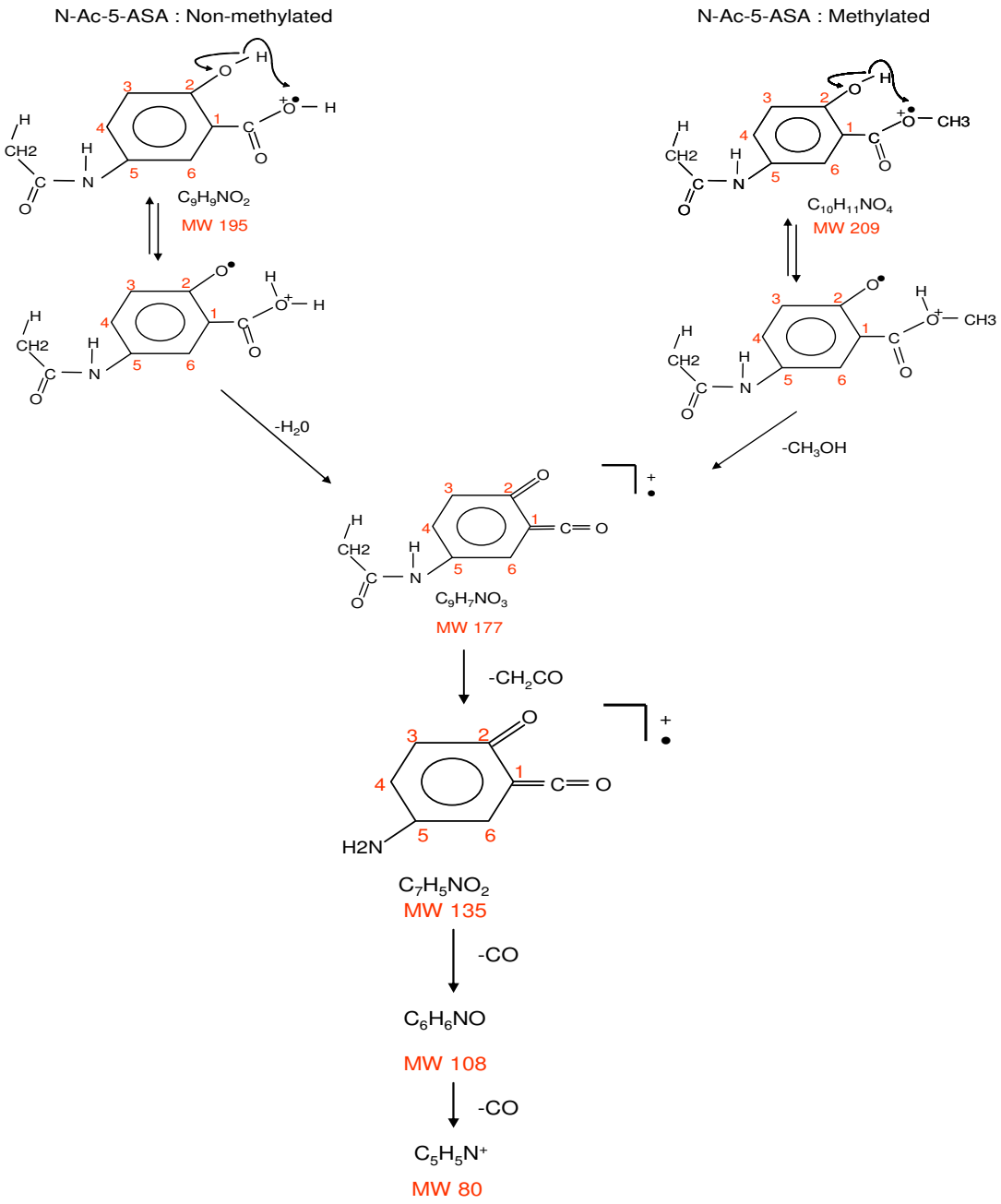

Figure 44: Purposed mechanism of fragmentation of N-Ac-5-ASA, both non-methylated (*m/z* 195) and methylated (*m/z* 209) respectively.

The LC-MS analysis of the urine sample found the metabolite N-Ac-5-ASA both as a monomer [M+H = *m/z* 196] and dimer [2M+H = *m/z* 391]. However, the complex of the dimer or dimerization process is still not known.

6 Conclusion

This master thesisdescribes the development of a robust method for global metabolomics in urine using LC ESI in positive ionization mode coupled to Q-TOF MS. An ACE C18 PFP (250 x 2.1 mm, 3 um) column separated most compounds compared to 6 other RP columns. Furthermore, the method was improved by using an hour long gradient with the result that approximately 6000 molecular features were detected in batch urine. Urine sample preparation was performed by using 0.22 µm cellulose acetate spin filters before injecting 8.0 µL onto the column. The method provided sufficiently promising characteristics to be a strong candidate method for future urinary metabolome analysis.

In order to study of the whole urinary metabolome, the method was also used successfully in negative ionization mode, where approximately 7000 molecular features were detected. A significant overlap is expected in metabolites found in normal urine samples such as the batch urine between positive and negative ionization. Attempts to investigate the overlap between the ionization modes were made using a range of different approaches, but the complexity and size of the data set made this comparison difficult. However, in the search for new diagnostic metabolites it is uncertain which ionization mode will provide measurable signals; therefore both modes must be measured in order to detect a complete metabolome.

The usefulness of this method was demonstrated by investigating intra- and inter- individual biological variation as a function of diet. The results in **Figure 21** show a significant clustering of human urinary metabolites after eating a standardized diet for 24 hours, a novel finding indicating a surprisingly fast change in the metabolome.

Using the method developed, in combination with a broad range of analytical techniques, an unknown compound in black urine from a patient was successfully identified as 5-ASA. Even though this compound turned out to originate from an anti-inflammatory drug, the workflow shows strong potential as a detection method for new diagnostic biomarkers in the future.

The ability to measure the entire metabolome has enormous diagnostic potential and the method developed here have been shown to work exactly as required. This is an exciting step

towards a diagnostic tool and the test results already provide new insights into the dynamics of the human metabolome.

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8 Appendix

8.1 Poster presented at the 20th Norwegian symposium on Chromatography in Sandefjord 2012

LC/MS Q-TOF MED POSITIV IONISERING FOR METABOLOMIKKSTUDIE I URIN

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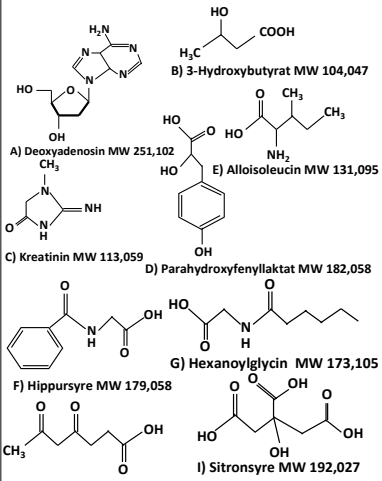
Innledning

Medfødte stoffskiftesykdommer (IEM – "Inborn errors of metabolism") utgjør en stor gruppe genetiske sykdommer som skyldes feil i metabolismen og medfører dermed en endring av metabolomet.[1] Nye IEM oppdages og karakteriseres ved nye diagnostiske metabolitter blir funnet. For å få en større forståelse av de biokjemiske endringene ved IEM, må først normalmetabolomet beskrives. Uselektert metabolomikk i urin skal utføres ved hjelp av massespektrometri (MS) av typen quadrupol-time of fight (Q-TOF) i kombinasjon med væskekromatografi (LC). Elektronsprayionisasjon (ESI) med positiv ionisering vil generelt gi god følsomhet fordi en stor del av metabolomet vil la seg ionisere ved protonering. Det skal utvikles en LC Q-TOF metode som er både robust og reproduserbar, og ulike kolonner og mobilfaser skal testes og sammenliknes ved analyse av referansestoffer og batchurin. Metoden som skal utvikles, vil være et kompromiss som fungerer godt for både positiv og negativ ionisering, se poster "Utvikling av LC QTOF metode med negativ ionisering for metabolomikkstudie av urin i forbindelse med medfødte stoffskiftesykdommer".

Metode

En standard blanding av 9 endogene stoffer (Figur 1) som skilles ut i urin ble laget for å undersøke hvordan kjente stoffer med ulike kjemiske egenskaper kromatograferer. Standard blanding ble laget i henholdsvis vandig prøve og i kunstig urin (salter) for å kunne avdekke eventuelle matrixeffekter. En reell batchurin ble samlet fra 2 friske personer. Standard blanding i vann, standard blanding i kunstig urin og batchurin ble filtrert gjennom 0,22 µm sprøytefilter før injeksjon på LC Q-TOF.



Figur 1: Komponenter i standard blanding i vann og standard blanding i kunstig urin

Omvendt fase kromatografi ble benyttet for 3 ulike analysekolonner (SP vist i i poster "Utvikling av LC QTOF metode av LC QTOF metode med negativ ionisering for metabolomikkstudie av urin i forbindelse med medfødte stoffskiftesykdommer") valgt ut fra rådføring med leverandører:

1. Atlantis T3 C18 3 µm 2,1*150 mm (Waters)
2. Kinetex C18 2,6 µm 2,1*100 mm (Phenomenex)
3. ACE C18 PFP 3 µm 2,1*150 mm, med C18 PFP forkolonne (TeknoLab)

Anvendt mobilfase: A (100 % H_2O + 0,1 % FA) og B (100% MeOH). Gradienteluering ble benyttet for de 3 kolonnene i henhold til Tabell 1.

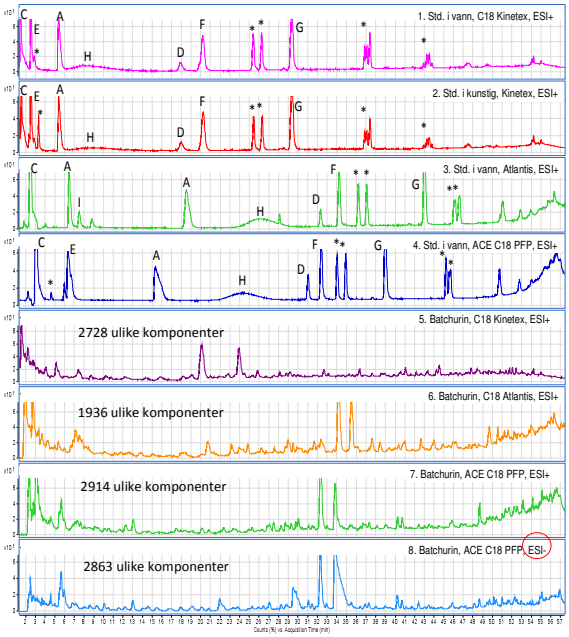
Tabell 1: Gradient eluering av batchurin, standard blanding i vann og standard blanding i urin

Tid (min)	0	10	40	50	54	64
MeOH B (%)	2	2	50	90	2	2

Funksjon "Find by molecular feature" ble benyttet for å undersøke hvor mange unike metabolitter som blir funnet ved analyse av batchurinen med de ulike kolonnene.

Foreløpige resultater

Ulike komponenter i std. blanding og batchurin ble separert med kolonnene Kinetex, Atlantis og ACE C18 PFP med LC/MS (Figur 2). Deoxyadenosin (A), Kreatinin (C), Parahydroxyfenyllaktat (D), Hippursyre (F), Hexanoylglycin (G) og Succinylaceton (H) ble separert og identifisert i std. blanding i vann og i kunstig urin (kromatogram 1-4). Sitronsyre (I) ble kun identifisert med Atlantis (kromatogram 3). Succionylaceton (H) kromatograferte dårlig på alle kolonnene. Kromatogram 1 og 2 av std. blanding i hhv. vann og kunstig urin tyder ikke på store matrixeffekter. Toppene som er merket * skyldes kontaminering i standarden av 3-hydroxybutyrat. Ukjente komponenter i batchurin ble separert med alle kolonnene (Figur 5-7), hvorav 2728 med Kinetex, 1936 med Atlantis og 2914 med ACE C18 PFP. Batchurin med negativ ionisering er hentet fra "Utvikling av LC QTOF metode med negativ ionisering for metabolomikkstudie av urin i forbindelse med medfødte stoffskiftesykdommer" har til sammenligning funnet 2863 ulike komponenter.



Figur 2: Kromatogram av hhv. batchurin, std. blanding i vann, std. blanding i kunstig urin, analysert på LC QTOF med ESI positiv ionisering, unntatt fig. 4 med negativ ionisering. Analysekolonnene Atlantis T3 C18, Kinetex C18 og ACE C18 PFP er testet med lik gradient, se tabell 1.

Diskusjon og konklusjon

Metabolitter med ulike kjemiske egenskaper er best egnet til å la seg ionisere positivt (eksempel kreatinin) eller negativt (eksempel sitronsyre) og begge metodene er derfor nødvending når urinmetabolomet skal kartlegges og nye diagnostiske metabolitter identifiseres. Det er små forskjeller i kromatografering av komponentene i std. blanding med de 3 kolonnene. For videre arbeid er det ønskelig å teste nye kolonner og ulike filtreringssteknikker før endelig metode bestemmes.

Kilder: 1. Janeckova H, Hron K, et al. Targeted metabolomic analysis of plasma samples for the diagnosis of inherited metabolic disorders. Journal of Chromatography A. In press 2010.

8.2 Example of class specific analysis

Acylcarnitines will fragment in reproducible patterns and analysis by MS/MS is highly selective. A chemical modification involving butyl esterification of the aculcarnitines, fragments to produce a charged loss of 85 mass units, see **Figure 44.** The R-group is detected in a precursor ion scan.

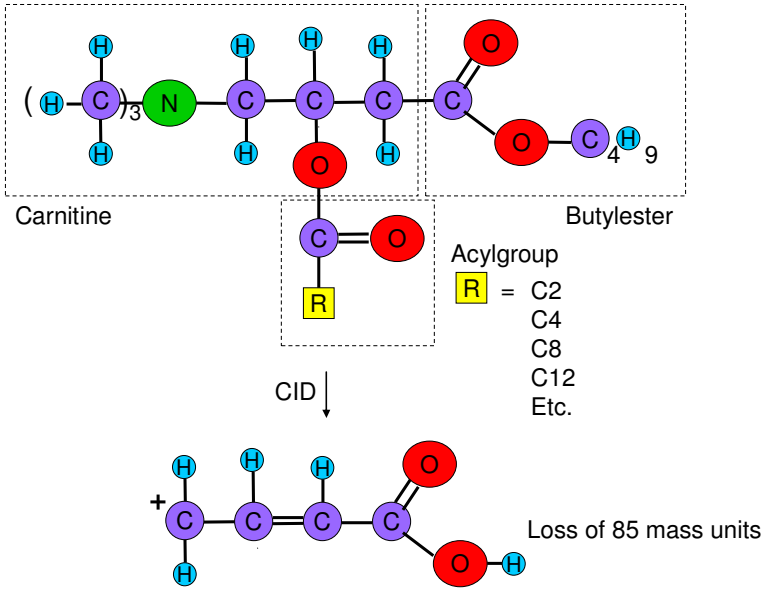


Figure 44: Butyl esterification of acylcarnitines produces a loss of 85 mass units and a neutral R-group, by collision induced ionization (CID).

In the first mass separator (Q1) the intact molecular mass is measured. The molecules are then fragmented in a collision cell (Q2) before charged ions are measured in a second mass separator (Q3). **Figure 45** illustrates the MS/MS set up for analysis of octanoylcarnitine (C8-carnitine). Intact butylated ion with mass of the specific acylcarnitine passes Q1. Q3 is set at *m/z* 85.

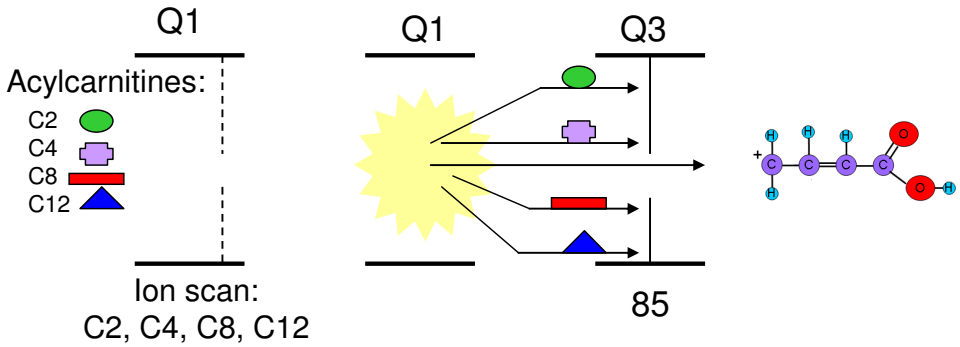


Figure 45. Precursor ion scan of AC C8-carnitine analysis by MS/MS. Mass separator Q3 is set to scan *m/z* 85.

8.3 Artificial urine recipe obtained at OUS-RH 2012.

Artificial urine. Chemical dissolved in 500mL water.	
Chemical (in Norwegian)	Amount (g)
Kaliumsulfat (K_2SO_4)	0,85
Urea (CH_4N_2O)	10,0
Dikaliumhydrogenfosfat K_2HPO_4 x $3H_2O$	1,15
Natriumklorid (NaCl)	5,0
Magnesiumsulfat ($MgSO_4$ x $7H_2O$)	0,6
Kaliumdihydrogenfosfat KH_2PO_4	0,7

8.4 Out-handed protocols for the volunteers participating in the experiment for intra- individual biological variation

(In norwegian)

Innsamling av urinprøver for bestemmelse av intra- individuell biologisk variasjon

Bakgrunn

Medfødte stoffskiftesykdommer (IEM - Inborn Errors of Metabolism) utgjør et stort antall av genetiske sykdommer som involverer feil i metabolismen. ”Metabolom” er det totale settet av metabolitter i en biologisk prøve, for eksempel urin, og gir opphav til en såkalt metabolsk profil. For å få en større forståelse av de biokjemiske endringene ved IEM, må først ”normalmetabolomet” beskrives. Utfordringen ved å undersøke normalmetabolomet er såkalt inter- og intra- individuell biologisk variasjon. Intraindividuell biologisk variasjon innebærer endringer av urinmetabolomet over korte eller lengre tidsrom hos ett enkelt individ. Endringene av metabolomet skjer kontinuerlig og kan gjenspeile ulikt kosthold, fysisk aktivitet og fysiologiske tilstander. Interindividuell biologisk variasjon innebærer forskjeller i urinmetabolomet mellom individer som kan skyldes ulik livsstil og genetiske forskjeller. I dette forsøket skal inter- og intraindividuell biologisk variasjon undersøkes ved analyse av urinprøver fra friske forsøkspersoner.

Beskrivelse av prøvetakingen

Prøvetakingen går over 10 testdager fra mandag til fredag i 2 uker, med vanlig kosthold og mosjon. Prosedyren for prøvetaking er illustrert i figur 1. På testdagene skal det samles inn **midtstrøms**morgenurin (første urinering om morgenen, etter kl 05:00). Midtstrømsurin er den midterste porsjonen som kommer ut under urinering. Den første porsjonen av urinen skal rett i toalettet, deretter plasseres et prøverør under urinstrålen, og til slutt skal den siste porsjonen gå i toalettet. Urin skal samles direkte i utleverte prøvetakingsrør. Utleverte klistrelapper skal fylles ut og festes på prøverøret. Alle prøvene skal settes i fryseren umiddelbart etter prøvetaking. Inntak av mat og drikke, trening og eventuelle kommentarer skal fylles ut i innsamlingsskjema for hver prøve, som er gitt i tabell 3 og 4. Et eksempel på utfylt skjema er vist i tabell 2. Søndag før testukene er det ingen prøvetaking, men innsamlingsskjema skal fylles ut. Dersom det oppstår endringer ved prøveinnsamling som går utenom oppgitt prosedyre er det viktig at dette blir notert og at man fortsetter etter planen.

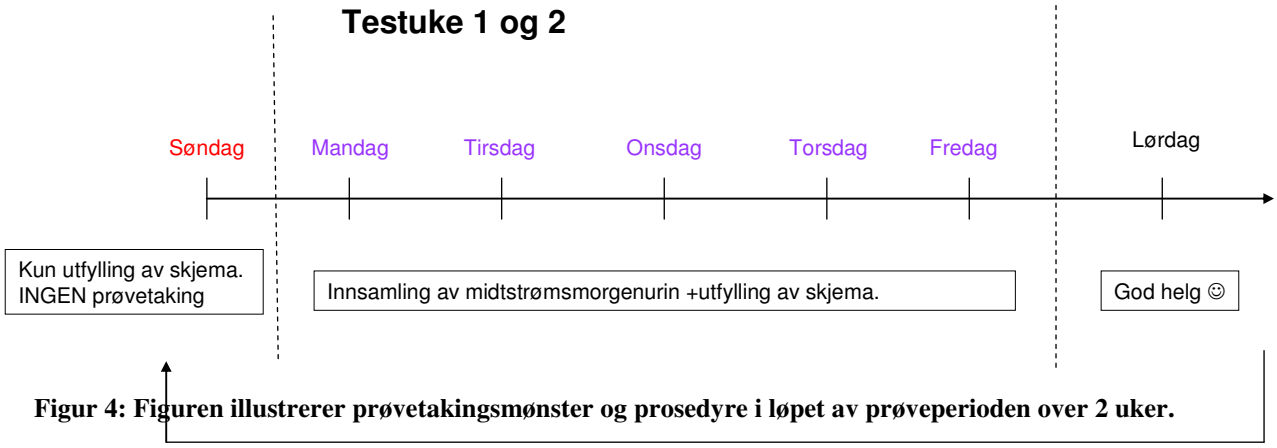
Tips: Sett røret på toalettskålen på kvelden for ikke å glemme innsamling neste morgen!

Kostholds- og treningsparametere vil IKKE bli linket sammen med personopplysninger i databasen.

LEV SOM NORMALT I FORHOLD TIL KOSTHOLD OG TRENING.

Prosedyre for testdagene (man-fre)

1. Prøvetaking i utlevert prøverør av midtstrømsmorgenurin etter kl. 06:00
2. Notér forsøksperson, prøvenummer og tid på utlevert klistrelapp og fest på prøverør
3. Sett prøverøret i fryser -70 ºC
4. Fyll ut innsamlingsskjema i tabell 3 / 4
5. Kommenter eventuelle avvik fra prosedyren



Figur 4: Figuren illustrerer prøvetakingsmønster og prosedyre i løpet av prøveperioden over 2 uker.

Tabell 1. Generell informasjon om forsøkspers

Forsøksperson:		
Dato ved start (søn)		
Fødselsdato:		
Kjønn:	Kvinne:	Mann:
Vekt (kg):		
Høyde (cm):		
Kosttilskudd (medisiner) :		
Vegeterianer:	Ja :	Nei:

Tabell 2. Eksempel på utfylling av innsamlingsskjema

Eksempel				
Dag	**Prøvenr.**	**Kosthold**	**Trening (kl)**	**Evt. kommentarer**
Mandag Dato: 01/01	1	Kokt torsk, poteter, gulrøtter, smeltet smør, 2 kaffe, 1 glass rødvin. Grovbrød med gulost og paprika	09:00	Klarte ikke å samle midtstrømsurin i prøverøret, bare siste porsjon av urineringen.

Tabell 3. Innsamlingsskjema av urinprøver uke 1

Prosedyre	Dag	Prøvenr.	Kosthold	Trening (kl)	Evt. kommentarer
Kun utfylling av skjema	Søndag Dato: ____/____	-			
Testuke 1 Prøvetaking av midtstrøms morgenurin og utfylling av skjema	Mandag Dato: ____/____	1			
	Tirsdag Dato: ____/____	2			
	Onsdag Dato: ____/____	3			
	Torsdag Dato: ____/____	4			
	Fredag Dato: ____/____	5	Ikke oppgi kosthold eller trening		

Tabell 4. Innsamlingsskjema av urinprøver uke 2

Prosedyre	Dag	Prøvenr.	Kosthold	Trening (kl)	Evt. kommentarer
Kun utfylling av skjema	Søndag Dato: ____/____	-			
Testuke 2 Prøvetaking av midtstrøms morgenurin og utfylling av skjema	Mandag Dato: ____/____	6			
	Tirsdag Dato: ____/____	7			
	Onsdag Dato: ____/____	8			
	Tor Dato: ____/____	9			
	Fredag Dato: ____/____	10	Ikke oppgi kosthold eller trening		

Kontakt Siv per tlf. 46935053 eller Anja per tlf. 98022811 ved endt prøvetaking eller evt. spørsmål.

Takk for ditt bidrag til vårt masterprosjekt!! ☺ ☺

8.5 Out-handed protocols for the volunteers participating in the experiment for inter- individual biological variation

(In norwegian)

Innsamling av urinprøver for bestemmelse av inter- individuell biologisk variasjon ved likt kosthold

Bakgrunn
Medfødte stoffskiftesykdommer (IEM - Inborn Errors of Metabolism) utgjør et stort antall av genetiske sykdommer som involverer feil i metabolismen. ”Metabolom” er det totale settet av metabolitter i en biologisk prøve, for eksempel urin, og gir opphav til en såkalt metabolsk profil. For å få en større forståelse av de biokjemiske endringene ved IEM, må først ”normalmetabolomet” beskrives. Utfordringen ved å undersøke normalmetabolomet er såkalt inter- og intra- individuell biologisk variasjon. Intraindividuell biologisk variasjon innebærer endringer av urinmetabolomet over korte eller lengre tidsrom hos ett enkelt individ. Endringene av metabolomet skjer kontinuerlig og kan gjenspeile ulikt kosthold, fysisk aktivitet og fysiologiske tilstander. Interindividuell biologisk variasjon innebærer forskjeller i urinmetabolomet mellom individer som kan skyldes ulik livsstil og genetiske forskjeller. I dette forsøket skal interindividuell biologisk variasjon undersøkes ved analyse av urinprøver fra friske forsøkspersoner som har likt kosthold ut i fra en forhåndsbestemt meny.

Beskrivelse av prøvetakingen
Prøvetakingen går over 3 testdager, hvor prosedyren er illustrert i figur 1. På dag 1 skal kosthold og mosjon være som NORMALT og det skal ikke samles inn urin, se tabell 1. På dag 2 skal kosthold og mosjon bestemmes i henhold til en utarbeidet ”meny” hvor alle forsøkspersonene skal spise og drikke det samme, samt unngå trening, se tabell 2. På dag 3 er det ingen kostholds- eller mosjonsregulering, se tabell 3. På testdag 2 og 3 skal det samles inn **midtstrøms**morgenurin (første urinering om morgenen) etter kl 05:00. Midtstrømsurin er den midterste porsjonen som kommer ut under urinering. Den første porsjonen av urinen skal rett i toalettet, deretter plasseres et prøverør under urinstrålen, og til slutt skal den siste porsjonen gå i toalettet. Urin skal samles direkte i utleverte prøvetakingsrør. Utleverte klistrelapper skal fylles ut og festes på prøverøret. Alle prøvene skal settes i fryseren umiddelbart etter prøvetaking.
Tips: Sett røret på toalettskålen på kvelden for ikke å glemme innsamling neste morgen!

Eventuelle kommentarer som avviker fra prosedyre skal fylles ut i innsamlingsskjema for alle dagene, som er gitt i tabell 5, 6 og 7. Et eksempel på utfylt skjema er vist i tabell 1, 2 og 3. Dersom det oppstår endringer ved prøveinnsamling som går utenom oppgitt prosedyre er det viktig at dette blir notert og at man videre fortsetter etter planen.

OBS! Kostholdsparametere vil IKKE bli linket sammen med personopplysninger i databasen.

Prosedyre

Dag 1:

Lev normalt. Oppgi kosthold og eventuell trening i tabell 5.

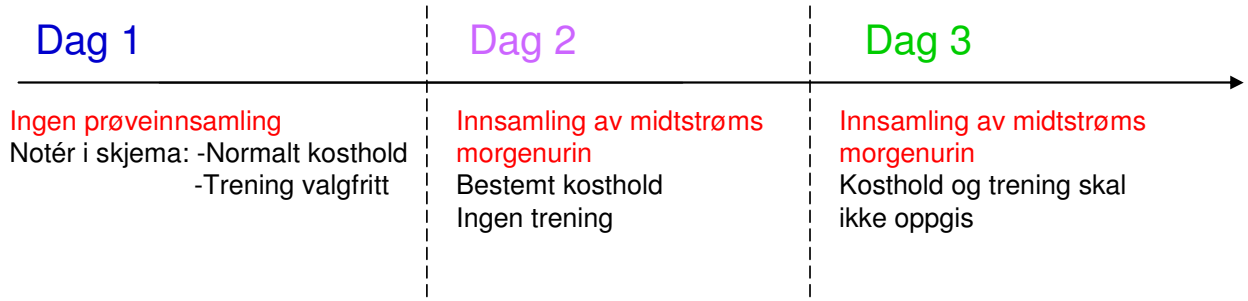
Dag 2:

1. Prøvetaking i utlevert prøverør av midtstrømsmorgenurin etter kl. 05:00
2. Notér forsøksperson, prøvenummer og tid på utlevert klistrelapp og fest på prøverør. Bruk vannfast markør!
3. Sett prøverøret i fryser (-70 ºC hvis mulig)
4. Fyll ut innsamlingsskjema i tabell 6.
5. Følg forhåndsbestemt kostholdsmeny. Ikke tren.
6. Kommenter eventuelle avvik fra prosedyren

Dag 3:

1. Prøvetaking i utlevert prøverør av midtstrømsmorgenurin etter kl. 05:00
2. Notér forsøksperson, prøvenummer og tid på utlevert klistrelapp og fest på prøverør
3. Sett prøverøret i fryser (-70 ºC hvis mulig)
4. Fyll ut innsamlingsskjema i tabell 7.
5. Kommenter eventuelle avvik fra prosedyren

Figur 1: Illustrert prosedyre for utførelse for testdagene 1-3



Eksempler på utfylling av skjema for testdag 1-3

Eksempel

Testdag 1	Prøvenr.	Kosthold (i kronologisk rekkefølge)	Trening (kl)	Evt. kommentarer
Dato: 01/01/12	Ingen prøvetaking	Morgen Frokost: Havrergyn med melk og sukker. Kaffe. Lunsj: Knekkebrød med gulost. Eplejucie. Banan. Middag: Spaghetti med kjøttdeigsaus og tomater. Kaffe. Eple Kveld	Spinning (16.00)	Begynner å bli forskjølet

Eksempel

Testdag 2	Prøvenr.	Kosthold	Trening (kl)	Evt. kommentarer
Dato: 02/01/12	1	Forhåndsbestemt meny	Uteblir	Glemte meg og spiste Statros selv om den ikke var oppgitt i forhåndsbestemt meny

Eksempel

Testdag 3	Prøvenr.	Kosthold	Trening (kl)	Evt. kommentarer
Dato: 03/01/12	2	Ikke oppgi	Ikke oppgi	Glemte å ta midtstrømsurin og samlet kun første "klunk"

Prøveinnsamling

Tabell 4: Generell informasjon om forsøksperson

Forsøksperson:	
Kjønn:	Kvinne: Mann:
Alder	
Vekt (kg):	
Høyde (cm):	
Spesiell kost: (vegeterianer)	Ja : Nei:
Faste kosttilskudd / medisiner :	
Allergier	
Røyk / snus	Ja : Nei:

Tabell 5. Innsamlingsskjema for testdag 1

Testdag 1	Prøvenr.	Kosthold	Trening (kl)	Evt. kommentarer
Dato:	Ingen prøvetaking			

Tabell 6. Innsamlingsskjema for testdag 2

Testdag 2	Prøvenr.	Kosthold	Trening (kl)	Evt. kommentarer
Dato:	1	Forhåndsbestemt meny	Uteblir	

Tabell 7. Innsamlingsskjema for testdag 3

Testdag 3	Prøvenr.	Kosthold	Trening (kl)	Evt. kommentarer
Dato:	2	Ikke oppgi kosthold	Ikke oppgi trening	

Kontakt Siv per tlf. 46935053 eller Anja per tlf. 98022811 ved endt prøvetaking eller evt. spørsmål.
Takk for ditt bidrag til vårt masterprosjekt!! ☺

Menu:

Breakfast: Bread (medium dark), cheese (Norvegia), butter (Bremykt) and pepper (red)
Lunch: Bread (medium dark), cheese (Norvegia), butter (Bremykt), pepper (red) and banana
Dinner: Stew, pasta and salad
Supper: Bread (medium dark), cheese (Norvegia), butter (Bremykt), pepper (red), banana
(The participant were specifically asked to eat the banana after 21 pm)

Only water was allowed to drink throughout the whole day.

There was not put any restrictions about the size of portions. However, 2 slices of bread qualifies as 1 whole banana. If you only had one slice of bread for breakfast, you were only allowed to eat half a banana.

8.6 Isotope calculator constructed by Per Ola Rønning for identification of UK460 by GC-MS spectrum

Analytt: UK460
Analyse-ID: C:\msdchem\1\DTA\FFAPrutine.D\20120123\1101001.D
Peak: 68,870 - 71,475 min (average)

Ion	Abundance	Norm	Type
209,00	310220	100,00	M
210,00	36219	11,68	M+1
211,00	4498	1,45	M+2
212,00	362	0,12	M+3

Antall karbon fra binomialfomel
Antall, n 10,8

	M+1	**M+2**				
I-ratio	11,68	1,45	Avrund	mass	**Molekylformel**	**Umetylert**
a C	10,2		10,0	120,0	C10H11NO4	C9H9NO4
b H	11,0		11,0	11,0	DBE = 6	
c O	4,4		4,0	64,0		
d N	1,0		1,0	14,0		
e S	0,0		0,0	0,0		
sum ratio	11,70	1,45 %		209,0		
DBE	6,0		Forventet	209		
			Differanse	0,0		

Intervall permited:		10 %
Low	10,51	
High	12,84	

Isotope abubdance					
12C	98,93 %	13C	1,07		
1H	99,988 %	2H	0,012		
16O	99,757 %	17O	0,038	18O	0,205 %
14N	99,632 %	15N	0,368		
32S	94,93 %	33S	0,76		
34S	4,29 %	36S	0,02		

8.7 NMR sample preparation and analysis for identification of UK460

Three samples were analyzed: black patient urine sample with UK460, yellow patient urine sample with UK460 and a healthy batch urine control. 500 ul of each sample was mixed with 100 µl distilled water and 50 ul buffer (KH2PO4/KOH, pH 7.4; D2O; TMSP-d4; NaN3) in an Eppendorf tube. The samples were centrifuged at 13400 g and 4 °C for 5 minutes, and 550 µl of each sample was transferred to 5 mm NMR tubes.

The analyses were performed by a 1H NMR AV 600 Hz at temperature of 300.0 K.

COSY spectrum, see **Figure 46,** of black urine shows that the six peaks are connected in two peak systems called A and B. (A: 7.7, 7.4 and 6.9 ppm; B: 7.3, 7.0 og 6.8 ppm; therefore: AABBAB). In yellow urine, most A was more abundant than B.

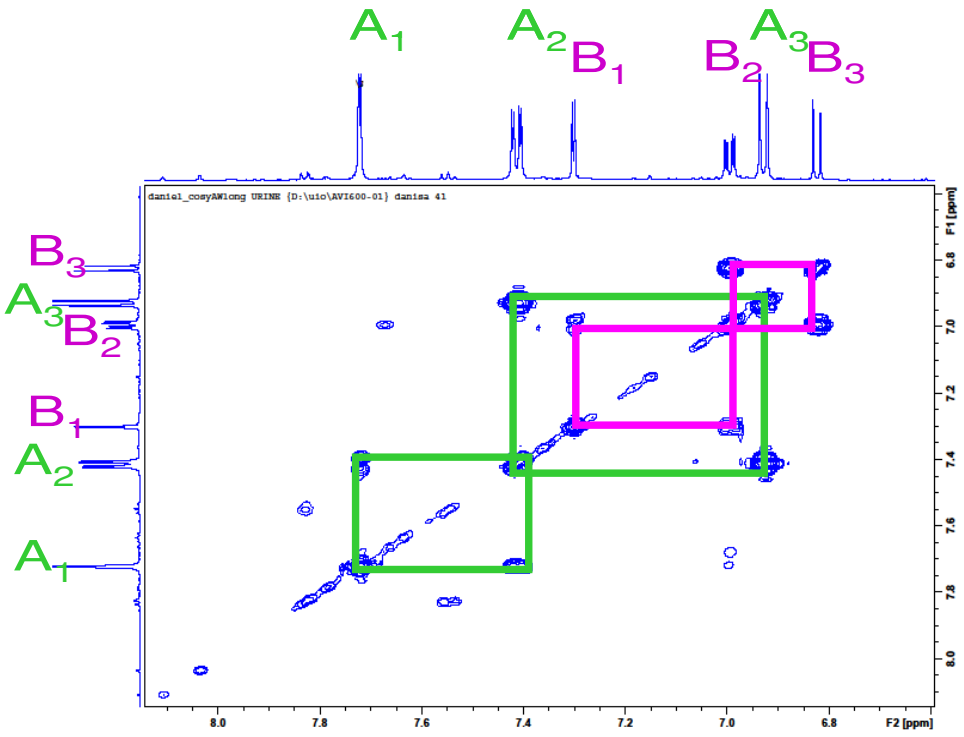


Figure 46: COSY-spectrum from black urine analyzed by 1H NMR,